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An integrated approach to assess the impacts of zinc pyrithione at different levels of biological organisation in marine mussels

Lorna J. Dallas,[†] Andrew Turner,*,[¶] Tim P. Bean,[‡] Brett P. Lyons,[‡] and Awadhesh N. Jha[†]

†*School of Biological and Marine Sciences, University of Plymouth, Plymouth, UK*

‡*Centre for Environment, Fisheries and Aquaculture Science, Weymouth, UK*

¶*School of Geography, Earth & Environmental Sciences, University of Plymouth, Plymouth, UK*

E-mail: aturner@plymouth.ac.uk

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48   Abstract
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50   The mechanisms of sublethal toxicity of the antifouling biocide, zinc pyrithione (ZnPT),

51   have not been well-studied. This investigation demonstrates that 14-d sublethal

52   exposure to ZnPT (0.2 or 2 μM, alongside inorganic Zn and sea water controls) is

53   genotoxic to mussel haemocytes but suggests that this is not caused by oxidative

54   DNA damage as no significant induction of oxidised purines was detected by Fpg-

55   modified comet assay. More ecologically relevant endpoints, including decreased

56   clearance rate (CR), cessation of attachment and decreased tolerance of stress on

57   stress (SoS), also showed significant response to ZnPT exposure. Our integrated

58   approach was underpinned by molecular analyses (qRT-PCR of stress-related genes,

59   2D gel electrophoresis of proteins) that indicated ZnPT causes a decrease in

60   phosphoenolpyruvate carboxykinase (PEPCK) expression in mussel digestive glands,

61   and that metallothionein genes are upregulated; PEPCK downregulation suggests

62   that altered energy metabolism may also be related to the effects of ZnPT. Significant

63   relationships were found between % tail DNA (comet assay) and all higher level

64   responses (CR, attachment, SoS) in addition to PEPCK expression. Principal

65   component analyses suggested that expression of selected genes described more

66   variability within groups whereas % tail DNA reflected different ZnPT concentrations.

67

68   Keywords: zinc pyrithione; sublethal toxicity; marine mussels; genotoxicity; DNA damage

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70 **1. Introduction**

71 Zinc pyrithione (ZnPT, 317.70 g mol^{-1}), an organic complex with two pyrithione

72 ligands chelated to Zn^{2+}, is used as a booster biocide in many copper-based

73 antifouling paints (AFPs). Its role in such products is to increase broad-spectrum

74 efficacy via antifungal action (Thomas et al., 2000), which also makes it ideal for use

75 in outdoor paints for resistance to mould. ZnPT also has a variety of household and

76 medical uses (for example, it is used in the treatment of psoriasis and is the only

77 active ingredient in medicated anti-dandruff shampoos; Reeder et al., 2011a)

78 resulting in multiple, additional sources from which ZnPT can enter the environment.

79

80 Although ZnPT is moderately hydrophobic (log K_{OW} = 0.93 at 25°C, solubility in

water = 8 mg L^{-1} at 20°C) and has the potential to accumulate in sediments, it is favoured over many other booster biocides because it can degrade relatively quickly when exposed to UV light (Price and Readman, 2013). Although there are uncertainties about the kinetics of ZnPT photo-degradation in sea water, its degradation products and the mechanisms by which it adsorbs to sediments (Maraldo and Dahllof, 2004; Turley et al., 2005; Bones et al., 2006), it is clear that the compound has potential to cause detrimental effects in aquatic species at concentrations typically encountered in environments where boating activities occur (up to 100 nM; Mackie et al., 2004; Madsen et al., 2000). For example, EC50 values for developmental abnormalities in zebrafish and medaka are 28 and 15 nM, respectively (Goka, 1999), and embryo toxicity is also reported in sea urchins (EC50 = 7.7 nM) and mussels (*M. edulis*, EC50 = 8 nM) (Bellas et al., 2005). Growth of diatoms is significantly reduced by as little as 5.9 nM ZnPT over 96 h (Bao et al., 2008), while cultured ascidian haemocytes show compromised immunity after exposure to ≤ 0.5 μM ZnPT (Cima and Ballarin, 2015) and treatment of paddy fields with ZnPT anti-dandruff shampoo decreased growth in juvenile medaka (Sánchez-Bayo and Goka, 2005). Recently, Marcheselli et al. (2013) demonstrated that ZnPT induced genotoxicity in marine mussels using the lethal-effect TUNEL assay. Thus, after 7-d exposure, adult *Mytilus galloprovincialis* showed increased DNA fragmentation and induced heat shock protein expression at ZnPT concentrations of 0.2 and 0.4 μM, with the higher concentration also causing a reduction in anoxia tolerance.

Despite these observations , there are still many unanswered mechanistic questions regarding ZnPT toxicity to aquatic biota, including mussels as ecologically important bioindicator species (Dallas et al., 2016). Furthermore, no study has attempted to connect alterations in genomic integrity with changes in protein expression by looking at intermediate processes such as transcriptional expression of relevant genes. In this context, we exposed adult *M. galloprovincialis* to ZnPT over a two-week time period to examine the effects of ZnPT across several levels of biological organization in the mussel. This included assessing the potential induction of oxidative DNA damage (Fpg-modified comet assay) and linking changes in genomic integrity throughout the mussel with transcriptional and protein expression (qRT-PCR and 2D gel electrophoresis, respectively) and with

114 changes to higher level, more ecologically relevant responses, including clearance

115 rate [CR] and attachment.. Where appropriate, observed biological responses were

116 analysed using Principal Component Analysis (PCA) and Cluster analyses which

117 effectively integrate multidimensional biomarker data into a more readily

118 interpretable two dimensional format.

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121 **2. Methods and experimental design**

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123 *2.1. Reagents and mussel collection and maintenance*

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125 Adult *M. galloprovincialis* (48.03 ± 2.43 mm) were collected from Trebarwith Strand

126 (50° 38' 40" N, 4° 45' 44" W), a reference site remote from significant boating activity,

127 and maintained in the laboratory as previously described (Dallas et al., 2013; Banni

128 et al., 2017). Sea water used in the exposures was sourced from Plymouth Sound,

129 stored on site and filtered on line (< 10 μm). Measured Cu concentrations (< 10 μg

130 L^{-1}) were considerably less than added Zn concentrations (see below), ensuring that

131 significant Cu(II)-Zn transchelation of the pyrithione ion (PT^-) was unlikely (Holmes

132 and Turner, 2009).

133

134 Unless otherwise stated, all reagents used in the exposures and for sample

135 processing and analysis were purchased from Sigma-Aldrich Ltd (Gillingham, UK).

136 Ultrapure water was obtained using a Milli-Q RG (Merck Millipore, Billerica, MA,

137 USA) or Elga Purelab Option system (Elga LabWater, Marlow, UK) and had a

138 resistivity of > 18.2 MΩ cm^{-1} at 25 °C.

139

140 *2.2. Exposure scenario and determination of higher level responses*

141

142 Concentrations used were based on acute range-finding exposures that compared

143 ZnPT to $ZnCl_2$ and the pyrithione ion (as NaPT) at equimolar concentrations of Zn or

144 PT and between 0.20 and 16.20 μM. These produced a 96-h LC50 for *M.*

145 *galloprovincialis* of 14.50 ± 1.45 μM for PT as ZnPT (50 % mortality not achieved for

146 the other treatments), 7-d LC50s of 24.58 ± 1.57 μM for PT as NaPT and 8.94 ± 1.30

147 μM for PT as ZnPT, and 14-d LC50s of 2.54 ± 1.32 (as NaPT) and 2.97 ± 1.36 μM

148   (as ZnPT), with 50 % mortality not achieved for inorganic Zn (Fig. S1). Degradation

149   of ZnPT into inorganic Zn was also assessed under our experimental conditions by

150   retention and subsequent 3 ml min^{-1} methanol elution of Zn on conditioned octadecyl

151   silane (C18) columns (15 mL; Chromabond, Macherey Nagel GmbH, Düren,

152   Germany) (Holmes and Turner, 2009) (Fig. S2 A, S2 B).

153

154   Depurated (28 d) and acclimatized (48 h) *M. galloprovincialis* (*n* = 16) were exposed

155   for 14 d and under a 12:12 h photoperiod to 0.2 μM ZnPT (low), 2 µM ZnPT (high), 2

156   µM ZnCl$_2$ (inorganic) and no added Zn (control) in triplicate and in pre-washed

157   plastic tanks containing 20 L of sea water (salinity 31.82 ± 0.34, pH 8.16 ± 0.21,

158   dissolved oxygen 90.02 ± 2.84 % and temperature 15.88 ± 0.52 ∘C). ZnPT

159   concentrations were either chronic (< 10% of 14-d LC50) or acute (67% of 14-d

160   LC50) as determined by the range-finding experiment described above. As the

161   degradation of ZnPT approached 80% at 48 h (Fig. S2 C), full water changes were

162   performed every 2 d with replenishment of ZnPT. Mussels were fed 2 h prior to water

163   changes with *Isochrysis galbana* (∼1.0^5 x 10^6 cells mL^{-1}), and during changes on

164   days 4, 6 and 12 (i.e. those closest to the sampling days), mussel attachment was

165   recorded where adherence to the interior surface or to other mussels was evident

166   when the emptied tank was tilted 45°. Clearance rate was determined for nine

167   mussels per treatment (and three from each replicate) on day 14, as previously

168   described (Devos et al., 2015), and after this time period the stress on stress (SoS)

169   test was performed in continuous anoxic conditions for a further 20 d (Viarengo et

170   al., 1995). Thus, tanks were drained and remaining mussels blotted dry before being

171   transferred to open petri dishes where they were maintained without water but in a

172   humid environment at 15 ^{o}C. Mortality, defined as persistent opening of the valves

173   and failure to respond when tapped, was recorded daily and subsequently used to

174   calculate LT50 values (median lethal time for 50 % mortality).

175

176   *2.3. Genotoxicity in circulating haemocytes*

177

178   After 0, 4, 7 and 14 d, haemolymph was extracted from nine mussels per treatment,

179   and stored on ice pending assay, whilst 5 mm^2 sections of gill and digestive gland

180   were removed and stored at -80 ∘C pending analysis. The enzyme-modified comet

181   assay was performed using the haemocytes according to methods previously

182   validated using H_2O_2 as a positive control (Dallas et al., 2013), with two slides per

183   sample: one control containing buffer only (40 mM HEPES, 0.1 M KCl, 0.5 mM

184   EDTA, 0.20 mg mL^{-1} BSA, pH 8.0) and one with the bacterial enzyme,

185   formamidopyrimidine glycosylase (Fpg), to detect oxidised purine and pyrimidine

186   bases. Prior to the performance of the comet assay, cell viability was checked using

187   Eosin Y stain and was found be > 80 % for all treatments (data not shown). The

188   micronucleus (MN) assay was performed on haemocytes as also described

189   previously (Dallas et al., 2013) with 1000 cells scored on each coded and

190   randomized slide.

191

192   *2.4. Alterations in transcriptional expression of candidate genes*

193

194   Total RNA was isolated from gill and digestive gland using the RNeasy mini kit

195   (Qiagen Ltd, UK). Two µg from samples with OD260:OD280 > 1.95 and crisp bands

196   was used for reverse transcription with M-MLV reverse transcriptase and random

197   primers (Promega Corporation, USA). Real-time- (q-)PCR was performed on

198   samples in duplicate (Applied Biosystems Step-One Plus RT-PCR system, StepOne

199   Software v2.2.2) in reactions containing 7.5 µL SYBR Green Jumpstart Taq

200   ReadyMix, 0.2 µM forward and reverse primers (as detailed in Table S1), 4.44 µL

201   ultrapure water and 3 µL template cDNA. Initial denaturation was at 95 $^{\circ}$C for 2 min,

202   followed by 40 cycles of 95 $^{\circ}$C for 15 s and 60 $^{\circ}$C for 1 min, plus a melt curve to

203   verify PCR-product purity. Relative expression ratio (RER) of 3 genes (*mt10*, *mt20*

204   and *hsp70*) was quantified using REST 2009 (v2.0.13; Qiagen Ltd) from PCR

205   efficiency (measured using LinRegPCR) and threshold cycle (C_q), relative to the

206   reference genes *atub* (alpha tubulin) and *ef1* (elongation factor 1) with control

207   samples as calibrators (Pfaffl et al., 2002; Ramakers et al., 2003). Both reference

208   genes showed low variability across samples (*Cq atub* = 20.45 ± 1.26, *ef1* = 18.01 ±

209   1.01).

210

211   *2.5. Alterations in protein expression*

212

213   Digestive glands from three mussels per exposure tank were pooled and

214   homogenised with four parts homogenisation buffer (10 mM Tris-HCl, 0.5 M sucrose,

215 0.15 M KCl, 1 mM EDTA, 1 mM PMSF) to provide sufficient protein and reduce inter-

216 individual variation (Karp and Lilley, 2005). Samples were prepared for 2D gel

217 electrophoresis (2DGE) using methods of Schmidt et al. (2003). Briefly, total protein

218 was extracted with TCA-acetone, rehydrated and loaded onto a 7 cm IPG strip

219 overnight (pH 3-10; GE Healthcare). Iso-electric focusing was performed using a

220 Protean IEF cell (Bio-Rad) at 300 V for 3 h, 1000 V for 6 h and 8000 V for 3 h,

221 followed by 8000 V for 20 kVh and hold at 500 V. Strips were equilibrated firstly with

222 2% DTT and secondly with 2.5% iodoacetamide. After transfer of strips to 12%

223 polyacrylamide gels (Mini-PROTEAN TGX, Bio-Rad), electrophoresis was carried

224 out at 90 V for 1 h, followed by 120 V for 30 min (in 0.25 M Tris, 1.92 M glycine, 0.03

225 M SDS). Gels were stained with 0.2% Coomassie brilliant blue (R-250 in 30%

226 methanol, 10% acetic acid) for 1 h, destained overnight (40% methanol, 10% acetic

227 acid) and visualised using a Gel Doc XR+ (Bio-Rad).

228

229 Spots of interest (i.e. those not evident in the control) were excised using sterile

230 razor blades, dried and sent for identification (Mass Spectrometry Unit, Instituto de

231 Tecnologia Qumica e Biolgica, Universidade Nova de Lisboa). After in-gel tryptic

232 digestion, extracted peptides were loaded onto a R2 micro column (RP-C18

233 equivalent), desalted, concentrated, and eluted directly onto a MALDI plate using α-

234 cyano-4-hydroxycinnamic acid as the matrix solution in 50% acetonitrile and 5%

235 formic acid. Mass spectra were acquired in positive reflectron MS and MS/MS

236 modes using a 4800plus MALDI TOF/TOF analyser with an exclusion list of trypsin

237 autolysis peaks (842.51, 1045.56, 2211.11 and 2225). Resulting spectra were

238 analysed in combined mode using the Mascot search engine and NCBI database

239 (restricted to 50 ppm peptide mass tolerance with no taxonomy restrictions).

240

241 *2.6. Statistical analysis*

242

243 Statistics were performed in R (i386, v2.15.2; www.R-project.org) and 2DGE data

244 were analysed using Progenesis Samespots software (v4.5.4325.32621; Nonlinear

245 Dynamics Ltd, UK). Two-way ANOVA was undertaken on micronuclei data, after

246 square root transformation, and comet assay data (as medians for each slide), with

247 LC50 values determined by probit analysis and survival curves and lethal time to

248 50% mortality (LT50) generated according to Kaplan-Meier and compared by log-

249 rank test. PCA ('prcomp' in R) and Cluster Analysis was performed for the six

250 biomarkers common to samples from days 4 and 14 (i.e. C_q for *hsp70*, *mt20* and

251 *mt10*; % tail DNA [buffer]; % tail DNA [Fpg]; MN/1000 cells). Proteomic results and

252 higher level responses were excluded from PCA due to pooling/non-paired samples,

253 but relationships between these parameters were examined by generating Pearson's

254 moment correlation coefficients.

255

256 **3. Results and discussion**

257

258 *3.1. Higher level responses*

259 Equimolar concentrations of PT in both ZnPT and NaPT induced similar mortality to

260 adult *M. galloprovincialis* after exposure for 14 d (Fig. S1), suggesting that PT$^-$ ions

261 are largely responsible for the effect. However, the fact that ZnPT was notably more

262 toxic than NaPT after 4-d exposure suggests the complex as a whole has a higher

263 acute toxicity, possibly due to its greater hydrophobicity and propensity to

264 accumulate than the PT$^-$ ion alone.

265

266 Control mussels showed increasing attachment with time, peaking at 100% for the

267 sea water control and $\sim$ 98% for the inorganic Zn treatment (Fig. 1A). In contrast,

268 exposure to both low and high concentrations of ZnPT significantly reduced

269 attachment at all time points compared to the control or inorganic Zn ($p < 0.0001$),

270 and never exceeded 5 % throughout the time course. This effect may be the result of

271 a generalised stress response that causes a reduction in the capacity for byssus

272 production (Babarro et al., 2008) or a specific chemical interaction effected between

273 ZnPT or PT$^-$ and byssus proteins. Regardless of the cause, these observations

274 suggest that ZnPT is an effective repellant in antifouling formulations for hard-fouling

275 organisms.

276

277 Clearance rate was also affected by ZnPT, with a reduction from > 1.75 L h^{-1} in the

278 control and inorganic Zn exposure to < 0.3 L h^{-1} at both concentrations of the

279 complex tested (Fig. 1B). The magnitude of this decrease is comparable to that

280 observed for mussels exposed to 18 - 56 µg L^{-1} Cu for 5 d (Al-Subiai et al., 2011), 32

281 mg L^{-1} methyl methanesulfonate for up to 7 d (Canty et al., 2009) and 56 µg L^{-1}

282 benzo(α)pyrene for 12 d (Di et al., 2011), and is in excess of that reported for 14-d

283 exposure to branched alkyl benzenes from crude oil (Scarlett et al., 2008) and 3-d

284 exposure to C60 fullerene nanoparticles (Al-Subiai et al., 2012). Reduced filtration in

285 mussels causes both decreased gas exchange at the gills and reduced food intake

286 (Bayne, 1976), and either of these parameters could have significant consequences

287 for the energy stores that result in weakened anoxia tolerance (SoS).

288

289 During the SoS test, mussels exposed to the control or inorganic Zn declined

290 steadily after 8 - 10 d (Fig. 1C), with similar LT50 values defining the two curves

291 (14.00 ± 0.58 and 14.00 ± 0.55, respectively). In contrast, mussels treated with either

292 concentration of ZnPT declined rapidly between 3 and 6 d and reached total

293 mortality earlier, with LT50 values for 0.2 and 2 µM ZnPT that were significantly

294 lower than the controls (6.00 ± 0.40 and 6.00 ± 0.34, respectively; $p < 0.0001$). Our

295 LT50 values are higher than those reported by Marcheselli et al. for *M.*

296 *galloprovincialis* exposed to 0.4 µM ZnPT for 7 d (Marcheselli et al., 2011), indicating

297 that mussels exposed to the higher concentration of ZnPT (2 µM) in the current

298 experiment survived longer under anoxic conditions. However, it must be borne in

299 mind that control, harbour mussels in the earlier study were defined by an LT50 of

300 10.70 d, suggesting that either these mussels were stressed before the exposures or

301 there is an inherent difference between the anoxia tolerance of *M. galloprovincialis*

302 collected for the two studies. The latter could be an artefact of the mussels' biological

303 situation, such as current reproductive status (Bignell et al, 2008). Significant

304 variation in LT50 values for mussels sampled from different sites and in different

305 seasons supports this assertion (Hellou and Law, 2003; Koukouzika and Dimitriadis,

306 2005) and until natural variability is fully characterised, SoS should be reported as

307 impact relative to control organisms. On this basis, mussels used here showed a

308 greater decrease (-62.50%) than those of Marcheselli et al. (2011) (-49.53%),

309 presumably because of the longer exposure to ZnPT employed in the present study.

310

311 *3.2. Genotoxicity in circulating haemocytes*

312

313 There was no significant effect of Fpg for any treatment and concurrent validation

314 with in vitro exposure to hydrogen peroxide (Dallas et al., 2013) showed positive

315 results. This confirmed that the Fpg enzyme had no significant effect on the

316 chemicals tested (data not shown); for brevity, therefore, only buffer-treated data are

317 shown and discussed. Concentrations of 2 µM ZnPT induced significantly elevated

318 DNA strand breaks in haemocytes at all time points compared with the control and

319 inorganic Zn ($p < 0.001$), with the lower concentration of ZnPT exhibiting elevated

320 strand breaks on day 14 only (Fig. 2). Control mussel haemocytes showed normal

321 levels of micronuclei (~3 per 1000 cells) whereas those treated with 2 µM inorganic

322 Zn exhibited a non-significant ($p > 0.05$) increase. In contrast, ZnPT caused a

323 concentration- and time-dependent increase in micronuclei (MN; $p < 0.0001$), with a

324 maximum of 22.42 MN per 1000 cells in the 2 µM ZnPT exposure on day 14 (Fig. 2).

325

326 These results provide independent confirmation that ZnPT is genotoxic to mussels,

327 as reported by Marcheselli et al. (2011) in gills and digestive gland using the TUNEL

328 assay. This is, however, the first report of ZnPT-induced sublethal genotoxicity in

329 mussel haemocytes, which does not appear to be caused by purine oxidation (based

330 on the enzyme-modified comet assay results). Similar results were obtained when

331 potential genotoxicity in haemocytes were determined following exposure of mussels

332 to nickel (Dallas et al., 2013). These studies suggest that mussel haemocytes might

333 have some inherent limitations to express metal- or organometallic-induced oxidative

334 damage to DNA. In this context, enzymatic repair of DNA oxidation induced by

335 chromium (VI) in mussel gill cells have suggested that DNA repair processes could

336 mask the oxidative damage and this could be influenced as a function of sampling

337 time (Emmanouil et al., 2006). Results from this and earlier studies (Dallas et al.,

338 2013) suggest that mussel haemocytes are able to maintain homoeostasis with

339 respect to DNA oxidation but further studies involving repeated sampling would be

340 required to shed light on the persistence of DNA oxidation. Furthermore, our results

341 suggest good correlations between induction of DNA strand breaks (as determined

342 by the comet assay) and cytogenetic damage (as determined by the MN assay). This

343 is in line with many in vivo and in vitro studies reported previously (Canty et al.,

344 2009; Dallas et al., 2013)., strengthening the case for the concomitant use of these

345 genotoxicity endpoints.

346

347 Although Nunes et al. (2015) studied oxidative stress in the freshwater fish,

348 *Gambusia holbrooki*, after exposure to ZnPT, to our knowledge the present

349 investigation is the first to examine such effects in a marine organism., with both

350 studies finding no increase in catalase or glutathione-s-transferase activity effected

351 by the chemical. The current comet assay results are also in agreement with in vitro

352 data from rat cardiomyocytes, where ZnPT reduced superoxide generation and

353 oxidative injury (as both LDH release and cell survival) after reperfusion (Kasi et al.,

354 2011). However, the mammalian literature is somewhat contradictory in that ZnPT

355 causes increased susceptibility to ROS-induced damage in rat thymocytes co-

356 exposed to hydrogen peroxide (Oyama et al. 2012). Furthermore, a study on human

357 skin cells reported upregulation of metallothionein genes, commonly associated with

358 oxidative stress (Lamore and Wondrak, 2011). The latter observation may be

359 explained by the known metal binding capacity of metallothionein proteins, i.e. the

360 effect may be induced by Zn itself rather than ZnPT. Further research to directly

361 quantify ROS in mussel cells (of different tissues) is recommended to elucidate the

362 mechanisms behind the genotoxic effects of ZnPT.

363

364 *3.3. Alterations in transcriptional expression of key genes*

365

366 Whilst many studies have been carried out to determine the toxicity of metals to

367 mussels (Lemoine et al., 2000; Banni et al., 2007; Al-Subiai et al., 2011; Dallas et al.,

368 2013; Varotto et al., 2013), there is limited information on their response to ZnPT or

369 other pyrithione compounds. Although the anti-dandruff characteristics of ZnPT may

370 result from indirect effects on skin cells (e.g. suppressed DNA synthesis; Imokawa et

371 al., 1983), in yeast the complex exhibits specific anti-fungal activity via iron starvation

372 or decreased iron-containing protein activity as a result of elevated intracellular Cu

373 (Yasokawa et al., 2010; Reeder et al., 2011b). There are also several reports of

374 genotoxicity and stress response induction (especially HSPs and increased p53

375 expression) in human skin cells exposed to ZnPT (Lamore and Wondrak, 2011;

376 Rudolf and Cervinka, 2011; Lamore et al., 2010). The molecular approaches used

377 here were designed to elucidate which - if any - of these potential mechanisms

378 contribute to ZnPT-induced toxicity in marine mussels.

379

380 In the present study, qPCR efficiencies were *hsp70* 1.750/1.798, *mt10* 1.791/1.830

381 and *mt20* 1.838/1.846 for gill/digestive gland of *M. galloprovincialis.* Significant

382 changes in expression were tissue-specific and only seen in digestive gland at day 4,

383 where all three target genes showed upregulation at the low ZnPT concentration but

384 only *hsp70* and *mt20* showed upregulation at the high concentration (Fig. 3); in gill,

385 upregulation of *hsp*70 and *mt20* occurred for 2 µM ZnPT at day 14 only (Fig. 3).

386 Temporal differences in *hsp70* and *mt20* expression observed between the gill and

387 digestive gland may be linked to time-dependent variations in the location of ZnPT

388 accumulation by mussels; for example, Marcheselli et al. (2010b) report increased

389 accumulation in digestive gland compared with gill of *M. galloprovincialis* after ≥ 2 d

390 exposure to 1.5 µM ZnPT. Regarding HSPs, both the present study and that of

391 Marcheselli et al.(2011) have identified these genes as components of the ZnPT

392 response in *M. galloprovincialis*. Furthermore, studies in human skin cells have

393 reported upregulation of HSPs and metalliothionein genes after ZnPT treatment,

394 suggesting that these genes and their toxic responses might be highly conserved

395 (Lamore and Wondrak, 2011; Lamore et al., 2010).

396

397 PCA and Cluster Analysis of the six common biomarkers showed that the first

398 component (PC1) explained 44.1 % and 59.0 % of total variance at day 4 and 14,

399 respectively (Fig. 4B and 4C), and was mostly related to *mt20* activity at day 4 but

400 with no defining biomarker at day 14. Treatment-specific clusters were evident at

401 both time points, but were more diffuse by day 14. However, the treatment-related

402 clusters were more clearly separated from each other at day 14, whereas there was

403 considerable overlap between treatments at day 4. Gene expression described more

404 variability within groups, while genotoxic parameters distinguished between

405 treatments. This suggests that either intra-individual variability is masking treatment-

406 specific effects on these genes, or that investigation of other genes (e.g. p53 and

407 those for other DNA repair proteins) may provide a better understanding of the

408 mechanistic aspects of the genotoxicity of ZnPT.

409

410 It is well accepted that the toxicity of a chemical or environmental stressor is a cell-

411 or tissue-specific phenomenon (Jha, 2008; 2004; Di et al., 2011). In the present

412 study, DNA damage using the comet assay was determined in the circulating

413 lymphocytes whereas the expression of genes were determined in different tissues.

414 While assessing the biological responses at the cellular and tissue level due to

415 technical and tissue-specific inherent limitations, it is not always possible to apply the

416 same assay across the biological samples obtained. For example, circulating

417 lymphocytes which could be obtained in small amounts from mussels are ideal for

418 the analysis of DNA damage using the comet assay whilst other tissues are ideal for

gene expression analyses. For the application of comet assay, it is a prerequisite to obtain a single cell suspension, which is not necessary for circulating haemocytes. On the other hand, application of the comet assay on solid tissues (e.g. digestive glands, gills etc.) require mechanical and enzymatic treatments, which could induce DNA damage in their own right (Jha, 2008). It is also important to point out that gene expression results could give different results in different tissues (Di et al., 2011). In addition, since different tissues have different turnover rates (cell cycle durations) and metabolic properties and the fact that contaminants could induce cell cycle stage specific changes for gene expression (Di et al., 2011), it is difficult to justify selection of a particular cell or tissue type for different biological assays. In this study, therefore, we used a range of cell types and the observed biological responses were analysed using PCA and Cluster analysis to obtain a holistic picture.

3.4. Alterations in protein expression

Digestive gland proteins were well-separated on the gel, with minimal streaking or smearing (Fig. S3). Only spots showing significant fold change (≥ 1.5 and $p < 0.05$) relative to both the sea water control and inorganic Zn exposure were considered as ZnPT-specific effects (5 spots and all on day 4, Table 1). Two such spots were positively identified by mass spectroscopy; specifically, spot *355* was significantly homologous to a predicted protein sequence for phosphoenolpyruvate carboxykinase (PEPCK) in barley (GenBank BAK02183.1), and spot *550* was a small HSP from *M. galloprovincialis* (GenBank AEP02968.1). Of the remaining spots, 1 could not be identified due to low protein content (*607*) and the other 2 had no matching sequences, possibly due to extensive post-translational modification.

Although several studies have examined PEPCK levels in mussels in response to physical stressors like temperature and salinity (Anestis et al., 2010; Lockwood and Somero, 2011; Tomanek and Zuzow, 2010), our investigation appears to be the first to demonstrate a negative impact on PEPCK by a contaminant in a model marine organism. If decreased PEPCK protein after 4-d ZnPT exposure corresponds to decreased PEPCK activity, this could compromise the ability of mussels to cope with anoxic conditions, as seen in the SoS assay. In contrast, Widdows et al. (1982) reported increased PEPCK activity in *M. galloprovincialis* after 140 d exposure to 30

453   µg L^{-1} water-accommodated hydrocarbons. The discrepancies between these

454   two responses may be attributed to a number of factors. Thus, firstly our study did

455   not measure enzyme activity but determined expression of protein, and post-

456   translational modification to activate or suppress PEPCK may result in differences

457   between expression and activity. Secondly, given that changes in PEPCK

458   expression appeared herein at day 4 but were not evident by day 14, it is possible

459   that temporal shifts in expression-activity patterns may have occurred in our

460   experiment, suggesting we are observing baseline variation rather than biological

461   effect. Thirdly, responses are often contaminant- or tissue-specific; for instance,

462   kinetic studies of glycolytic enzymes in *M. edulis* have shown significant variation in

463   activity between adductor muscle (catabolic tissue) and mantle (anabolic tissue)

464   (Churchill and Livingstone, 1989).

465

466

467   *3.5. Environmental risk assessment and future work*

468

469   Given the detrimental effects of ZnPT on *M. galloprovincialis* at all levels of biological

470   organization, it is perhaps surprising that mussels are found in abundance in many

471   marinas where the biocide has the potential to reach levels considerably higher than

472   in pristine waters through leaching from boat hulls and from spent antifouling waste

473   (Holmes and Turner, 2009). It is possible, however, that mussels from environments

474   impacted by boating have adapted to elevated ZnPT concentrations through some

475   resistance mechanism or compensatory response. For instance, it is known that *M.*

476   *edulis* exhibit a high adaptive capacity to the impact of a variety of metallic and

477   lipophilic contaminants (Bakhmet et al., 2009). To this end, a useful extension to the

478   current work would be a direct comparison of the condition and response of mussels

479   from a reference site with those from a region where boat storage or maintenance is

480   significant.

481

482   There are several issues that need to be addressed before the general

483   environmental risk from ZnPT can be more accurately estimated. Regarding

484   environmental concentrations, there exist only limited data, with a reported range in

485   the aqueous phase (as PT) between about 2 and 100 nM for marinas and navigation

486   channels (Mackie et al., 2004; Madsen et al., 2000). While this range is lower than

concentrations employed in the present study, environmental concentrations may be elevated locally by point sources and more generally in turbid or shaded sea water (e.g. under pontoons and boats) where the biocide is more persistent (Marcheselli et al., 2010b). ZnPT induces considerable physiological and behavioural effects in mussels in addition to its genotoxicity, and emphasizing the need for integrated studies that examine a wide range of effects. Also, as mussels appear to be more tolerant of ZnPT than crustaceans and polychaetes (Bao et al., 2008; Mochida et al., 2006; Marcheselli et al., 2010b), the wide-ranging effects demonstrated here also suggest a greater potential risk to other marine biota. Studying the effects of this compound in a wider range of aquatic invertebrate species is, therefore, essential. Indirectly, ZnPT has additional impacts through transchelation of PT^- ions with Cu^{2+} (the primary ion formed during the ablation of most contemporary antifouling formulations) (Holmes and Turner, 2009; Grunnet and Dahllof, 2005). Studies suggest that CuPT is more toxic than its Zn counterpart (Bao et al., 2011) and that mixtures exhibit synergistic effects (Bao et al., 2014), but more experimental studies would be required to explore these effects in a broader context.

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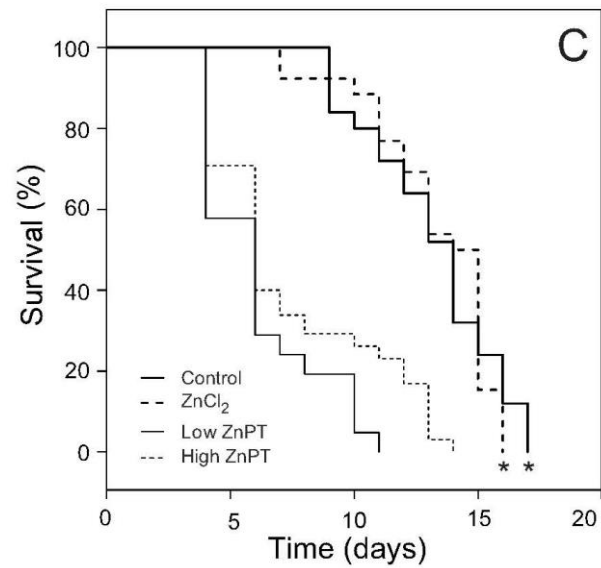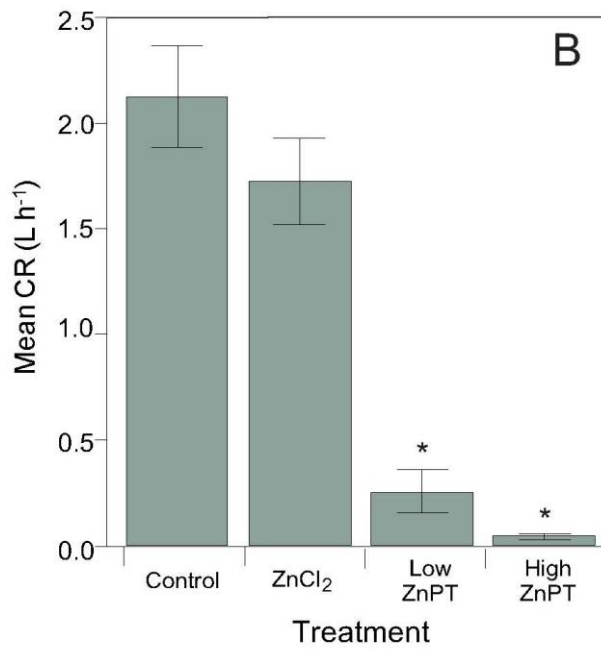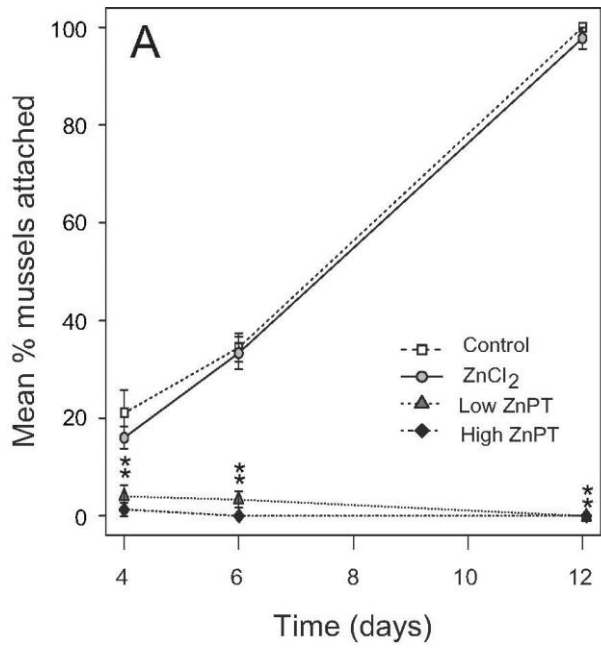
Figure 1: Higher level responses of *M. galloprovincialis* exposed to inorganic Zn or

ZnPT. (A) Percentage of mussels showing attachment during 14 d exposure; (B)

clearance rate (CR) after 14 d exposure; (C) survival (SoS; $n = 25$) in anoxic

conditions during 20 d after exposure. Error bars are ± one SE and asterisks indicate

significant differences ($p < 0.05$) from both sea water and inorganic Zn treatments.

Figure 2: Genotoxicity in *M. galloprovincialis* haemocytes after exposure to inorganic Zn or ZnPT for (A) 0, (B) 4, (C) 7 or (D) 14 d, and as determined by the enzyme-modified comet assay (% tail DNA; bars) and micronuclei assay (per 1000 cells; black circles). Error bars are ± one SE and asterisks indicate significant differences ($p < 0.05$) from both sea water and inorganic Zn treatments on the same day, whereas daggers indicate differences from day 0.

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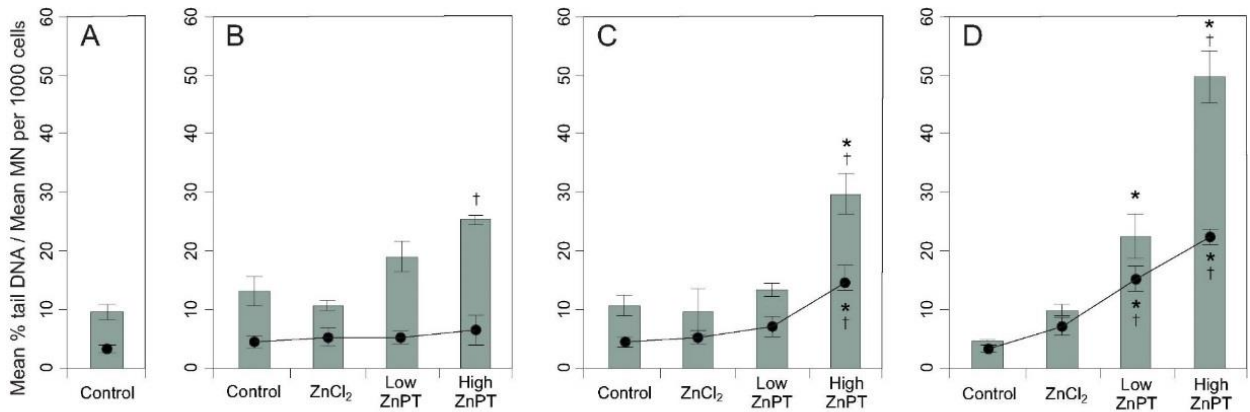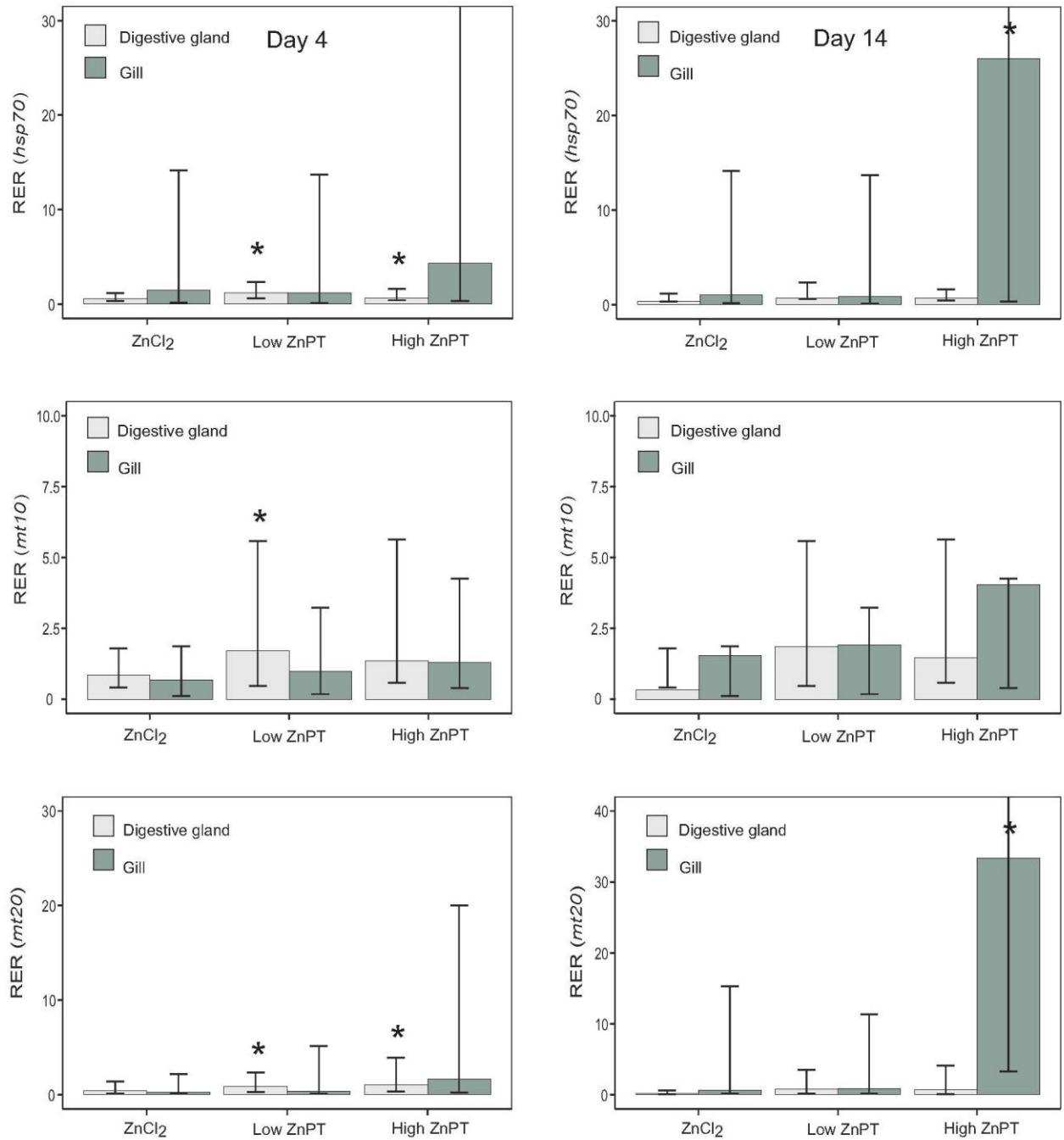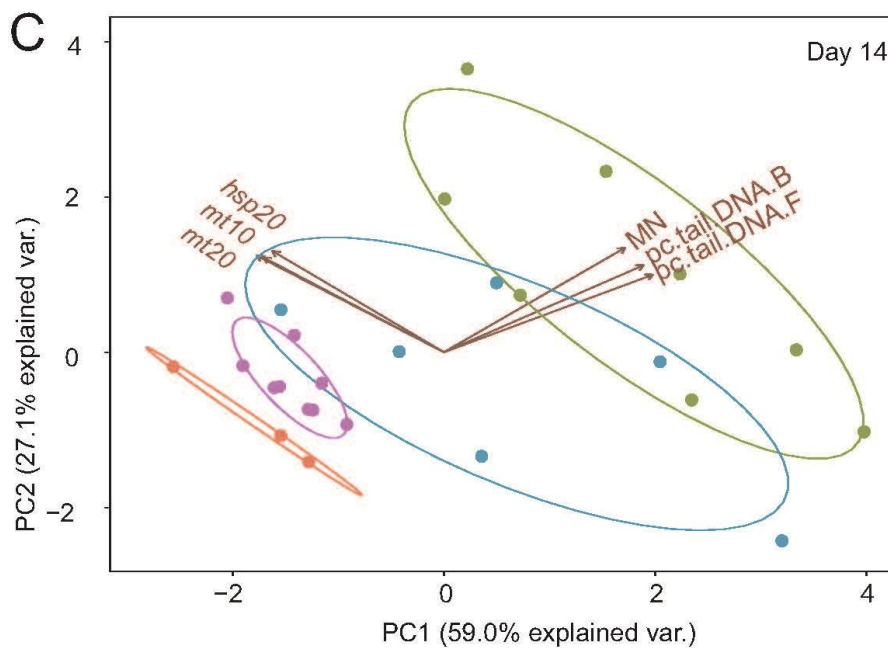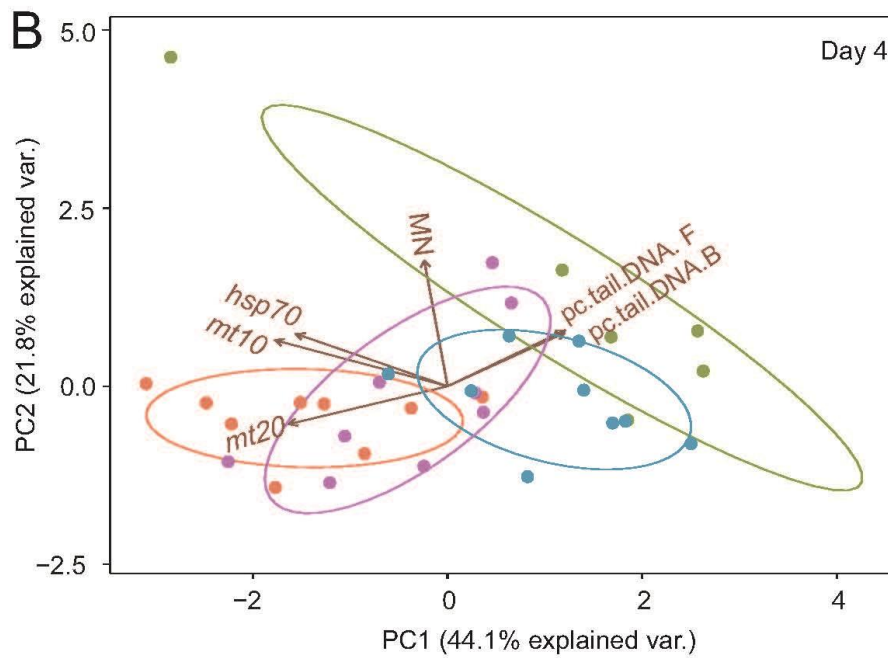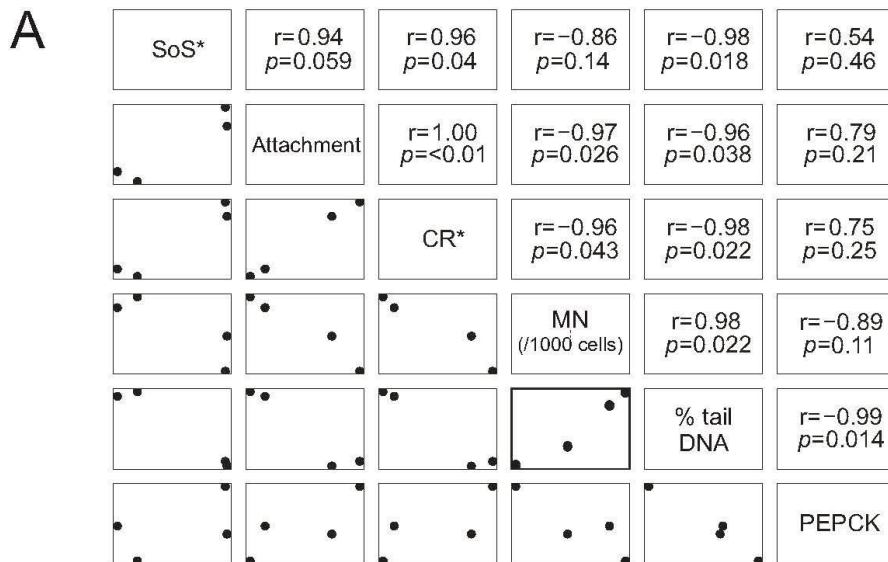816 Figure 3: Relative expression ratios (RER) of three genes (*hsp70*, *mt10* and *mt20*) in

817 digestive gland and gill tissue of *M. galloprovincialis* after exposure to inorganic Zn or

818 ZnPT. Data are normalised for two reference genes (*atub* and *ef1*) and the sea water

819 control treatment and are shown with ± 95% confidence intervals. Values above 1

820 indicate upregulation and those below downregulation. Significant differences

821 (PFRRT, $p < 0.05$) from the corresponding tissue control at day 0 are indicated by

822 asterisks.

A

	SoS*	r=0.94 p=0.059	r=0.96 p=0.04	r=−0.86 p=0.14	r=−0.98 p=0.018	r=0.54 p=0.46
Attachment			r=1.00 p=<0.01	r=−0.97 p=0.026	r=−0.96 p=0.038	r=0.79 p=0.21
CR*				r=−0.96 p=0.043	r=−0.98 p=0.022	r=0.75 p=0.25
MN (/1000 cells)					r=0.98 p=0.022	r=−0.89 p=0.11
% tail DNA						r=−0.99 p=0.014
PEPCK						

B Day 4

PC2 (21.8% explained var.) vs PC1 (44.1% explained var.)

C Day 14

PC2 (27.1% explained var.) vs PC1 (59.0% explained var.)

Control   High ZnPT   Low ZnPT   Inorganic Zn

823

824   Figure 4: Links between responses at different levels of biological organisation in *M.*
825   *galloprovincialis* across all treatments. (A) Correlation matrix showing relationships
826   between higher level responses, genotoxicity and expression of PEPCK. Data were
827   averaged for each treatment and parameters were measured from day 14 onwards
828   except where noted with an asterisk, with lower left panels showing data plots and
829   upper diagonal panels showing corresponding *r* and *p* values. (B) and (C) Principal
830   component analysis of 6 genotoxicity and gene expression parameters (ΔC_q values
831   for *hsp70*, *mt20* and *mt10*, % tail DNA [buffer], % tail DNA [Fpg] and MN/1000 cells)
832   for 4 and 14 d, respectively.

833 Table 1: Differentially expressed proteins in the digestive gland of *M.*

834 *galloprovincialis* exposed to 0.2 or 2 µM ZnPT for 4 d. Non-significant fold changes

835 ($p > 0.05$) have been omitted.

836

Spot id	protein id	Fold change relative to sea water control		Fold change relative to inorganic Zn	
		0.2 µM	2 µM	0.2 µM	2 µM
239	unidentified	+2.10	+3.91		+1.89
355	phosphoenolpyruvate carboxykinase		-2.91		-1.94
412	unidentified	-1.68	-2.04	-1.73	-2.10
550	small heat shock 24.1	+1.78		+1.90	
607	unidentified		+1.59		+2.00