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3 An integrated approach to assess the impacts
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5 organisation in marine mussels

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Abstract

The mechanisms of sublethal toxicity of the antifouling biocide, zinc pyrithione (ZnPT), have not been well-studied. This investigation demonstrates that 14-d sublethal exposure to ZnPT (0.2 or 2 μ M, alongside inorganic Zn and sea water controls) is genotoxic to mussel haemocytes but suggests that this is not caused by oxidative DNA damage as no significant induction of oxidised purines was detected by Fpg-modified comet assay. More ecologically relevant endpoints, including decreased clearance rate (CR), cessation of attachment and decreased tolerance of stress on stress (SoS), also showed significant response to ZnPT exposure. Our integrated approach was underpinned by molecular analyses (qRT-PCR of stress-related genes, 2D gel electrophoresis of proteins) that indicated ZnPT causes a decrease in phosphoenolpyruvate carboxykinase (PEPCK) expression in mussel digestive glands, and that metallothionein genes are upregulated; PEPCK downregulation suggests that altered energy metabolism may also be related to the effects of ZnPT. Significant relationships were found between % tail DNA (comet assay) and all higher level responses (CR, attachment, SoS) in addition to PEPCK expression. Principal component analyses suggested that expression of selected genes described more variability within groups whereas % tail DNA reflected different ZnPT concentrations.

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

1. Introduction

Zinc pyrithione (ZnPT, $317.70 \text{ g mol}^{-1}$), an organic complex with two pyrithione ligands chelated to Zn^{2+} , is used as a booster biocide in many copper-based antifouling paints (AFPs). Its role in such products is to increase broad-spectrum efficacy via antifungal action (Thomas et al., 2000), which also makes it ideal for use in outdoor paints for resistance to mould. ZnPT also has a variety of household and medical uses (for example, it is used in the treatment of psoriasis and is the only active ingredient in medicated anti-dandruff shampoos; Reeder et al., 2011a) resulting in multiple, additional sources from which ZnPT can enter the environment.

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 Dahllöf, 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

changes to higher level, more ecologically relevant responses, including clearance rate [CR] and attachment.. Where appropriate, observed biological responses were analysed using Principal Component Analysis (PCA) and Cluster analyses which effectively integrate multidimensional biomarker data into a more readily interpretable two dimensional format.

2. Methods and experimental design

2.1. Reagents and mussel collection and maintenance

Adult *M. galloprovincialis* (48.03 ± 2.43 mm) were collected from Trebarwith Strand ($50^{\circ} 38' 40''$ N, $4^{\circ} 45' 44''$ W), a reference site remote from significant boating activity, and maintained in the laboratory as previously described (Dallas et al., 2013; Banni et al., 2017). Sea water used in the exposures was sourced from Plymouth Sound, stored on site and filtered on line ($< 10 \mu\text{m}$). Measured Cu concentrations ($< 10 \mu\text{g L}^{-1}$) were considerably less than added Zn concentrations (see below), ensuring that significant Cu(II)-Zn transchelation of the pyrrithione ion (PT^{-}) was unlikely (Holmes and Turner, 2009).

Unless otherwise stated, all reagents used in the exposures and for sample processing and analysis were purchased from Sigma-Aldrich Ltd (Gillingham, UK). Ultrapure water was obtained using a Milli-Q RG (Merck Millipore, Billerica, MA, USA) or Elga Purelab Option system (Elga LabWater, Marlow, UK) and had a resistivity of $> 18.2 \text{ M}\Omega \text{ cm}^{-1}$ at 25°C .

2.2. Exposure scenario and determination of higher level responses

Concentrations used were based on acute range-finding exposures that compared ZnPT to ZnCl_2 and the pyrrithione ion (as NaPT) at equimolar concentrations of Zn or PT and between 0.20 and $16.20 \mu\text{M}$. These produced a 96-h LC_{50} for *M. galloprovincialis* of $14.50 \pm 1.45 \mu\text{M}$ for PT as ZnPT (50 % mortality not achieved for the other treatments), 7-d LC_{50} s of $24.58 \pm 1.57 \mu\text{M}$ for PT as NaPT and $8.94 \pm 1.30 \mu\text{M}$ for PT as ZnPT, and 14-d LC_{50} s of 2.54 ± 1.32 (as NaPT) and $2.97 \pm 1.36 \mu\text{M}$

(as ZnPT), with 50 % mortality not achieved for inorganic Zn (Fig. S1). Degradation of ZnPT into inorganic Zn was also assessed under our experimental conditions by retention and subsequent 3 ml min⁻¹ methanol elution of Zn on conditioned octadecyl silane (C18) columns (15 mL; Chromabond, Macherey Nagel GmbH, Düren, Germany) (Holmes and Turner, 2009) (Fig. S2 A, S2 B).

Depurated (28 d) and acclimatized (48 h) *M. galloprovincialis* ($n = 16$) were exposed for 14 d and under a 12:12 h photoperiod to 0.2 µM ZnPT (low), 2 µM ZnPT (high), 2 µM ZnCl₂ (inorganic) and no added Zn (control) in triplicate and in pre-washed plastic tanks containing 20 L of sea water (salinity 31.82 ± 0.34 , pH 8.16 ± 0.21 , dissolved oxygen 90.02 ± 2.84 % and temperature 15.88 ± 0.52 °C). ZnPT concentrations were either chronic (< 10% of 14-d LC50) or acute (67% of 14-d LC50) as determined by the range-finding experiment described above. As the degradation of ZnPT approached 80% at 48 h (Fig. S2 C), full water changes were performed every 2 d with replenishment of ZnPT. Mussels were fed 2 h prior to water changes with *Isochrysis galbana* ($\sim 1.0^5 \times 10^6$ cells mL⁻¹), and during changes on days 4, 6 and 12 (i.e. those closest to the sampling days), mussel attachment was recorded where adherence to the interior surface or to other mussels was evident when the emptied tank was tilted 45°. Clearance rate was determined for nine mussels per treatment (and three from each replicate) on day 14, as previously described (Devos et al., 2015), and after this time period the stress on stress (SoS) test was performed in continuous anoxic conditions for a further 20 d (Viarengo et al., 1995). Thus, tanks were drained and remaining mussels blotted dry before being transferred to open petri dishes where they were maintained without water but in a humid environment at 15 °C. Mortality, defined as persistent opening of the valves and failure to respond when tapped, was recorded daily and subsequently used to calculate LT50 values (median lethal time for 50 % mortality).

2.3. Genotoxicity in circulating haemocytes

After 0, 4, 7 and 14 d, haemolymph was extracted from nine mussels per treatment, and stored on ice pending assay, whilst 5 mm² sections of gill and digestive gland were removed and stored at -80 °C pending analysis. The enzyme-modified comet

assay was performed using the haemocytes according to methods previously validated using H₂O₂ as a positive control (Dallas et al., 2013), with two slides per sample: one control containing buffer only (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.20 mg mL⁻¹ BSA, pH 8.0) and one with the bacterial enzyme, formamidopyrimidine glycosylase (Fpg), to detect oxidised purine and pyrimidine bases. Prior to the performance of the comet assay, cell viability was checked using Eosin Y stain and was found to be > 80 % for all treatments (data not shown). The micronucleus (MN) assay was performed on haemocytes as also described previously (Dallas et al., 2013) with 1000 cells scored on each coded and randomized slide.

2.4. Alterations in transcriptional expression of candidate genes

Total RNA was isolated from gill and digestive gland using the RNeasy mini kit (Qiagen Ltd, UK). Two µg from samples with OD₂₆₀:OD₂₈₀ > 1.95 and crisp bands was used for reverse transcription with M-MLV reverse transcriptase and random primers (Promega Corporation, USA). Real-time- (q-)PCR was performed on samples in duplicate (Applied Biosystems Step-One Plus RT-PCR system, StepOne Software v2.2.2) in reactions containing 7.5 µL SYBR Green Jumpstart Taq ReadyMix, 0.2 µM forward and reverse primers (as detailed in Table S1), 4.44 µL ultrapure water and 3 µL template cDNA. Initial denaturation was at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, plus a melt curve to verify PCR-product purity. Relative expression ratio (RER) of 3 genes (*mt10*, *mt20* and *hsp70*) was quantified using REST 2009 (v2.0.13; Qiagen Ltd) from PCR efficiency (measured using LinRegPCR) and threshold cycle (C_q), relative to the reference genes *atub* (alpha tubulin) and *ef1* (elongation factor 1) with control samples as calibrators (Pfaffl et al., 2002; Ramakers et al., 2003). Both reference genes showed low variability across samples (C_q *atub* = 20.45 ± 1.26, *ef1* = 18.01 ± 1.01).

2.5. Alterations in protein expression

Digestive glands from three mussels per exposure tank were pooled and homogenised with four parts homogenisation buffer (10 mM Tris-HCl, 0.5 M sucrose,

0.15 M KCl, 1 mM EDTA, 1 mM PMSF) to provide sufficient protein and reduce inter-individual variation (Karp and Lilley, 2005). Samples were prepared for 2D gel electrophoresis (2DGE) using methods of Schmidt et al. (2003). Briefly, total protein was extracted with TCA-acetone, rehydrated and loaded onto a 7 cm IPG strip overnight (pH 3-10; GE Healthcare). Iso-electric focusing was performed using a Protean IEF cell (Bio-Rad) at 300 V for 3 h, 1000 V for 6 h and 8000 V for 3 h, followed by 8000 V for 20 kVh and hold at 500 V. Strips were equilibrated firstly with 2% DTT and secondly with 2.5% iodoacetamide. After transfer of strips to 12% polyacrylamide gels (Mini-PROTEAN TGX, Bio-Rad), electrophoresis was carried 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 M SDS). Gels were stained with 0.2% Coomassie brilliant blue (R-250 in 30% methanol, 10% acetic acid) for 1 h, destained overnight (40% methanol, 10% acetic acid) and visualised using a Gel Doc XR+ (Bio-Rad).

Spots of interest (i.e. those not evident in the control) were excised using sterile razor blades, dried and sent for identification (Mass Spectrometry Unit, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa). After in-gel tryptic digestion, extracted peptides were loaded onto a R2 micro column (RP-C18 equivalent), desalted, concentrated, and eluted directly onto a MALDI plate using α -cyano-4-hydroxycinnamic acid as the matrix solution in 50% acetonitrile and 5% formic acid. Mass spectra were acquired in positive reflectron MS and MS/MS modes using a 4800plus MALDI TOF/TOF analyser with an exclusion list of trypsin autolysis peaks (842.51, 1045.56, 2211.11 and 2225). Resulting spectra were analysed in combined mode using the Mascot search engine and NCBI database (restricted to 50 ppm peptide mass tolerance with no taxonomy restrictions).

2.6. Statistical analysis

Statistics were performed in R (i386, v2.15.2; www.R-project.org) and 2DGE data were analysed using Progenesis SameSpots software (v4.5.4325.32621; Nonlinear Dynamics Ltd, UK). Two-way ANOVA was undertaken on micronuclei data, after square root transformation, and comet assay data (as medians for each slide), with LC50 values determined by probit analysis and survival curves and lethal time to 50% mortality (LT50) generated according to Kaplan-Meier and compared by log-

rank test. PCA ('prcomp' in R) and Cluster Analysis was performed for the six biomarkers common to samples from days 4 and 14 (i.e. C_q for *hsp70*, *mt20* and *mt10*; % tail DNA [buffer]; % tail DNA [Fpg]; MN/1000 cells). Proteomic results and higher level responses were excluded from PCA due to pooling/non-paired samples, but relationships between these parameters were examined by generating Pearson's moment correlation coefficients.

3. Results and discussion

3.1. Higher level responses

Equimolar concentrations of PT in both ZnPT and NaPT induced similar mortality to adult *M. galloprovincialis* after exposure for 14 d (Fig. S1), suggesting that PT^- ions are largely responsible for the effect. However, the fact that ZnPT was notably more toxic than NaPT after 4-d exposure suggests the complex as a whole has a higher acute toxicity, possibly due to its greater hydrophobicity and propensity to accumulate than the PT^- ion alone.

Control mussels showed increasing attachment with time, peaking at 100% for the sea water control and ~ 98% for the inorganic Zn treatment (Fig. 1A). In contrast, exposure to both low and high concentrations of ZnPT significantly reduced attachment at all time points compared to the control or inorganic Zn ($p < 0.0001$), and never exceeded 5 % throughout the time course. This effect may be the result of a generalised stress response that causes a reduction in the capacity for byssus production (Babarro et al., 2008) or a specific chemical interaction effected between ZnPT or PT^- and byssus proteins. Regardless of the cause, these observations suggest that ZnPT is an effective repellant in antifouling formulations for hard-fouling organisms.

Clearance rate was also affected by ZnPT, with a reduction from $> 1.75 \text{ L h}^{-1}$ in the control and inorganic Zn exposure to $< 0.3 \text{ L h}^{-1}$ at both concentrations of the complex tested (Fig. 1B). The magnitude of this decrease is comparable to that observed for mussels exposed to $18 - 56 \mu\text{g L}^{-1}$ Cu for 5 d (Al-Subiai et al., 2011), 32 mg L^{-1} methyl methanesulfonate for up to 7 d (Canty et al., 2009) and $56 \mu\text{g L}^{-1}$ benzo(α)pyrene for 12 d (Di et al., 2011), and is in excess of that reported for 14-d

exposure to branched alkyl benzenes from crude oil (Scarlett et al., 2008) and 3-d exposure to C60 fullerene nanoparticles (Al-Subiai et al., 2012). Reduced filtration in mussels causes both decreased gas exchange at the gills and reduced food intake (Bayne, 1976), and either of these parameters could have significant consequences for the energy stores that result in weakened anoxia tolerance (SoS).

During the SoS test, mussels exposed to the control or inorganic Zn declined steadily after 8 - 10 d (Fig. 1C), with similar LT50 values defining the two curves (14.00 ± 0.58 and 14.00 ± 0.55 , respectively). In contrast, mussels treated with either concentration of ZnPT declined rapidly between 3 and 6 d and reached total mortality earlier, with LT50 values for 0.2 and 2 μ M ZnPT that were significantly lower than the controls (6.00 ± 0.40 and 6.00 ± 0.34 , respectively; $p < 0.0001$). Our LT50 values are higher than those reported by Marcheselli et al. for *M. galloprovincialis* exposed to 0.4 μ M ZnPT for 7 d (Marcheselli et al., 2011), indicating that mussels exposed to the higher concentration of ZnPT (2 μ M) in the current experiment survived longer under anoxic conditions. However, it must be borne in mind that control, harbour mussels in the earlier study were defined by an LT50 of 10.70 d, suggesting that either these mussels were stressed before the exposures or there is an inherent difference between the anoxia tolerance of *M. galloprovincialis* collected for the two studies. The latter could be an artefact of the mussels' biological situation, such as current reproductive status (Bignell et al, 2008). Significant variation in LT50 values for mussels sampled from different sites and in different seasons supports this assertion (Hellou and Law, 2003; Koukouzika and Dimitriadis, 2005) and until natural variability is fully characterised, SoS should be reported as impact relative to control organisms. On this basis, mussels used here showed a greater decrease (-62.50%) than those of Marcheselli et al. (2011) (-49.53%), presumably because of the longer exposure to ZnPT employed in the present study.

3.2. Genotoxicity in circulating haemocytes

There was no significant effect of Fpg for any treatment and concurrent validation with in vitro exposure to hydrogen peroxide (Dallas et al., 2013) showed positive results. This confirmed that the Fpg enzyme had no significant effect on the chemicals tested (data not shown); for brevity, therefore, only buffer-treated data are

shown and discussed. Concentrations of 2 μ M ZnPT induced significantly elevated DNA strand breaks in haemocytes at all time points compared with the control and inorganic Zn ($p < 0.001$), with the lower concentration of ZnPT exhibiting elevated strand breaks on day 14 only (Fig. 2). Control mussel haemocytes showed normal levels of micronuclei (~ 3 per 1000 cells) whereas those treated with 2 μ M inorganic Zn exhibited a non-significant ($p > 0.05$) increase. In contrast, ZnPT caused a concentration- and time-dependent increase in micronuclei (MN; $p < 0.0001$), with a maximum of 22.42 MN per 1000 cells in the 2 μ M ZnPT exposure on day 14 (Fig. 2).

These results provide independent confirmation that ZnPT is genotoxic to mussels, as reported by Marcheselli et al. (2011) in gills and digestive gland using the TUNEL assay. This is, however, the first report of ZnPT-induced sublethal genotoxicity in mussel haemocytes, which does not appear to be caused by purine oxidation (based on the enzyme-modified comet assay results). Similar results were obtained when potential genotoxicity in haemocytes were determined following exposure of mussels to nickel (Dallas et al., 2013). These studies suggest that mussel haemocytes might have some inherent limitations to express metal- or organometallic-induced oxidative damage to DNA. In this context, enzymatic repair of DNA oxidation induced by chromium (VI) in mussel gill cells have suggested that DNA repair processes could mask the oxidative damage and this could be influenced as a function of sampling time (Emmanouil et al., 2006). Results from this and earlier studies (Dallas et al., 2013) suggest that mussel haemocytes are able to maintain homeostasis with respect to DNA oxidation but further studies involving repeated sampling would be required to shed light on the persistence of DNA oxidation. Furthermore, our results suggest good correlations between induction of DNA strand breaks (as determined by the comet assay) and cytogenetic damage (as determined by the MN assay). This is in line with many in vivo and in vitro studies reported previously (Canty et al., 2009; Dallas et al., 2013)., strengthening the case for the concomitant use of these genotoxicity endpoints.

Although Nunes et al. (2015) studied oxidative stress in the freshwater fish, *Gambusia holbrooki*, after exposure to ZnPT, to our knowledge the present investigation is the first to examine such effects in a marine organism., with both studies finding no increase in catalase or glutathione-s-transferase activity effected

by the chemical. The current comet assay results are also in agreement with in vitro data from rat cardiomyocytes, where ZnPT reduced superoxide generation and oxidative injury (as both LDH release and cell survival) after reperfusion (Kasi et al., 2011). However, the mammalian literature is somewhat contradictory in that ZnPT causes increased susceptibility to ROS-induced damage in rat thymocytes co-exposed to hydrogen peroxide (Oyama et al. 2012). Furthermore, a study on human skin cells reported upregulation of metallothionein genes, commonly associated with oxidative stress (Lamore and Wondrak, 2011). The latter observation may be explained by the known metal binding capacity of metallothionein proteins, i.e. the effect may be induced by Zn itself rather than ZnPT. Further research to directly quantify ROS in mussel cells (of different tissues) is recommended to elucidate the mechanisms behind the genotoxic effects of ZnPT.

3.3. Alterations in transcriptional expression of key genes

Whilst many studies have been carried out to determine the toxicity of metals to mussels (Lemoine et al., 2000; Banni et al., 2007; Al-Subiai et al., 2011; Dallas et al., 2013; Varotto et al., 2013), there is limited information on their response to ZnPT or other pyrithione compounds. Although the anti-dandruff characteristics of ZnPT may result from indirect effects on skin cells (e.g. suppressed DNA synthesis; Imokawa et al., 1983), in yeast the complex exhibits specific anti-fungal activity via iron starvation or decreased iron-containing protein activity as a result of elevated intracellular Cu (Yasokawa et al., 2010; Reeder et al., 2011b). There are also several reports of genotoxicity and stress response induction (especially HSPs and increased p53 expression) in human skin cells exposed to ZnPT (Lamore and Wondrak, 2011; Rudolf and Cervinka, 2011; Lamore et al., 2010). The molecular approaches used here were designed to elucidate which - if any - of these potential mechanisms contribute to ZnPT-induced toxicity in marine mussels.

In the present study, qPCR efficiencies were *hsp70* 1.750/1.798, *mt10* 1.791/1.830 and *mt20* 1.838/1.846 for gill/digestive gland of *M. galloprovincialis*. Significant changes in expression were tissue-specific and only seen in digestive gland at day 4, where all three target genes showed upregulation at the low ZnPT concentration but only *hsp70* and *mt20* showed upregulation at the high concentration (Fig. 3); in gill,

upregulation of *hsp70* and *mt20* occurred for 2 μ M ZnPT at day 14 only (Fig. 3). Temporal differences in *hsp70* and *mt20* expression observed between the gill and digestive gland may be linked to time-dependent variations in the location of ZnPT accumulation by mussels; for example, Marcheselli et al. (2010b) report increased accumulation in digestive gland compared with gill of *M. galloprovincialis* after ≥ 2 d exposure to 1.5 μ M ZnPT. Regarding HSPs, both the present study and that of Marcheselli et al. (2011) have identified these genes as components of the ZnPT response in *M. galloprovincialis*. Furthermore, studies in human skin cells have reported upregulation of HSPs and metallothionein genes after ZnPT treatment, suggesting that these genes and their toxic responses might be highly conserved (Lamore and Wondrak, 2011; Lamore et al., 2010).

PCA and Cluster Analysis of the six common biomarkers showed that the first component (PC1) explained 44.1 % and 59.0 % of total variance at day 4 and 14, respectively (Fig. 4B and 4C), and was mostly related to *mt20* activity at day 4 but with no defining biomarker at day 14. Treatment-specific clusters were evident at both time points, but were more diffuse by day 14. However, the treatment-related clusters were more clearly separated from each other at day 14, whereas there was considerable overlap between treatments at day 4. Gene expression described more variability within groups, while genotoxic parameters distinguished between treatments. This suggests that either intra-individual variability is masking treatment-specific effects on these genes, or that investigation of other genes (e.g. p53 and those for other DNA repair proteins) may provide a better understanding of the mechanistic aspects of the genotoxicity of ZnPT.

It is well accepted that the toxicity of a chemical or environmental stressor is a cell- or tissue-specific phenomenon (Jha, 2008; 2004; Di et al., 2011). In the present study, DNA damage using the comet assay was determined in the circulating lymphocytes whereas the expression of genes were determined in different tissues. While assessing the biological responses at the cellular and tissue level due to technical and tissue-specific inherent limitations, it is not always possible to apply the same assay across the biological samples obtained. For example, circulating lymphocytes which could be obtained in small amounts from mussels are ideal for 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

$\mu\text{g L}^{-1}$ water-accommodated hydrocarbons. The discrepancies between these two responses may be attributed to a number of factors. Thus, firstly our study did not measure enzyme activity but determined expression of protein, and post-translational modification to activate or suppress PEPCK may result in differences between expression and activity. Secondly, given that changes in PEPCK expression appeared herein at day 4 but were not evident by day 14, it is possible that temporal shifts in expression-activity patterns may have occurred in our experiment, suggesting we are observing baseline variation rather than biological effect. Thirdly, responses are often contaminant- or tissue-specific; for instance, kinetic studies of glycolytic enzymes in *M. edulis* have shown significant variation in activity between adductor muscle (catabolic tissue) and mantle (anabolic tissue) (Churchill and Livingstone, 1989).

3.5. Environmental risk assessment and future work

Given the detrimental effects of ZnPT on *M. galloprovincialis* at all levels of biological organization, it is perhaps surprising that mussels are found in abundance in many marinas where the biocide has the potential to reach levels considerably higher than in pristine waters through leaching from boat hulls and from spent antifouling waste (Holmes and Turner, 2009). It is possible, however, that mussels from environments impacted by boating have adapted to elevated ZnPT concentrations through some resistance mechanism or compensatory response. For instance, it is known that *M. edulis* exhibit a high adaptive capacity to the impact of a variety of metallic and lipophilic contaminants (Bakhmet et al., 2009). To this end, a useful extension to the current work would be a direct comparison of the condition and response of mussels from a reference site with those from a region where boat storage or maintenance is significant.

There are several issues that need to be addressed before the general environmental risk from ZnPT can be more accurately estimated. Regarding environmental concentrations, there exist only limited data, with a reported range in the aqueous phase (as PT) between about 2 and 100 nM for marinas and navigation 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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References

- Al-Subiai, S. N.; Moody, A. J.; Mustafa, S. A.; Jha, A. N. A multiple biomarker approach to investigate the effects of copper on the marine bivalve mollusc, *Mytilus edulis*. *Ecotoxicology and Environmental Safety* 2011, 74, 1913–20.
- Al-Subiai, S. N.; Arlt, V. M.; Frickers, P. E.; Readman, J. W.; Stolpe, B.; Lead, J. R.; Moody, A. J.; Jha, A. N. Merging nano-genotoxicology with eco-genotoxicology: An integrated approach to determine interactive genotoxic and sub-lethal toxic effects of C-60 fullerenes and fluoranthene in marine mussels, *Mytilus sp.* *Mutation Research-Genetic Toxicology and Environmental Mutagenesis* 2012, 745, 92–103.

521
522 Anestis, A.; Pörtner, H. O.; Karagiannis, D.; Angelidis, P.; Staikou, A.; Michaelidis, B.
523 Response of *Mytilus galloprovincialis* (L.) to increasing seawater temperature and to
524 martellosis: Metabolic and physiological parameters. Comparative Biochemistry and
525 Physiology Part A: Molecular and Integrative Physiology 2010b, 156, 57–66.
526
527 Babarro, J. M.; Fernández Reiriz, M. J.; Labarta, U. Secretion of byssal threads and
528 attachment strength of *Mytilus galloprovincialis*: the influence of size and food avail-
529 ability. Journal of the Marine Biological Association of the United Kingdom 2008, 88,
530 783–791.
531
532 Bakhmet, I.N., Fokina, N.N., Nefedova, Z.A., Nemova, N.N. Physiological–
533 biochemical properties of blue mussel *Mytilus edulis* adaptation to oil contamination.
534 Environmental Monitoring and Assessment 2009, 155, 581–591.
535
536 Bao, V. W. W.; Leung, K. M. Y.; Kwok, K. W. H.; Zhang, A. Q.; Lui, G. C. S.
537 Synergistic toxic effects of zinc pyrithione and copper to three marine species:
538 Implications on setting appropriate water quality criteria. Marine Pollution Bulletin
539 2008, 57, 616–623.
540
541 Bao, V. W. W.; Leung, K. M. Y.; Qiu, J.-W.; Lam, M. H. W. Acute toxicities of five
542 commonly used antifouling booster biocides to selected subtropical and
543 cosmopolitan marine species. Marine Pollution Bulletin 2011, 62, 1147–1151.
544
545 Bao, V. W. W.; Lui, G. C. S.; Leung, K. M. Y. Acute and chronic toxicities of zinc
546 pyrithione alone and in combination with copper to the marine copepod *Tigriopus*
547 *japonicus*. Aquatic Toxicology 2014, 157, 81–93.
548
549 Banni, M.; Dondero, F.; Jebali, J.; Guerbej, H.; Boussetta, H.; Viarengo, A.
550 Assessment of heavy metal contamination using real-time PCR analysis of mussel
551 metallothionein *mt10* and *mt20* expression: a validation along the Tunisian coast.
552 Biomarkers 2007, 12, 369–383.
553
554 Banni M, S Sforzini, VM Arlt, A Barranger, LJ Dallas, C Oliveri, Y Aminot, B

555 Pacchioni, C Millino, G Lanfranchi, JW Readman, MN Moore, A Viarengo, AN Jha
 556 (2017) Assessing the impact of benzo[a]pyrene on marine mussels: Application of a
 557 novel targeted low density microarray complementing classical biomarker responses.
 558 PLoS ONE 12 (6): e0178460doi.org/10.1371/journal.pone.0178460
 559
 560 Bayne, B. Marine Mussels: Their Ecology and Physiology; Cambridge University
 561 Press: Cambridge, UK, 1976.
 562
 563 Bellas, J.; Granmo, A.; Beiras, R. Embryotoxicity of the antifouling biocide zinc
 564 pyrithione to sea urchin (*Paracentrotus lividus*) and mussel (*Mytilus edulis*). Marine
 565 Pollution Bulletin 2005, 50, 1382–1385.
 566
 567 Bignell, J.P., Dodge, M.J., Feist, S.W., Lyons, B., Martin, P.D., Taylor, N.G.H.,
 568 Stone, D., Travalent, L.; Stentiford, G.D. Mussel histopathology: effects of season,
 569 disease and species. Aquatic Biology 2008, 2, 1-15.
 570
 571
 572 Bones, J.; Thomas, K. V.; Paull, B. Improved method for the determination of zinc
 573 pyrithione in environmental water samples incorporating on-line extraction and pre-
 574 concentration coupled with liquid chromatography atmospheric pressure chemical
 575 ionisation mass spectrometry. Journal of Chromatography A 2006, 1132, 157–164.
 576
 577 Canty, M. N.; Hutchinson, T. H.; Brown, R. J.; Jones, M. B.; Jha, A. N. Linking
 578 genotoxic responses with cytotoxic and behavioural or physiological consequences:
 579 Differential sensitivity of echinoderms (*Asterias rubens*) and marine molluscs (*Mytilus*
 580 *edulis*). Aquatic Toxicology 2009, 94, 68–76.
 581
 582 Churchill, H. M.; Livingstone, D. R. Kinetic studies of the glycolytic enzymes from the
 583 mantle and posterior adductor muscle of the common mussel, *Mytilus edulis* L., and
 584 use of activity ratio (V_m/v) as an indicator of apparent K_m . Comparative
 585 Biochemistry and Physiology Part B: Comparative Biochemistry 1989, 94, 299–314.
 586
 587 Cima, F.; Ballarin, L. Immunotoxicity in ascidians: Antifouling compounds alternative
 588 to organotin IV. The case of zinc pyrithione. Comparative Biochemistry and

589 Physiology Part C: Toxicology and Pharmacology 2015, 169, 16–24.

590

591 Dallas, L. J.; Bean, T. P.; Turner, A.; Lyons, B. P.; Jha, A. N. Oxidative DNA damage
 592 may not mediate Ni-induced genotoxicity in marine mussels: Assessment of
 593 genotoxic biomarkers and transcriptional responses of key stress genes. Mutation
 594 Research/Genetic Toxicology and Environmental Mutagenesis 2013, 754, 22–31.

595

596 Dallas, L. J.; Devos, A.; Fievet, B.; Turner, A.; Lyons, B. P.; Jha, A. N. Radiation
 597 dose estimation for marine mussels following exposure to tritium: Best practice for
 598 use of the ERICA tool in ecotoxicological studies. Journal of Environmental
 599 Radioactivity 2016, 155/156, 1–6.

600

601 Devos, A.; Dallas, L. J.; Voiseux, C.; Lecomte-Pradines, C.; Jha, A. N.; Fivet, B.
 602 Assessment of growth, genotoxic responses and expression of stress related genes
 603 in the Pacific oyster *Crassostrea gigas* following chronic exposure to ionizing
 604 radiation. Marine Pollution Bulletin 2015, 95, 688-698.

605

606 Di, Y.; Schroeder, D. C.; Highfield, A.; Readman, J. W.; Jha, A. N. Tissue-Specific
 607 Expression of p53 and ras genes in response to the environmental genotoxicant
 608 benzo(a)pyrene in marine mussels. Environmental Science and Technology 2011,
 609 45, 8974-8981.

610

611 Emmanouil, C.; Smart, .J.; Hodges, N.J.; Chipman, J.K. Oxidative damage produced
 612 by Cr(VI) and repair in mussel (*Mytilus edulis* L.) gill. Marine Environ Research 2006,
 613 62 (Suppl. 1), S292-S296.

614

615 Goka, K. Embryotoxicity of zinc pyrithione, an antidandruff chemical, in fish.
 616 Environmental Research 1999, 81, 81–3.

617

618 Grunnet, K. S.; Dahllof, I. Environmental fate of the antifouling compound zinc
 619 pyrithione in seawater. Environmental Toxicology and Chemistry 2005, 24, 3001–6.

620

621 Jha A.N. Ecotoxicological applications and significance of the comet assay.
 622 Mutagenesis 2008, 23, 207-221.

623

624 Jha A.N. Genotoxicological studies in aquatic organisms: an overview. *Mutation*
625 *Research* 2004, 552, 1-17.

626

627 Hellou, J.; Law, R. J. Stress on stress response of wild mussels, *Mytilus edulis* and
628 *Mytilus trossulus*, as an indicator of ecosystem health. *Environmental Pollution* 2003,
629 126, 407–416.

630

631 Holmes, L.; Turner, A. Leaching of hydrophobic Cu and Zn from discarded marine
632 antifouling paint residues: Evidence for transchelation of metal pyrrithiones.
633 *Environmental Pollution* 2009, 157, 3440–3444.

634

635 Imokawa, G.; Okamoto, K. The effect of zinc pyrrithione on human skin cells in vitro.
636 *Journal of the Society of Cosmetic Chemists* 1983, 34, 1–11.

637

638

639 Karp, N. A.; Lilley, K. S. Maximising sensitivity for detecting changes in protein
640 expression: experimental design using minimal CyDyes. *Proteomics* 2005, 5, 3105–
641 15.

642

643 Kasi, V.; Bodiga, S.; Kommuguri, U. N.; Sankuru, S.; Bodiga, V. L. Zinc pyrrithione
644 salvages reperfusion injury by inhibiting NADPH oxidase activation in
645 cardiomyocytes. *Biochemical and Biophysical Research Communications* 2011, 410,
646 270–275.

647

648 Koukouzika, N.; Dimitriadis, V. K. Multiple biomarker comparison in *Mytilus*
649 *galloprovincialis* from the Greece coast: “lysosomal membrane stability, neutral red
650 retention, micronucleus frequency and stress on stress”. *Ecotoxicology* 2005, 14,
651 449–63.

652

653 Lamore, S. D.; Wondrak, G. T. Zinc pyrrithione impairs zinc homeostasis and
654 upregulates stress response gene expression in reconstructed human epidermis.
655 *Biometals* 2011, 24, 875–890.

656

657 Lamore, S. D.; Cabello, C. M.; Wondrak, G. T. The topical antimicrobial zinc
 658 pyrithione is a heat shock response inducer that causes DNA damage and PARP-
 659 dependent energy crisis in human skin cells. *Cell Stress and Chaperones* 2010, 15,
 660 309–322.
 661
 662 Lemoine, S.; Bigot, Y.; Sellos, D.; Cosson, R. P.; Laulier, M. Metallothionein isoforms
 663 in *Mytilus edulis* (Mollusca, Bivalvia): Complementary DNA characterization and
 664 quantification of expression in different organs after exposure to cadmium, zinc, and
 665 copper. *Marine Biotechnology* 2000, 2, 195–203.
 666
 667 Lockwood, B. L.; Somero, G. N. Transcriptomic responses to salinity stress in
 668 invasive and native blue mussels (genus *Mytilus*). *Molecular Ecology* 2011, 20, 517–
 669 529.
 670
 671 Mackie, D. S.; van den Berg, C. M. G.; Readman, J. W. Determination of pyrithione
 672 in natural waters by cathodic stripping voltammetry. *Analytica Chimica Acta* 2004,
 673 511, 47–53.
 674
 675 Madsen, T.; Gustavsson, K.; Samsøe-Petersen, L.; Simonsen, F.; Jakobsen, J.;
 676 Foverskov, S.; Larsen, M. M. Ecotoxicological assessments of antifouling biocides
 677 and nonbiocidal paints. Environmental Project No. 531 ; Danish Environmental
 678 Protection Agency: Copenhagen, Denmark, 2000; pp 1–112.
 679
 680 Marcheselli, M.; Rustichelli, C.; Mauri, M. Novel antifouling agent zinc pyrithione:
 681 determination, acute toxicity, and bioaccumulation in marine mussels (*Mytilus*
 682 *galloprovincialis*). *Environmental Toxicology and Chemistry* 2010a, 29, 2583–92.
 683
 684 Marcheselli, M.; Conzo, F.; Mauri, M.; Simonini, R. Novel antifouling agent-Zinc
 685 pyrithione: Short- and long-term effects on survival and reproduction of the marine
 686 polychaete *Dinophilus gyrotilatus*. *Aquatic Toxicology* 2010b, 98, 204–210.
 687
 688 Marcheselli, M.; Azzoni, P.; Mauri, M. Novel antifouling agent-zinc pyrithione: Stress
 689 induction and genotoxicity to the marine mussel *Mytilus galloprovincialis*. *Aquatic*
 690 *Toxicology* 2011, 102, 39–47.

691

692 Maraldo, K.; Dahllof, I. Indirect estimation of degradation time for zinc pyrrithione and
693 copper pyrrithione in seawater. *Marine Pollution Bulletin* 2004, 48, 894–901.

694

695

696 Mochida, K.; Ito, K.; Harino, H.; Kakuno, A.; Fujii, K. Acute toxicity of pyrrithione
697 antifouling biocides and joint toxicity with copper to red sea bream (*Pagrus major*)
698 and toy shrimp (*Heptacarpus futilirostris*). *Environmental Toxicology and Chemistry*
699 2006, 25, 3058–3064.

700

701

702 Moon, T. W.; Johnston, I. A. Starvation and the activities of glycolytic and
703 gluconeogenic enzymes in skeletal muscles and liver of the plaice, *Pleuronectes*
704 *platessa*. *Journal of Comparative Physiology* 1980, 136, 31–38.

705

706

707 Nunes, B.; Braga, M. R.; Campos, J. C.; Gomes, R.; Ramos, A. S.; Antunes, S. C.;
708 Correia, A. T. Ecotoxicological effect of zinc pyrrithione in the freshwater fish
709 *Gambusia holbrooki*. *Ecotoxicology* 2015, 24, 1896–905.

710

711 Oyama, T. M., Saito, M., Yonezawa, T., Okano, Y., Oyama, Y. Nanomolar
712 concentrations of zinc pyrrithione increase cell susceptibility to oxidative stress
713 induced by hydrogen peroxide in rat thymocytes, *Chemosphere*, 2012, 87, 1316–
714 1322.

715

716 Pfaffl, M. W.; Horgan, G. W.; Dempfle, L. Relative expression software tool (REST)
717 for group-wise comparison and statistical analysis of relative expression results in
718 real-time PCR. *Nucleic Acids Research* 2002, 30 .

719

720 Price, A. R. G.; Readman, J. Late lessons from early warnings: science, precaution,
721 innovation. EEA Report No 1/2013 ; European Environment Agency: Copenhagen,
722 Denmark, 2013; pp 265–278.

723

724 Ramakers, C.; Ruijter, J. M.; Deprez, R. H.; Moorman, A. F. Assumption-free
725 analysis of quantitative real-time polymerase chain reaction (PCR) data.
726 *Neuroscience Letters* 2003, 339, 62–6.

727

728 Reeder, N. L.; Xu, J.; Youngquist, R. S.; Schwartz, J. R.; Rust, R. C.; Saunders, C.

729 W. The antifungal mechanism of action of zinc pyrithione. *British Journal of*
730 *Dermatology* 2011a, 165, 9–12.

731

732 Reeder, N. L., Kaplan, J., Xu, J., Youngquist, R. S., Wallace, J., Hu, P., Juhlin, K. D.,
733 Schwartz, J. R., Grant, R. A., Fieno, A., Nemeth, S., Reichling, T., Tiesman, J. P.,
734 Mills, T., Steinke, M., Wang, S. L., Saunders, C. W. Zinc pyrithione inhibits yeast
735 growth through copper influx and inactivation of iron-sulfur proteins. *Antimicrobial*
736 *Agents and Chemotherapy* 2011b, 55, 5753– 5760.

737

738 Rudolf, E.; Cervinka, M. Stress responses of human dermal fibroblasts exposed to
739 zinc pyrithione. *Toxicology Letters* 2011, 204, 164–173.

740

741 Sánchez-Bayo, F.; Goka, K. Unexpected effects of zinc pyrithione and imidacloprid
742 on Japanese medaka fish (*Oryzias latipes*). *Aquatic Toxicology* 2005, 74, 285–293.

743

744 Scarlett, A.; Rowland, S. J.; Galloway, T. S.; Lewis, A. C.; Booth, A. M. Chronic
745 sublethal effects associated with branched alkylbenzenes bioaccumulated by
746 mussels. *Environmental Toxicology and Chemistry* 2008, 27, 561–567.

747

748 Schmidt, W.; Rainville, L.-C.; McEneff, G.; Sheehan, D.; Quinn, B. A proteomic
749 evaluation of the effects of the pharmaceuticals diclofenac and gemfibrozil on
750 marine mussels (*Mytilus spp.*): evidence for chronic sublethal effects on stress-
751 response proteins. *Drug Testing and Analysis* 2013, doi: 10.1002/dta.1463.

752

753 Thomas, K. V.; Blake, S. J.; Waldock, M. J. Antifouling paint booster biocide
754 contamination in UK marine sediments. *Marine Pollution Bulletin* 2000, 40, 739–745.

755

756 Tomanek, L.; Zuzow, M. J. The proteomic response of the mussel congeners *Mytilus*
757 *galloprovincialis* and *M. trossulus* to acute heat stress: implications for thermal
758 tolerance limits and metabolic costs of thermal stress. *Journal of Experimental*
759 *Biology* 2010, 213, 3559–74.

760

761 Turley, P. A.; Fenn, R. J.; Ritter, J. C.; Callow, M. E. Pyrithiones as antifoulants:
762 Environmental fate and loss of toxicity. *Biofouling* 2005, 21, 31–40.

Varotto, L.; Domeneghetti, S.; Rosani, U.; Manfrin, C.; Cajaraville, M. P.; Raccanelli, S.; Pallavicini, A.; Venier, P. DNA damage and transcriptional changes in the gills of *Mytilus galloprovincialis* exposed to nanomolar doses of combined metal salts (Cd, Cu, Hg). Plos One 2013, 8, e54602.

Viarengo, A.; Cannes, L.A.; Pertica, M.; Mancinelli, G.; Accomando, R.; Smaal, A. C.; Orunesu, M. Stress on stress response: A simple monitoring tool in the assessment of a general stress syndrome in mussels. Marine Environmental Research 1995, 39, 245–248.

Widdows, J.; Bakke, T.; Bayne, B. L.; Donkin, P.; Livingstone, D. R.; Lowe, D. M.; Moore, M. N.; Evans, S. V.; Moore, S. L. Responses of *Mytilus edulis* on exposure to the water-accommodated fraction of North Sea oil. Marine Biology 1982, 67, 15–31.

Yasokawa, D.; Murata, S.; Iwahashi, Y.; Kitagawa, E.; Kishi, K.; Okumura, Y.; Iwahashi, H. DNA microarray analysis suggests that zinc pyrithione causes iron starvation to the yeast *Saccharomyces cerevisiae*. Journal of Bioscience and Bioengineering 2010, 109, 479–486.

798 Figure 1: Higher level responses of *M. galloprovincialis* exposed to inorganic Zn or
799 ZnPT. (A) Percentage of mussels showing attachment during 14 d exposure; (B)
800 clearance rate (CR) after 14 d exposure; (C) survival (SoS; $n = 25$) in anoxic
801 conditions during 20 d after exposure. Error bars are $\pm$ one SE and asterisks indicate
802 significant differences ($p < 0.05$) from both sea water and inorganic Zn treatments.
803

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 $\pm$ 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.

Figure 3: Relative expression ratios (RER) of three genes (*hsp70*, *mt10* and *mt20*) in digestive gland and gill tissue of *M. galloprovincialis* after exposure to inorganic Zn or ZnPT. Data are normalised for two reference genes (*atub* and *ef1*) and the sea water control treatment and are shown with $\pm$ 95% confidence intervals. Values above 1 indicate upregulation and those below downregulation. Significant differences (PFRRT, $p < 0.05$) from the corresponding tissue control at day 0 are indicated by asterisks.

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.

Table 1: Differentially expressed proteins in the digestive gland of *M. galloprovincialis* exposed to 0.2 or 2 μ M ZnPT for 4 d. Non-significant fold changes ($p > 0.05$) have been omitted.

Spot id	protein id	Fold change relative to sea water control		Fold change relative to inorganic Zn	
		0.2 μ M	2 μ M	0.2 μ M	2 μ M
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