Detecting the Effects of Chronic Metal Exposure on Benthic Systems: Importance of Biomarker and Endpoint Selection

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Abstract

Understanding metal toxicity to benthic systems is still an ecotoxicological priority and, although numerous biomarkers exist, a multi-biomarker and endpoint approach with sediment as the delivery matrix combined with life-history relevant exposure timescales is missing. Here we assess potential toxicity by measuring a suite of biomarkers and endpoints after exposing the ecologically important polychaete *Alitta virens* to sediment spiked with environmentally relevant concentrations of copper and zinc (and in combination) for 3, 6 and 9 months. We compared biomarker and endpoint sensitivity providing a guide to select the appropriate endpoints for the chosen time frame (exposure period) and concentration (relevant to Sediment Quality Guidelines) needed to identify effects for benthic polychaetes such as *A. virens*. Target bioavailable sediment and subsequent porewater concentrations reflect the global contamination range, whilst tissue concentrations, although elevated, were comparable with other polychaetes. Survival reduced as concentrations increased, but growth was not significantly different between treatments. Metabolic changes were restricted to significant reductions in protein after 9 months exposure across all copper concentrations, and reductions in lipid at high copper concentrations (3 months). Significant changes in feeding behaviour and increases in metallothionein-like protein concentration were limited to the medium and high copper and zinc concentrations, respectively, both after 6 months exposure. Despite data highlighting *A. virens* metal tolerance, DNA damage and protein concentrations are the most sensitive biomarkers. Copper and zinc cause biomarker responses at concentrations routinely found in coastal sediments that are characterised as low contamination, suggesting a reappraisal of the current input sources (especially copper) is required.

Keywords

Heavy metal, bioavailability, genotoxic, antifouling, nanoparticles

1. Introduction

Metals are often considered pollutants of the past, yet industrial use is still extensive with new applications e.g. nanotechnology and ship emissions, causing significant amounts to enter the natural environment (Tourinho et al. 2012; Heggelund et al. 2014; Turner et al. 2017). Coastal waters, in particular, receive contaminants from anthropogenic sources and via riverine inputs (Eggleton and Thomas 2004), with copper and zinc being common pollutants (Walker et al. 2006; Johnson et al. 2017). As sediments are considered sinks, these inputs often lead to substantially elevated concentrations (Bryan and Langston 1992), with recent studies indicating that coastal concentrations are stable or increasing (Watson et al. 2018). These metals, therefore, remain of great concern in terms of ecotoxicological risk assessment for coastal benthic systems (Walker et al. 2006; Luoma and Rainbow 2008).

There is extensive literature on metals in the aquatic environment (see Rainbow 2018) yet understanding the biological effects for benthic organisms under natural conditions is still limited. Biomarkers are valuable tools, but as sediment-dwelling macrofauna are constantly exposed to sediment-bound contaminants assessing toxicity over long periods (e.g. several months) and using sediment as the delivery matrix is essential. Exposure to metals can have many effects on benthic organisms including: DNA damage (e.g. Martins and Costa 2015); metal-specific metallothionein-like protein (MTLP) production (e.g. Luoma and Rainbow 2008) and changing key behaviours (e.g. Bonnard et al. 2009). In addition, they can be

neurotoxic (e.g. inhibits acetylcholinesterase [AChE] enzyme) (e.g. Davies and Vethaak 2012; Ghribi et al. 2019); destabilise membranes (e.g. Luoma and Rainbow 2008) and modify energy allocation (e.g., Durou et al. 2005; Pook et al. 2009). Whilst studies investigating biomarker responses as indicators of toxicity have been comprehensive, and there have been significant advances in sediment toxicity testing methods (see Simpson et al., 2016), to date none have utilised those from a range of organisation levels (e.g. biochemical, physiological, and individual) to assess long term, sediment-mediated metal exposure impacts in a key benthic system. Previously, Watson et al. (2018) evaluated copper and zinc's chronic toxicity using a 9-month sediment-spiking exposure, whilst monitoring tissue, porewater and the bioavailable sediment concentration. However, they only used mortality, growth and DNA damage for *Alitta* (*Nereis*) *virens* as endpoints. Here we build on their study by reporting toxicity using a suite of additional biomarkers and endpoints at different organisational levels including: metabolic responses (protein, lipid and carbohydrate levels), lysosomal membrane stability, AChE activity, MTLP production and feeding behaviour. In addition, we compare responses of worms collected from field sites representing the full contamination range. Together these data enable us to compare multiple biomarkers and endpoints under controlled, but chronic exposure conditions that are environmentally relevant; providing important data for monitoring the long-term effects and the potential tolerance of benthic polychaete species to metal exposure.

2. Materials and methods

2.1. Mesocosm study: sediment and organism collection and experimental setup

Sediment (upper 10 cm) was collected from Chichester Harbour, UK (50 48’43.23"N, 0 52’30.78"W) and stored at 4°C in the dark until spiking. As worm weight is important in bioaccumulation processes (Poirier et al. 2006), 1-2 g cultured *A. virens* were used (purchased from Dragon Baits Ltd) and grown in sediment (copper: 1.5 mg kg^{-1}, zinc: 14 mg kg^{-1}). The target metal-spiking concentrations were based on presumed bioavailable concentrations measured by Pini et al. (2015) spanning the full range of contamination (see Table 1 and Watson et al. 2018). Ten treatments: control (C); low copper (LC); low zinc (LZ); low copper and zinc combined (LCZ); medium copper (MC); medium zinc (MZ), medium copper and zinc combined (MCZ); high copper (HC); high zinc (HZ) and high copper and zinc combined (HCZ) were created by spiking sediment, with nine exposure boxes per treatment (see Watson et al. [2018] for method). Sediments were spiked with copper chloride dihydrate ($CuCl_2.2H_2O$), zinc chloride (ZnCl) or a combination of both and with seawater as a control. A 5:1 ratio of sediment/seawater was used in each box, mixed for one minute and then left for a week at 4°C in the dark. After settlement, boxes were placed in three holding tanks (3 boxes per treatment per tank) with each box connected to seawater (mean flow rate per box: 20 l h^{-1}). The following day, eight worms were added to each box (a total of 24 worms per treatment), with any dead worms replaced within 48 hours. The worms were fed 1-2 % of their starting biomass twice a week using food pellets containing 7.7 µg g^{-1} copper and 65.4 µg g^{-1} zinc (dry weight). To provide field data comparisons worms were collected (see Pini et al. [2015] for method) from four field sites in 2013: Langstone and Poole Harbours, Tamar and Fal estuaries. Temperature, pH, salinity and dissolved oxygen of each box was monitored weekly.

2.2. Mesocosm study: sediment, porewater and worm tissue processing

Sediment, porewater and worms were sampled for metal analysis at each mesocosm sampling point. Sediment and porewater samples were extracted using a pore-extractor (Nayar et al. 2006) and subsamples of <63 µm sediment and porewater were stored at -20°C until analysis. Surviving worms were removed, counted, and left overnight at 4°C for gut depuration. Each worm was then weighed and coelomic fluid and tissue (2-3 cm of the anterior section) was collected. Samples were snap frozen in liquid nitrogen and stored at -80°C. Sediment samples were analysed using the BCR three-steps sequential extraction procedure developed by Standards, Measurements and Testing Programme (formerly BCR) of the European Commission (Mäkelä et al. 2011). This procedure is based on three steps and assesses the distribution of metals in the following fractions: (a) exchangeable; (b) reducible and (c) oxidizable. The sum of the three steps is described as the bioavailable concentration (Davidson et al. 1994; Zimmerman and Weindorf 2010) and we have used this term here, although we recognise the complexities around the terminology. Sediment samples from the BCR procedure were analysed for metals using a Varian Spectra AA 220FS Flame Atomic Absorption Spectrophotometer FAAS as detailed in Pini (2014). Percentage recoveries (mean ± SD) against BCR-701 sediment for copper for steps 1-3 were 110.64 ± 2.14, 97.58 ± 1.66 and 102.20 ± 0.80, respectively. Percentage recoveries for zinc for steps 1-3 were 99.02 ± 0.86, 92.5 ± 4.51 and 103.10 ± 0.21, respectively. Porewater and tissue samples were also processed according to Pini et al. (2015). Tissue analysis was verified with the reference material TORT-2 from the National Research Council Canada giving a recovery percentage (mean ± SD) of 91.63 ± 1.93 for copper and 99.52 ± 2.84 for zinc.

2.3. Mortality, growth and feeding activity

Mortality and growth assessments are described in Watson et al. (2018) with mortality defined as the number of dead worms in each box per sampling point and growth as a change in biomass per box divided by survivorship. Worm feeding behaviour was recorded using a camcorder mounted on a tripod above each box. Activity was recorded for twenty minutes immediately following the addition of food. The time taken for each worm to successfully grab a pellet from initially emerging from a burrow (Time to Grab Pellet, TGP) was recorded.

2.4. Energetic responses (protein, lipid and carbohydrate)

Energy reserves analysis was performed after 3, 6 and 9 months of exposure and on field-collected worms. 0.1 g of tissue was homogenized on ice in phosphate buffered saline at pH 7.5 before storage at -20°C. Total protein was determined based on the Biuret method (Gornall and David 1949) and modified from Murray (2010). Three millilitres of Biuret solution (3 g of Na, K-Tartrate, 0.75 g of copper sulphate and 150 mL of 10 % NaOH dissolved in 250 mL of distilled water, then topped up with distilled water to a final volume of 500 mL) was added to 50 µL of crude homogenate and 50 µL of protein standards. Protein standard concentrations of 0, 2, 4, 6, 8 and 10 mg mL−1 were prepared by dissolving 0.1 g of Bovine Serum Albumin (BSA) stock in 10 mL distilled water to obtain a final concentration of 10 mg mL−1 of stock solution. All samples and standards were heated at 37 °C for 30 minutes before being transferred in disposable polystyrene cuvettes and analysed on the Biomate3 spectrophotometer at an absorbance of 570 nm.

Total lipid content was determined based on the method developed by Bligh and Dyer (1959) with some modifications (Murray 2010). Acid salt solution (25 µl of 5 M NaCl, 1 M phosphoric acid) was added to 125 µL of crude homogenate, 300 µL of methanol and 600 µL of chloroform, followed by vortexing. Then, a further 100 µL of methanol, 200 µL of chloroform, 120 µL of distilled water and 30 µL of acid salt solution were added to the homogenate before being centrifuged at 0 °C (3500 g) for 10 minutes. Triglyceride tripalmitin standards were prepared using chloroform for 5, 2.5, 1.25, 0.625, 0.3125, 0.150625 mg mL^{-1} as well as a chloroform blank. Samples and standards were heated at 200ºC for 15 minutes after addition of 500 µL of 95.0-98.0 % sulphuric acid. After cooling, 1.5 ml of distilled water was added, and absorbance measured at 340 nm.

Total carbohydrate (carbohydrate and glycogen) content was determined using the method described by Carrol et al. (1956) with slight modifications from Murray (2010). Crude homogenate (200 µL) was ultrasonicated before vortexing with 15 % trichloroacetic acid followed by centrifugation at 4°C (3500 g) for 10 minutes. The supernatant from a second centrifuge process was added to the first and left on ice while glucose standards were prepared. Finally, 4 ml of anthrone reagent was added to 400 µL of each sample/standard before heating at 100°C for 10 minutes and absorbance measured at 590 nm.

2.5. Metallothionein-like Proteins (MTLPs)

MTLP levels were measured spectrophotometrically based on the method of Viarengo et al. (1997). In order to have sufficient tissue amounts, worm samples (n) were pooled per treatment (0.4 g per pool needed) for 3 months (all treatments n = 3), 6 months (all treatments n = 3, except HCZ, n= 2) and 9 months (all treatments n = 3, except LC, n = 2 and

HCZ, n= 1) with insufficient tissue from field worms for analysis. Each pool of tissues was homogenised in solution containing 0.5 M sucrose, 20 mM tris-HCl, 3.0 µL mL−1 leupeptin, 1.5 µL mL−1 phenylmethylsulphonyl fluoride (PMSF) and 0.1 µL mL−1 -mercaptoethanol (equivalent to 0.01 %). The homogenate was then centrifuged at 30000 g for 20 minutes to obtain a supernatant that contained MTLPs. For each sample, 1.05 mL of ethanol (-20 °C) and 80 µL of chloroform were added to 1 mL aliquot of supernatant and vortexed for a few seconds. After further centrifugation at 6000 g for 10 minutes, the supernatant was collected and 40 µL of 37 % HCl and 10 µL of a solution of RNA (1 mg/10 µL) followed by 3 volumes of cold ethanol were added and placed at -20 °C for 1 hour. The pellet was then washed in 87 % ethanol and 1 % chloroform homogenizing buffer solution and dried under N_2 gas for 10 minutes. Then, 150 µL of 0.25 M NaCl solution and 150 µL of destabilizing solution (1N HCl containing 4 mM EDTA) were added to resuspend the MT enriched fraction. Finally, DTNB (5,5-dithiobis-2-nitrobenzoic acid, pH 8) was added to samples at room temperature. Absorbance was measured at 412 nm and the MTLP concentration was calculated using reduced glutathione as a reference standard.

2.6. AChE, Lysosomal membrane stability and DNA damage

The AChE activity was determined using coelomic fluid samples based on the method of Ellman et al. (1961). First, 10 µL of coelomic fluid was added in duplicate in a 96 well flat bottom microplate. Then, 30 µL of the reagent DTNB (5',5'-Dithiobis 2-Nitrobenzoic acid) was added to each well and read at 405 nm each minute for 5 minutes on a multi-well plate reader LT-400 Labtech. Finally, 10 µL of acetylthiocholine iodine (3 mM in 50 mL of distilled water) was added to each sample and read again at 405 nm for 5 minutes. The assay was

standardised (Bradford, 1976) with Bio-Rad reagent and protein standards, and absorbance measured at 595 nm.

Lysosomal membrane stability of coelomocytes was measured by a neutral red assay based on Babich and Borenfreund (1992) and Repetto et al. (2008) with adaptations for *A. virens* (C. Lewis personal communication). First, 50 µL of coelomic fluid was added to microplates pre-treated with poly-L-lysine. Non-adhered cells were discarded, and 50 µL of neutral red working solution (0.2 % neutral red stock solution in physiological saline) was added and left at room temperature to incubate for 3 hours. Excess solution was removed before adding 50 µL of acidified ethanol (1 % acetic acid, 50 % ethanol). The optical density of Neutral red was then measured spectrophotometrically at 540 nm. This assay was standardised using the same protein protocol as described in the AChE assay. DNA damage was measured using the alkaline version of the comet assay by detecting single/double strand breaks plus incomplete excision repair sites (Jha 2008) from coelomocytes as described in Lewis and Galloway (2008) with modifications by Pini (2014). Slides were scored blind under epifluorescence with an average of 20 cells scored per sample (including a 3-min UV light exposure as a positive control) with results presented as % DNA damage.

2.7. Statistical analysis

Data were analysed using Minitab v17. Data for biomarkers and endpoints were split by exposure length and metal treatment group and analysed using General Linear Models (GLMs) with 'tank' and 'treatment' as fixed factors followed by post Hoc Tukey HSD pairwise comparison tests on significant data. GLMs were also used to assess differences in biomarker and endpoint responses for field sites. All data were checked for normality and equal variances using Box-Cox tests, relevant transformations were applied as required.

3. Results and Discussion

3.1. Metal concentrations (sediment, porewater and tissue)

Metal concentrations in sediment, porewater and tissue (Tables 1, S1, S2, S3) reflect global coastal contamination (Eggleton and Thomas 2004; Tourinho et al. 2012; Heggelund et al. 2014; Turner et al. 2017). Whilst the metal concentrations approached the target values, the bioavailable metal concentrations in the sediment decreased over the 9-month experiment, which is likely due to the continuous water flow; permanent sediment submersion; ageing of the sediment and elevated bioturbation due to worm densities (Remaili et al. 2016). The loss of bioavailable metal could have been reduced by longer equilibrium times and increased mixing during the spiking process (Hutchins et al. 2008; Simpson et al. 2004). Despite system loss, the bioavailable sediment concentrations match real-world contamination levels (Table 1). Controls reflect the low contamination seen in Langstone Harbour, whilst low treatments at 3 months reflect contamination seen in Poole Harbour and the Tamar Estuary. Medium treatments represent higher contamination areas with the potential for toxic effects (e.g. Pan and Wang 2012; Briant et al. 2013). High concentrations reflect elevated contamination in the Fal Estuary and others (e.g. Bryan and Langston 1992; Miller et al. 2000; Pan and Wang 2012), with concentrations well above Sediment Quality Guidelines (SQGs) that will have significant adverse effects on benthic organisms. Generally, copper porewater concentrations in all low and medium treatments, and zinc in LZ and MZ, were not significantly different from each other and corresponded with concentrations found at field sites. Porewater concentrations in the high treatments exceed those from the most contaminated field site (Fal estuary). In contrast, porewater concentrations of zinc in the combined treatments were generally much higher and likely driven by the spiking process (U.S.EPA 2005).

Table 1. Bioavailable sediment, porewater and tissue concentrations for copper and zinc

Treatment	Mon	Control	LC	MC	HC	LZ	MZ	HZ	LCZ	MCZ	HCZ	Langstone Harbour	Poole Harbour	Tamar Estuary	Fal Estuary
Copper target		-	70	120	575	-	-	-	70	120	575	-	-	-	-
Bioavailable sediment	0	8 ± 0.3 [a]	121 ± 43 [b]	158 ± 47 [b]	654 ± 208 [c]	-	-	-	74 ± 7 [b]	97 ± 10 [b]	636 ± 30 [c]	11 ± 0.6	48 ± 5	88 ± 7	422 ± 64
	3	6 ± 0.3 [a]	77 ± 7 [b]	157 ± 11 [c]	614 ± 109 [d]	-	-	-	62 ± 13 [b]	81 ± 6 [b]	563 ± 25 [c]				
	6	6 ± 0.3 [a]	42 ± 7 [b]	93 ± 9 [c]	406 ± 42 [d]	-	-	-	64 ± 6 [b]	82 ± 5 [b]	463 ± 56 [b]				
	9	7 ± 0.6 [a]	49 ± 31 [a, b]	100 ± 25 [b]	364 ± 98 [c]	-	-	-	45 ± 8 [b]	72 ± 13 [b]	362 ± 78 [c]				
Porewater	0	0.4 ± 0.20 [a]	1.1 ± 0.69 [a]	1.4 ± 0.47 [a]	7.0 ± 2.41 [b]	-	-	-	0.6 ± 0.02 [b]	1.0 ± 0.20 [b]	7.3 ± 0.63 [c]	0.7 ± 0.14	0.8 ± 0.02	1.6 ± 0.30	1.9 ± 0.23
	3	0.8 ± 0.27 [a]	1.1 ± 0.19 [a]	1.9 ± 0.44 [a]	6.7 ± 1.09 [b]	-	-	-	0.9 ± 0.23 [a]	1.1 ± 0.20 [a]	3.5 ± 1.26 [a]				
	6	0.4 ± 0.01 [a]	0.7 ± 0.12 [b]	1.1 ± 0.25 [b]	3.0 ± 0.86 [c]	-	-	-	1.1 ± 0.09 [b]	1.2 ± 0.11 [b]	4.4 ± 0.93 [b]				
	9	0.2 ± 0.01 [a]	0.3 ± 0.04 [b]	0.3 ± 0.04 [b]	0.3 ± 0.01 [b]				0.2 ± 0.02 [a]	0.2 ± 0.03 [a]	1.2 ± 0.89 [b]				
Tissue	3	9 ± 1.1 [a]	16 ± 1.5 [a]	39 ± 4.3 [a]	177 ± 26.0 [b]	-	-	-	53 ± 25.9 [a]	36 ± 13.3 [a]	102.0 [a]	9 ± 0.9	10 ± 2.8	7 ± 1.4	10 ± 1.9
	6	10 ± 0.5 [a]	33 ± 5.4 [a]	120 ± 41.5 [b]	240 ± 16.0 [b]	-	-	-	38 ± 12.0 [b]	78 ± 14.5 [b]	313.0 [c]				
	9	6 ± 0.3 [a]	13 ± 2.9 [a]	27 ± 2.8 [b]	82 ± 31.4 [b]	-	-	-	15 ± 2.9 [a]	23 ± 4.2 [a]	NA				
Zinc target		-	-	-	-	200	270	1160	200	270	1160	-	-	-	-
Bioavailable sediment	0	23 ± 0.5 [a]	-	-	-	282 ± 20 [b]	353 ± 39 [b]	856 ± 74 [c]	212 ± 9 [b]	211 ± 21 [b]	873 ± 45 [c]	36 ± 5	159 ± 31	175 ± 24	671 ± 46
	3	28 ± 2 [a]	-	-	-	197 ± 42 [b]	322 ± 35 [b]	614 ± 26 [c]	173 ± 34 [b]	188 ± 21 [b]	607 ± 32 [c]				
	6	23 ± 0.5 [a]	-	-	-	163 ± 17 [b]	181 ± 40 [b]	590 ± 65 [c]	152 ± 19 [b]	143 ± 40 [b]	477 ± 51 [c]				
	9	23 ± 1 [a]	-	-	-	146 ± 25 [b]	172 ± 27 [b]	581 ± 40 [c]	87 ± 9 [b]	130 ± 26 [b]	475 ± 57 [c]				
Porewater	0	0.6 ± 0.15 [a]	-	-	-	2.2 ± 0.42 [b]	3.0 ± 1.09 [b]	76.3 ± 15 [c]	7.6 ± 1.10 [b]	13.8 ± 2.49 [b]	374 ± 130 [c]	3 ± 1.90	0.5 ± 0.04	1.4 ± 0.48	2.1 ± 0.55
	3	3.5 ± 1 [a]	-	-	-	2.6 ± 0.42 [a]	2.6 ± 0.15 [a]	3.1 ± 0.64 [a]	5.4 ± 0.47 [a]	6.3 ± 2.19 [a]	60.0 ± 14 [b]				
	6	0.9 ± 0.14 [a]	-	-	-	0.4 ± 0.07 [a]	0.5 ± 0.11 [a]	0.6 ± 0.14 [a]	1.7 ± 0.30 [a]	1.2 ± 0.28 [a]	28.1 ± 3.09 [b]				
	9	0.03 ± 0.02	-	-	-	0.2 ± 0.02 [b]	0.5 ± 0.02 [c]	0.5 ± 0.04 [c]	2.2 ± 0.6 [b]	3.2 ± 0.78 [b]	5.7 ± 1.3 [b]				
Tissue	3	54 ± 1.7 [a]	-	-	-	101 ± 4.6 [b]	126 ± 32.8 [b]	131 ± 26.0 [b]	84 ± 6.0 [a]	93 ± 9.5 [b]	106.0 [b]	62 ± 4.9	73 ± 22.8	69 ± 6.4	140 ± 61
	6	69 ± 15.3 [a]	-	-	-	71 ± 10.5 [a]	115 ± 14 [a, b]	173 ± 34.0 [b]	92 ± 11.0 [a]	95 ± 10.8 [a]	350.0 [c]				
	9	62 ± 8.3 [a]	-	-	-	62 ± 11.3 [a]	97 ± 24.3 [b]	67 ± 22.6 [a]	92 ± 4.2 [a]	71 ± 2.2 [a]	NA				

Footnote: Table already published in Watson et al., 2018 but required here to contextualise biomarker responses. Copper and zinc mesocosm target bioavailable concentrations for sediment (mg kg-1 dry weight) generated from Pini et al. (2015). Bioavailable sediment (mg kg-1 dry weight); porewater (µg l-1) and worm tissue (µg kg-1 dry weight) concentrations (mean ± SEM) of copper and zinc from mesocosm experiment treatments (control [C]; copper: Low [LC], Medium [MC] and High [HC]; zinc: Low [LZ], Medium [MZ] and High [HZ]; and copper and zinc combined: Low [LCZ], Medium [MCZ] and High [HCZ]), field sites. Mesocosm: N=3 boxes per treatment per month for sediment, porewater and tissue; N=2 worms per box for tissue. Field sites: N=3 samples for sediment and porewater per site with 10 worms per site for tissue concentrations. General Linear Models (GLMs) were used to compare treatments for each month separately (except month 0 for tissue). Treatments that share the same letters (compare rows only) are not significantly different from each other when analysed using Tukey HSD pairwise comparisons. NA: no data collected as no worms survived. For comparison: Sediment Quality Guideline (SQG) values (mg kg-1 dry sediment) from Simpson et al. (2013) are: 65 for Cu and 200 for Zn; and SQGH (Sediment Quality Guideline High value): 270 for Cu and 410 for Zn.

Tissue concentrations for the copper-only treatments generally followed the sediment concentrations, mostly separating into two distinct groups: Control and LC; and MC and HC treatments. In contrast, the copper tissue concentrations from the combined treatments were much more variable, although treatments were still elevated against the control. Consistently low tissue concentrations from field-collected worms support data of Pini et al. (2015) that *A. virens* can regulate copper, but only at lower concentrations. Elevated tissue concentrations from the MC and HC treatments match those found in *Nereis diversicolor* (e.g. Amiard et al. 2007; Rainbow et al. 2009). Zinc tissue concentrations were also similar to other polychaetes, such as *Nereis diversicolor* and *Marphysa sanguinea* (e.g. Amiard et al. 2007; Garcês and Costa 2009; Rainbow et al. 2009), but closely track field-collected worms and those of Pini et al. (2015), suggesting that *A. virens* is performing some zinc regulation when exposed to high concentrations. Although less evident in the combined treatments, tissue concentrations for both metals reduced over time. As suggested by Watson et al. (2018) further investigation to understand tissue accumulation and regulation in this species is required.

3.2. Sensitivity of biomarkers and endpoints

3.2.1 Mortality and growth

Mortality is the most obvious endpoint, and although it was not a planned endpoint, the data confirm that metal exposure reduces survivorship, but only significantly at the highest concentration of copper or in combination with zinc after three and six months of exposure (Figure S1, Table S4). The appropriateness of mortality as an endpoint for measuring the chronic effects of metals is limited to high concentrations and only then over extended

32 exposure periods. As a rapid initial test, it may still have relevance, but as stated by Watson
33 et al. (2018) one must account for underlying seasonality and biological interactions.

34 Reanalysed data from Watson et al. (2018) confirm that over the nine-month experiment
35 the worms gained weight in all treatments (Figure S2, Table S4). Generally, growth was lower
36 at 6 months compared to 3 months but was higher and more variable at 9 months compared
37 to 6 months. Whilst some studies have shown hormetic responses (e.g. Jenkins and Sanders
38 1986), a lack of significant differences for growth supports neither a positive nor negative
39 effect. Water temperature and photoperiod are critical in determining *A. virens* activity (Last
40 and Olive 1999). As stated by Watson et al. (2018) the differential treatment mortality is
41 likely to have offset any impacts on growth as the surviving worms in the high concentration
42 treatments would have access to more food. Therefore, future experiments should expose
43 individuals separately to control food rations.
44

45 *3.2.2 Metabolic responses (carbohydrate, protein and lipid)*

46 Analyses of metabolic changes for carbohydrate, protein and lipid tissue were conducted
47 separately, however, to give an overview of tissue concentrations, the percentage
48 contribution to worm energy reserves is presented in Figure 1. Over 50 % of reserves were
49 made up of carbohydrates for all treatments in month 3 and more than 80 % for treatments
50 in months 6 and 9. Carbohydrate concentrations increased from 3 months to 6 months and
51 decreased from 6 months to 9 months (Figure 1 and S3, Table S4), but GLMs for the separate
52 metal treatments per exposure period confirmed no significant difference. Concentrations
53 in the worms collected from the field sites reflected those exposed for 9 months, but a GLM
54 confirmed no significant difference between the sites.

55 Protein concentrations showed a decrease from 3 months to 9 months in all treatments
56 including the control group (Figures 1 and S4, Table S4). Concentrations of protein in the
57 control at month 9 reduced to 12.8 % of the total energy reserve, whereas all other
58 treatments (except HCZ) reduced further to between 3.3 % and 8.1 %. Protein
59 concentrations from the field sites were low (1.1 % - 10.1 %), similar to those from the 9-
60 month exposure treatments, and statistical analysis showed no significant differences
61 between field sites ($F_{3,11} = 3.07$, $p = 0.051$). GLMs for the separate metal treatments revealed
62 a significant difference in protein concentration for copper at 3 months ($F_{3,11} = 5.6$, $p = 0.036$)
63 and at 9 months ($F_{3,11} = 9.4$, $p = 0.017$), with Post hoc analysis revealing a significantly lower
64 protein concentration in the control than HC at 3 months. At 9 months, the control had a
65 significantly higher protein concentration than all other treatments. Pearson's correlations
66 confirm a significant positive relationship for protein and bioavailable sediment
67 concentration of copper at month 3 ($r = 0.733$; $p = 0.007$).
68 Lipid concentrations in tissues, similar to protein, decreased from month 3 to month 9 in all
69 treatments (Figure 1 and S5, Table S4). Concentrations in all treatments reduced to below
70 1.5% of the total energy reserve at month 9. Statistical analysis revealed a significant
71 difference between field sites ($F = 3.87$, $p = 0.029$), with a higher concentration in worms
72 from the Fal Estuary than Langstone Harbour. GLM for the separate metal treatments
73 revealed only a significant difference in lipid concentration for copper at 3 months ($F_{3,11} = 9.3$,
74 $p = 0.008$), with Post hoc analysis showing that HC had a significantly lower concentration
75 (mean 1.68 mg g^{-1}) than all other treatments. Pearson's correlations confirm a significant
76 positive relationship of lipids and bioavailable sediment concentration of zinc at 6 months
77 ($r = 0.724$; $p = 0.008$).

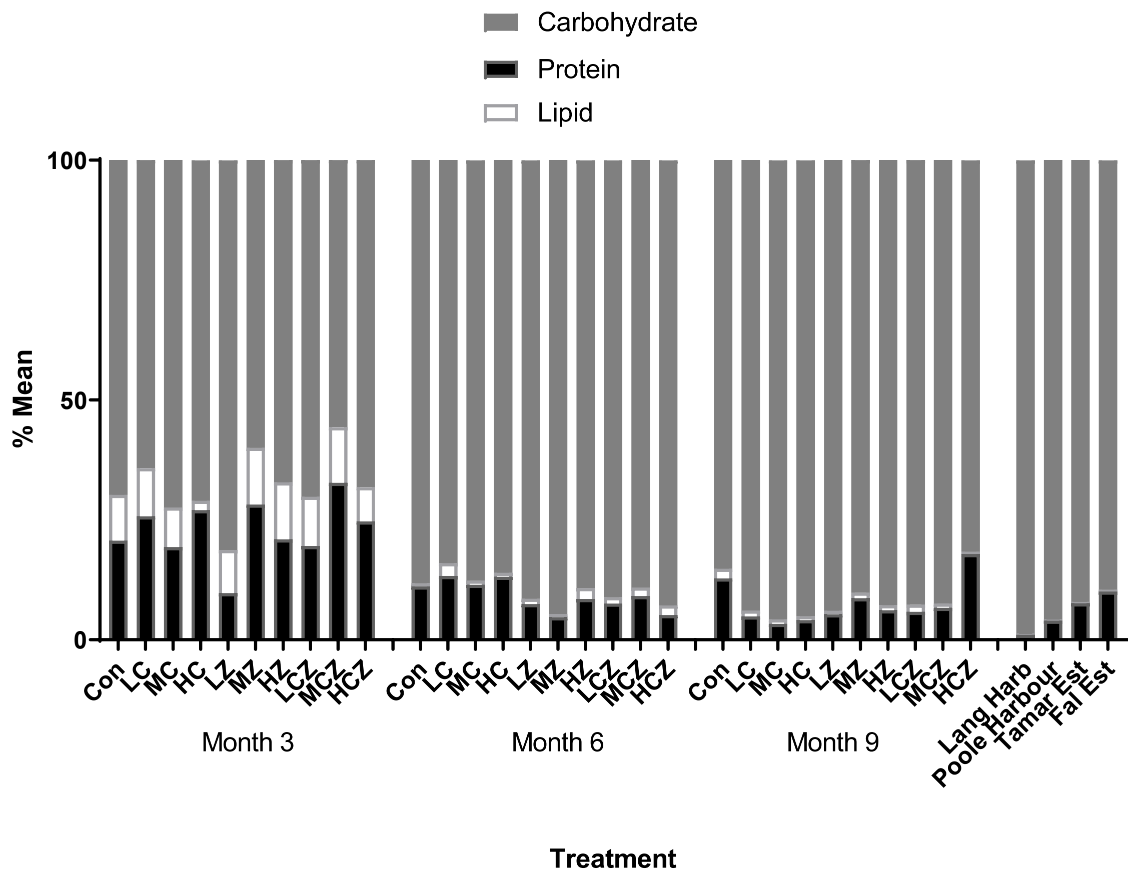
78 Invertebrates living in metal-contaminated sites can change their energy allocation as part
79 of their defence response (Hahn 1998; Gerber et al. 2018). Our data show that metabolic
80 changes do occur for *A. virens*, but are metal, exposure length and metabolite-specific.
81 Carbohydrate levels did not change significantly for any treatment, whilst lipid
82 concentrations were only significantly lower in the HC treatment compared to the Control
83 after 3 months of exposure. In contrast, protein level changes were more prevalent: LC, MC
84 and HC all had significantly lower concentrations than the Control after 9 months.

85 The presence of spawning individuals at about 6-months confirms gametogenic processes
86 for some individuals. Lipids and carbohydrate stores are essential for reproduction (Pook et
87 al. 2009) which could have increased inter-individual variability in metabolic concentrations
88 masking treatment-specific changes. Despite the onset of gametogenesis, it is clear that 9
89 months of copper exposure across a range of environmentally relevant concentrations
90 induces significant reductions in protein levels, indicating stress to the organisms, which
91 supports other studies from polychaetes using stressors such as ocean acidification (Freitas
92 et al. 2016). These decreases in protein levels and concomitant reduction in lipid at 3 months
93 under the highest concentration may link to energy-intensive toxicity responses (Calow
94 1991). In contrast, the increase in protein levels at month 3 may be associated with the onset
95 of MTLP production (as a metal detoxification process), which were higher than the controls,
96 although not significantly so.

97 Metabolic responses of polychaetes to contaminants have been shown to vary considerably.
98 For example, glycogen and lipids were higher in *H. diversicolor* from a clean site compared
99 to a contaminated site (Durou et al. 2005) whilst Mouneyrac et al. (2006) did not reveal any
100 differences. Individual weight can influence metabolic profiles in *A. virens* (Pellerin-

101 Massicotte et al. 1994) and Durou et al. (2005) showed that *H. diversicolor* lipid
102 concentrations were influenced by weight. Nevertheless, it is unlikely that weight or growth
103 affected our results as no significant differences in growth were observed.

104



105

106 Figure 1. Energy reserves (carbohydrate, protein and lipid) expressed as a mean percentage of total reserves
107 (SE omitted for clarity) for all treatments for month 3, 6 and 9 and for worms collected from field sites.
108 Calculated as a mean from survivors, which varied from 1 to 8 per box. n = 3 boxes per sampling point, expect
109 for HCZ month 6 and HC month 9 where n = 2 boxes and HCZ month 9 where n = 1. control (Con); copper Low
110 (LC), Medium (MC) and High (HC); zinc Low (LZ), Medium (MZ) and High (HZ); and copper and zinc combined
111 Low (LCZ), Medium (MCZ) and High (HCZ). Field sites were sampled between July–September 2013 with 6
112 worms sampled from each site, except Langstone Harbour with 5.

113

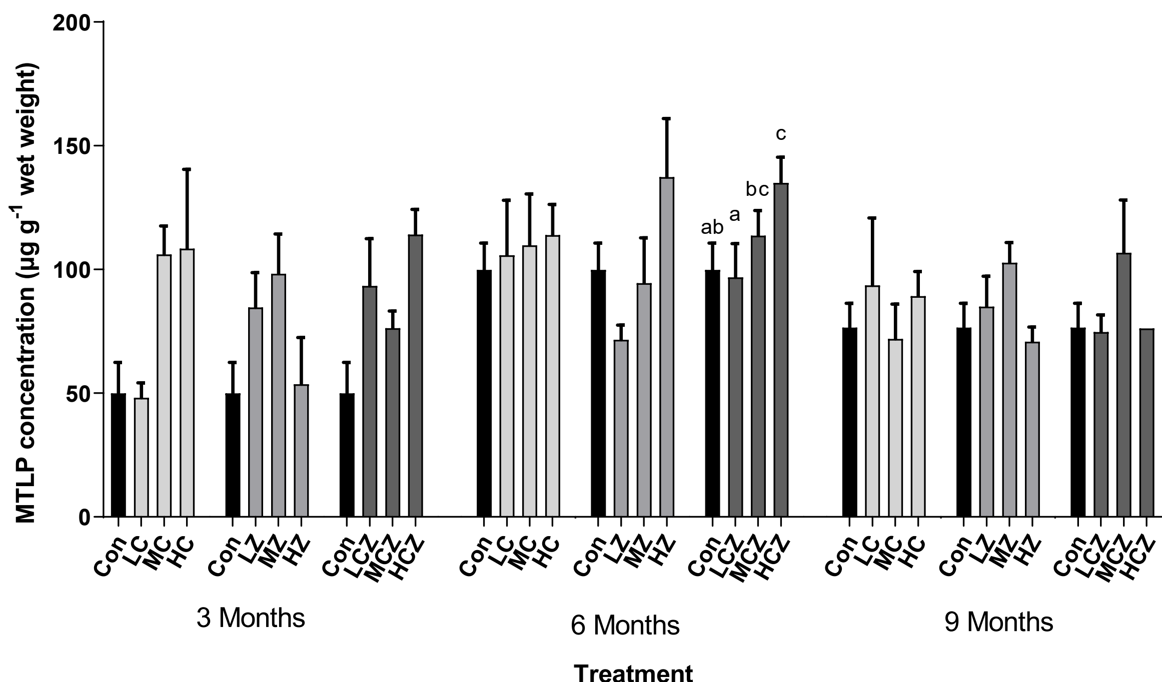
114 *3.2.3. MTLPs*

115 Significant changes in MTLP concentrations were only observed in worms after 6 months
116 (Figure 2, Table S4). GLMs for the separate metal treatments at each month revealed a
117 significant difference for the copper and zinc combined treatment at 6 months ($F_{3,11}$ =18.5,

118 p = 0.004) with post hoc analysis showing that HCZ had significantly more MTLP than LCZ
119 and the control, whilst MCZ had significantly higher concentrations than LCZ.

120 Many studies have reported MTLP-induction as a metal detoxification process (e.g. Amiard
121 et al. 2006; Monserrat et al. 2007; Won et al. 2008; 2012). However, MTLP concentrations
122 fluctuate depending on season, exposure time and reproductive cycle (Machreki-Ajmi et al.
123 2011; Won et al. 2012); can decrease with increasing levels of dissolved zinc (Bighiu et al.
124 2017); or do not change (Petrovic et al. 2001). The lack of a clear MTLP response and the
125 potential for multiple MTLP forms having different functions (Reddy et al. 2014) indicates
126 that the MTLP expression is not a dominant metal response in this species. Nevertheless,
127 assessing gene expression levels to account for pleiotropic genes would be an obvious next
128 step if MTLP induction is to be a chronic metal exposure biomarker.

129



130

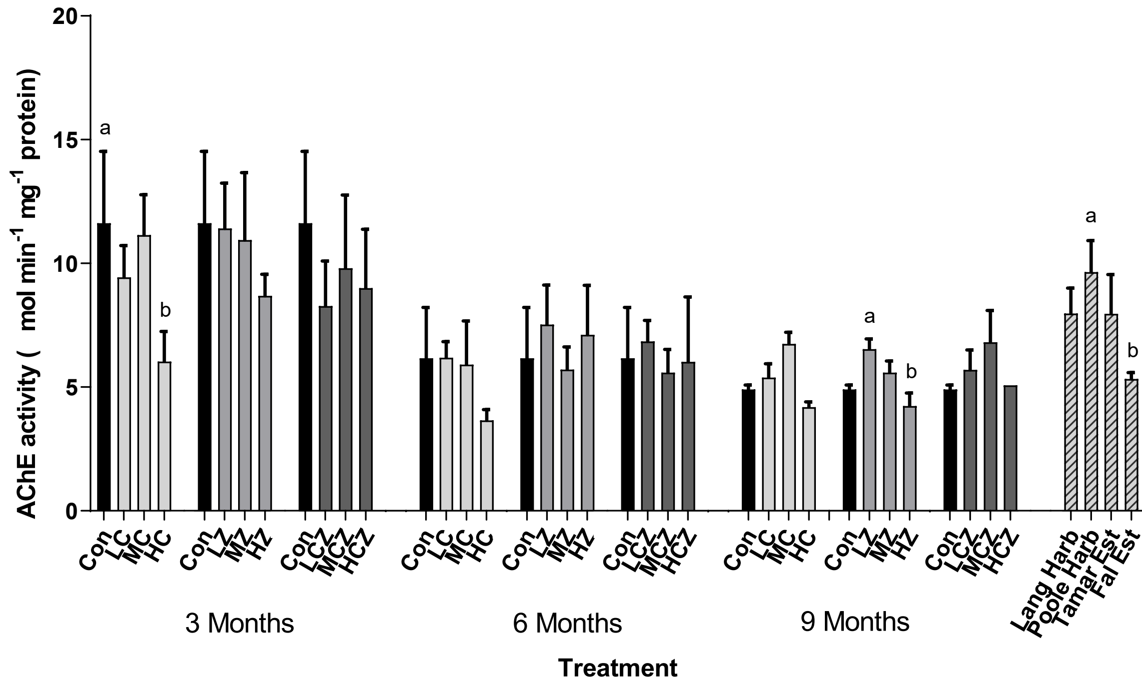
131 Figure 2. Mean metallothionein (MTLP) concentration expressed in µg g^{-1} of wet weight (±SEM) for all
132 treatments sampled at 3 months (n = 30), 6 (n = 29) and 9 (n = 27). No error bars are shown for HCZ at 9
133 months as only one individual was tested. The control values for each exposure period are presented multiple

134 times to enable post hoc analysis to be represented. No data is presented for field sites as there was insufficient
135 tissue from field worms for analysis.

136

137 *3.2.4 AChE*

138 AChE activity across all treatments generally declined from 3 months to 9 months (Figure 3,
139 Table S4). GLMs for the separate metal treatments per period of exposure revealed a
140 significant difference in AChE activity for copper treatments at 3 months ($F_{3,11}$ =5.4, p =
141 0.039), with post hoc analysis revealing that the control had significantly higher activity than
142 HC. Analysis also revealed a significant difference in activity for zinc treatments after 9
143 months of exposure ($F_{3,11}$ =7.3, p= 0.020), with post hoc analysis showing LZ had significantly
144 higher activity than HZ. Mean activity also differed between field sites (F = 3.19, p = 0.047),
145 with Poole Harbour (9.6 µmol min^{-1} mg^{-1}) showing the highest activity and being significantly
146 different from the Fal Estuary (5.3 µmol min^{-1} mg^{-1}) similar to that of medium to high
147 treatments at 3 months exposure. Despite significant changes in AChE activity, the effect of
148 metals on *A. virens* AChE inhibition is not clearly revealed. Seasonal temperature fluctuations
149 are reported as the major cause of AChE variability (Rank et al. 2007), but our analyses were
150 separated by exposure period to eliminate the effects of these fluctuations. Some studies
151 have reported the inhibition of AChE by copper in fish (Alves Costa et al. 2007), yet Cunha et
152 al. (2007) observed no effect at all in marine gastropods. The effect of metals on AChE
153 activity appears to be species-specific and dependent on the interaction of the metal ion and
154 binding sites.



155

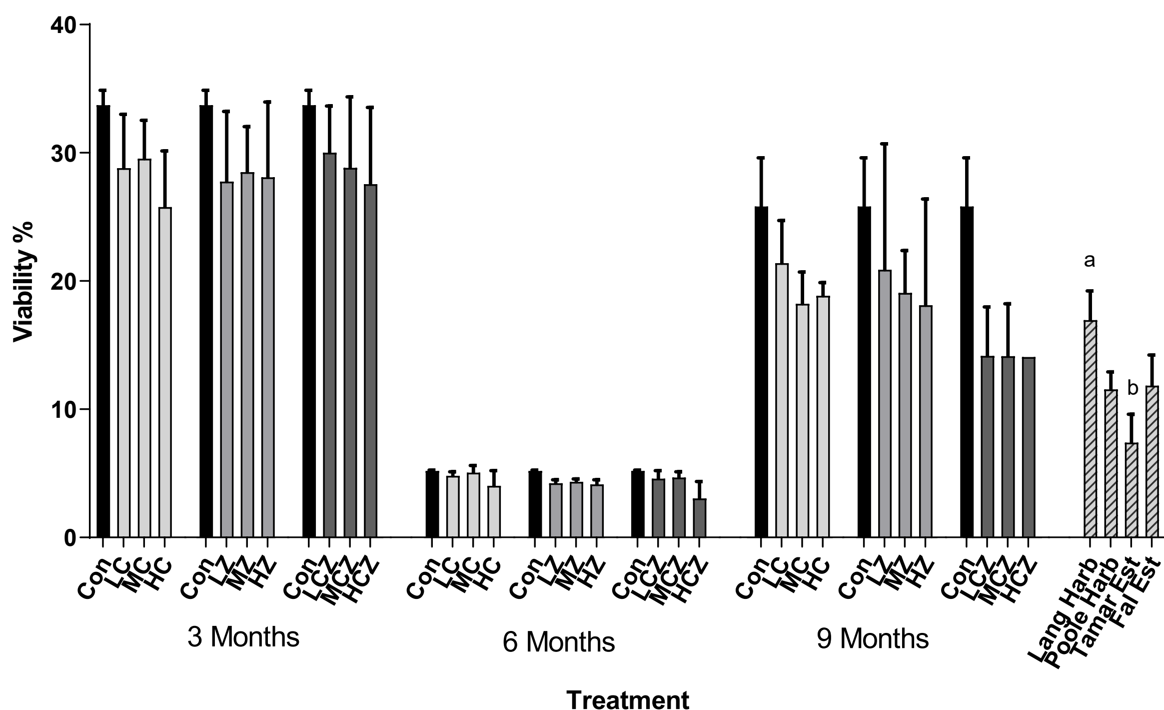
156 Figure 3. AChE activity expressed in µmol min^{-1} mg^{-1} of protein (±SEM) for all treatments at 3 months (n =
157 102), 6 (n = 114) and 9 (n = 56), and for worms collected at field sites, with 6 worms sampled from each site,
158 except Langstone Harbour with 5. The control values for each exposure period are presented multiple times to
159 enable post hoc analysis to be represented.

160

161 *3.2.5 Lysosomal membrane stability*

162 The neutral red assay showed a dramatic reduction in cell viability from 3 months to 6
163 months, followed by a partial recovery in 9 months (Figure 4, Table S4). GLMs for separate
164 metal and exposure period combinations revealed a significant difference in percentage
165 viability for zinc treatments at 6 months ($F_{3,11}$ =4.9, p = 0.048), although post hoc analysis
166 revealed no difference between treatments. A GLM for field sites also revealed a significant
167 difference ($F_{3,23}$ =3.9, p = 0.025) with post hoc analysis showing that worms from the Tamar
168 Estuary had a significantly lower percentage of cell viability than those from Langstone
169 Harbour. The neutral red test to assess the potential effect of metals on membrane stability
170 has been widely used in marine invertebrates (e.g. Brown et al. 2004; Moore et al. 2006;

171 Boughattas et al. 2016), so the lack of significant effects for treatments and exposure period
172 and differences for field sites is surprising.
173 Lysosomal instability can appear after only 3 days of exposure (Rocco et al. 2011) and when
174 coupled with a rapid recovery (14 days in the earthworm *Eisenia fetida* for copper exposure)
175 it could mean that instability will be shown with earlier and more frequent sampling which
176 we recommend be tested for *A. virens*. Our data indicate that *A. virens* has a considerable
177 ability to maintain/repair membranes, thus lysosomal stability is not considered an
178 appropriate biomarker for long-term chronic metal exposure studies unless sampling is more
179 frequent. Data also revealed dramatic changes in stability levels between months
180 (recognising that season is confounded in exposure period). Seasonal variations impact
181 membrane stability (Domouhtsidou and Dimitriadis 2001) with temperature and salinity
182 contributing to membrane changes (Lowe et al. 1992; Cho and Jeong 2005). Gametogenic
183 processes may also affect responses, with Davies and Vethaak (2012) hypothesising that
184 spawning-induced defence system disruption explains stability decrease.



185

186 Figure 4. Lysosomal membrane stability revealed by the neutral red assay expressed in percentage of cell
187 viability (±SEM) for all the treatments at 3 months (n = 101), 6 (n = 116) and 9 (n = 52) and for worms collected
188 from field sites. The control values for each exposure period are presented multiple times to enable post hoc
189 analysis to be represented.

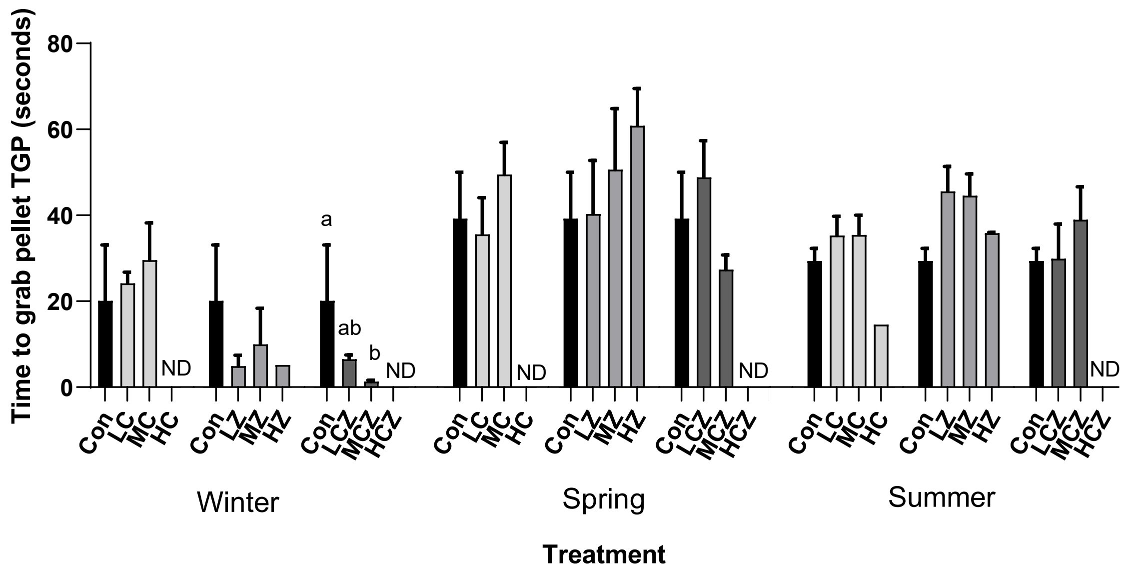
190

191 *3.2.6 Behaviour*

192 Differences in environmental conditions can alter *A. virens* activity (Last et al. 1999),
193 therefore behaviour recordings were categorised according to season instead of exposure
194 period. In terms of the corresponding exposure durations, winter corresponds with the 3
195 month exposure period, spring to the 6 month exposure period and summer to the 9 month
196 exposure period. No feeding activity (TGP, in seconds) was recorded in any season for the
197 HCZ treatment, or for HC in winter and spring due to no worms emerging from their burrows.
198 Generally, across all treatments, TGP was higher in spring and summer than winter (Figure
199 5, Table S4). When split by both metal and season, GLM analysis revealed significant
200 differences for copper and zinc combined in winter ($F_{2,7}$ = 15.3, p = 0.027), with post hoc
201 analysis revealing that worms in the control took significantly longer to grab a pellet than
202 those in the MCZ treatment. Generally, except for a significant reduction in TGP for the MCZ
203 treatment (winter), our data do not support any behavioural effects from metals. Water
204 temperature and photoperiod underpin activity levels in *A. virens* (Last and Olive 1999),
205 nevertheless our separate season-specific analysis failed to find consistent significant
206 effects.

207

208



209

210 Figure 5. Mean time to grab pellet in seconds (±SEM), per box for each treatment and month. n = 3 boxes per
211 treatment per sampling point, expect for HC in winter and spring, and HCZ in all three seasons, where no
212 activity was recorded. The control values for each exposure period are presented multiple times to enable post
213 hoc analysis to be represented. ND= no data due to no worms emerged.

214

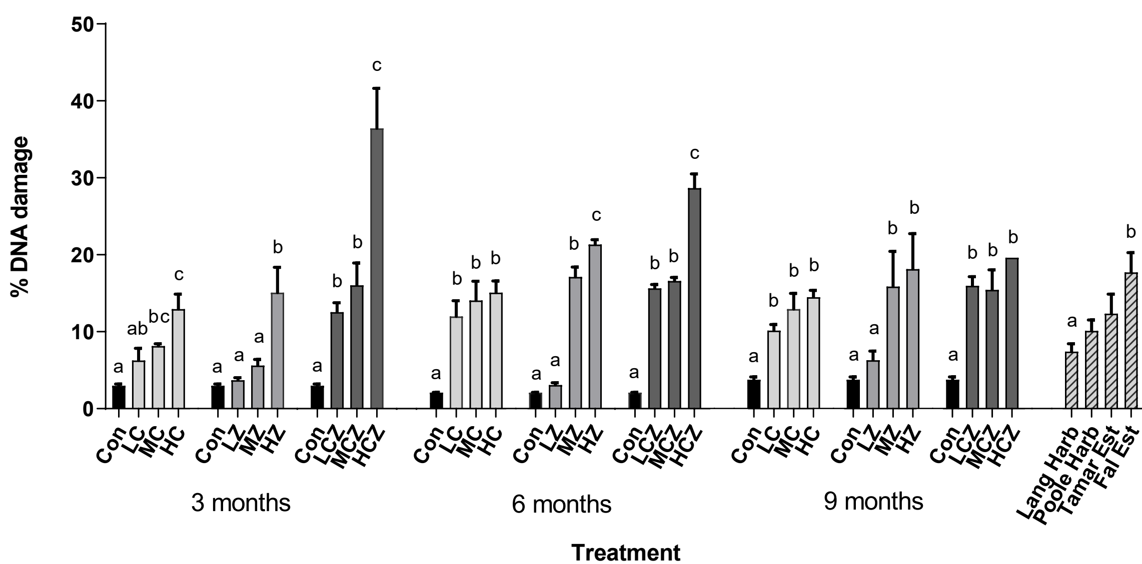
215 *3.2.7. DNA damage*

216 Of all the biomarkers and endpoints assessed, only DNA damage was consistently induced
217 across all three exposure periods for zinc and copper and in combination, which was
218 consistent with the DNA damage seen from worms collected at the field sites. Reanalysis of
219 Watson et al. (2018) data, split by exposure length and metal treatment (Figure 6, Table S4),
220 reveals that the HZ treatment induces significant DNA damage at all exposure periods, and
221 MZ induced significant increases compared to the control at 6 and 9 months. Analysis of the
222 copper and copper and zinc combined treatments reveals significant DNA damage for all
223 treatments and exposure periods, except for LC at 3 months.

224 DNA damage resulting from toxicant exposure has been reported in many invertebrates (see
225 review by Martins and Costa [2015]). Data here shows that copper and zinc produce
226 genotoxic effects at concentrations routinely found in coastal regions, however, the growth

227 assessment data confirm that *A. virens* still grows. A fundamental next step is to investigate
228 what level of DNA damage *A. virens* can tolerate and at what threshold it has other
229 physiological impacts. Elucidating the functional effect of DNA damage on an individual *A.*
230 *virens* and comparing this to other (potentially less tolerant) benthic species is critical to
231 scaling the effects to the population/ecological system level.

232



233

234 Figure 6. Mean percentage tail DNA damage (±SEM) per box for each treatment and month, and for worms
235 collected from field sites. n = 3 boxes per sampling point, expect for HCZ 6 month and HC 9 month which were
236 2 boxes and 1 box for HCZ 9 months. Number of worms sampled per box varies from 1 to 5 (mean of 2.4). The
237 control values for each exposure period are presented multiple times to enable post hoc analysis to be
238 represented.

239

240

241 *3.3 Multi-biomarker and endpoint comparisons*

242 The observed effects on biomarkers and endpoints in this study combined with those from
243 Watson et al. (2018) reveal changes at different biological organisation levels using chronic
244 ecotoxicological studies. *A. virens* was selected here for its ecological importance and as a
245 model species to test toxicity. The lack of definitive results for most biomarkers and

246 endpoints, combined with considerable growth, advocates that it is a relatively hardy species
247 in terms of metal contamination. Additional research specifically at the transcriptomic and
248 genomic levels using *A. virens* would begin to resolve the underlying mechanisms of
249 tolerance for this species. Comparing these mechanisms with less tolerant species will go
250 some way to understanding the whole community response to metal exposure.

251 A multiple biomarker/endpoint approach can also assess biomarker sensitivity. Various
252 indices have been developed to simplify the measured biological responses (e.g. Dagnini et
253 al. 2007; Hagger et al. 2008; Schettino et al. 2012). They can be used to rank the toxicity of
254 contaminants (e.g. Parolini et al. 2013) or evaluate organism health status from
255 contaminated sites (Hagger et al. 2008). Pini et al. (2014) used the Biomarker Response Index
256 (BRI) developed by Hagger et al. (2008) to evaluate biomarkers and endpoints used here,
257 but their analysis was limited as it compares all treatments and excluded mortality and
258 behaviour data. Although the BRI and other approaches have significant merit, we have
259 generated a simplified analysis presented in Table 2. It guides users in the selection of the
260 appropriate biomarker/endpoint and chronic exposure period needed to identify copper
261 and zinc effects (and most likely other metals) for benthic polychaetes such as *A. virens*. To
262 make them more comparable, concentrations have been converted to total metal
263 concentrations based on Sutherland et al. (2010) and colour coded to link specifically to
264 SQGs and SQGHs (Sediment Quality Guideline High) developed by Simpson et al. (2013)
265 (green: mean sediment concentration <SQG; amber: mean sediment concentration >SQG,
266 <SQGH; red: mean sediment concentration >SQGH). Finally, we have assigned our field sites
267 to the low, medium and high concentration based on the mean concentrations reported in
268 Table 2.

269

270 Table 2. Biomarker/endpoint selection for metal treatments (low, medium and high) and exposure

271 duration (3, 6 and 9 months).

Metal	Exposure (month)	Low (LH, PH)	Medium (TE)	High (FE)
Cu	3		DNA	Survivorship, DNA, Protein, Lipid, AChE
	6	DNA	DNA	Survivorship, DNA
	9	DNA, Protein	DNA, Protein	DNA, Protein
Zn	3			DNA
	6			DNA
	9		DNA	DNA
Cu/Zn	3	DNA	DNA	Survivorship, DNA
	6		DNA, Behaviour	Survivorship, DNA, MTLP
	9	DNA	Survivorship, DNA	Survivorship, DNA

272

273 Footnote: Data combined with Watson et al (2018) to provide an overview of all biomarker/endpoint
274 responses. Biomarkers/endpoints listed are those with statistically significant ($p<0.05$) effects from the control.
275 Green = measured bioavailable metal concentrations <SQG; Amber = >SQG, but <SQGH; Red = >SQGH. Red and
276 orange for medium Cu/Zn is where Cu is >SQGH, but Zn is >SQG, but <SQGH. SQG values (mg kg^{-1} dry sediment
277 adjusted for total digestion) are taken from Simpson et al. (2013): 65 for Cu and 200 for Zn; and SQGH: 270 for
278 Cu and 410 for Zn. Bioavailable metal concentrations of field sites (LH: Langstone Harbour; PH: Poole Harbour;
279 TE: Tamar estuary; FE: Fal estuary) are matched to target metal treatment concentrations.

280

281 It is clear from the 17 significant biomarker/endpoint responses for copper compared to four
282 for zinc, that copper is more toxic to *A. virens*. For copper, significant changes in biomarkers
283 and endpoints were seen in all concentration and exposure period combinations (except LC
284 3-month exposure), whilst biomarker/endpoint changes were only seen for HZ (all exposure
285 periods) and the 9-month exposure for MZ. These data support previous work of Watson et
286 al. (2008), using *A. virens* larval stages, that shows copper is more toxic, but also reflect
287 copper's higher aquatic toxicity ranking (Johnson et al. 2017).

288 The most sensitive biomarker for copper across all concentrations and exposure periods
289 (except 3 months) is DNA damage, especially in the copper/zinc combination. For zinc alone,
290 DNA damage is only sensitive for concentrations represented by HZ for all exposure periods,
291 but with an exposure of 9 months for the MZ concentration. Protein concentration will also
292 respond to all copper concentrations, but the exposure period must be extended. Mortality
293 is relevant for the MCZ concentration after a 9-month exposure but will only be sufficiently
294 sensitive for shorter exposures at high concentrations represented by HZ and HCZ. AChE
295 activity and lipid concentrations will only be sensitive to the highest copper concentration
296 (HC) and with the shortest exposure period, whilst increases in MTLP concentration occur
297 for HCZ and 6-month exposure. Finally, behavioural changes will only be sensitive at the MCZ
298 and during the winter period.

299 Table 2 also highlights *A. virens'* biomarker/endpoint responses in relation to the SQGs.
300 Exposure to sediment-bound copper at concentrations below the SQG threshold of 65 mg
301 kg^{-1} induces significant DNA damage and reduces the tissue protein concentration. In
302 contrast, the only significant biomarker response (DNA damage) for zinc is observed when
303 SQGHs are exceeded unless the exposure is extended to 9 months. These data show that
304 copper via sediment exposure produces genotoxic and metabolic effects at sediment
305 concentrations routinely found in coastal regions (e.g. Bryan and Langston 1992; Miller et al.
306 2000; Caplat et al. 2005; Larner et al. 2007; Naidu et al. 2012; Pan and Wang 2012; Briant et
307 al. 2013). The bioavailable sediment concentrations in the LC treatments equate to those
308 found in Langstone and Poole Harbours and represent low levels of contamination and,
309 according to the SQGs, should pose a minimal/limited toxic risk to benthos. It is, therefore,
310 highly concerning they induce DNA damage and protein concentration changes. Even for

311 concentrations represented by the MC treatment (Tamar Estuary), the SQGs suggest only a

312 *potential* for toxic effects.

313

314 **4. Conclusion**

315 Our data show that biomarker and endpoint changes in *A. virens* occur after chronic

316 exposure at environmentally relevant concentrations of copper and zinc. These data further

317 support the need to revisit SQGs at least for copper, however, the greater challenge remains:

318 to translate these multiple biomarker and endpoint effects at different organisational levels

319 into population and community effects. Our field data combined with those from Lewis and

320 Galloway (2008) show that *A. virens* is tolerant to high levels of contamination. Ragworms

321 are dominant benthic species, but tolerance is individual, population and species-specific

322 and so it should not be assumed that other benthic species are as tolerant. Although we are

323 beginning to understand the role of variability (inter-individual, inter-population and inter-

324 species) that discriminates a 'winner' from a 'loser'; the Anthropocene is taking

325 ecotoxicology, species and biotope communities into unchartered waters. Our data for

326 metal toxicity combined with others on climate change (e.g. Nielson et al. 2019) indicate

327 that *A. virens* could well be on the winning side, but we can only hope that many benthic

328 species also share its capacity to be a future 'winner'.

329

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336

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