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Cu
Zn
DNA
damage

Chronic exposure to copper and zinc induces DNA damage in the polychaete *Alitta virens* and the implications for future toxicity of coastal sites.

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Keywords

Nereis virens, porewater, bioavailability, genotoxic, antifouling, nanoparticles

Abstract

Copper and zinc are metals that have been traditionally thought of as past contamination legacies. However, their industrial use is still extensive and current applications (e.g. nanoparticles and antifouling paints) have become additional marine environment delivery routes. Determining a pollutant's genotoxicity is an ecotoxicological priority, but in marine benthic systems putative substances responsible for sediment genotoxicity have rarely been identified. Studies that use sediment as the delivery matrix combined with exposures over life-history relevant timescales are also missing for metals. Here we assess copper and zinc's genotoxicity by exposing the ecologically important polychaete *Alitta virens* to sediment spiked with environmentally relevant concentrations for 9 months. Target bioavailable sediment and subsequent porewater concentrations reflect the global contamination range for coasts, whilst tissue concentrations, although elevated, were comparable with other polychaetes. Survival generally reduced as concentrations increased, but monthly analyses show that growth was not significantly different between treatments. The differential treatment mortality may have enabled the surviving worms in the high concentration treatments to capture more food thus removing any concentration treatment effects for biomass. Using the alkaline comet assay we confirm that both metals via the sediment are genotoxic at concentrations routinely found in coastal regions and this is supported by elevated DNA damage in worms from field sites. However, combined with the growth data it also highlights the tolerance of *A. virens* to DNA damage. Finally, using long term (decadal) monitoring data we show stable or increasing sediment concentrations of these metals for many areas. This will potentially mean coastal sediment is a significant mutagenic hazard to the

benthic community for decades to come. An urgent reappraisal of the current input sources for these 'old pollutants' is, therefore, required.

Capsule

Chronic exposure of zinc and copper via sediment at environmentally relevant concentrations induces DNA damage in a marine polychaete.

Introduction

DNA damage impacts all processes as it affects metabolism; induces mutations; carcinogenesis and teratogenesis; and alters gene functions (Jha, 2008*;* Luoma and Rainbow, 2008; Martins and Costa, 2015). Determining a pollutant's genotoxicity, therefore, continues to be an environmental risk priority. Traditionally thought of as past contamination legacies (Walker et al., 2006) copper and zinc are highly toxic metals (Reish and Gerlinger, 1997; King, et al. 2004; Watson et al., 2008; 2013) that are still used extensively in industry, in addition to being released into coastal environments via new applications such as nanoparticles (Baker et al., 2014). As sediments are considered sinks for metals these inputs often lead to substantially elevated concentrations (Bryan and Langston, 1992). These metals, therefore, remain a great concern in terms of ecotoxicological risk assessment for economically and ecologically important coastal benthic systems (Walker et al., 2006; Luoma and Rainbow, 2008). Crucially, these metals are also predicted to be more bioavailable under ocean acidification scenarios (Millero et al., 2009).

Although copper and zinc have been shown to induce genotoxic damage in marine organisms, studies used direct seawater exposures that were also short-term (e.g. Caldwell et al., 2011; Mai et al., 2012; Gomes et al., 2013; Schwarz et al., 2013; Anjos et al., 2014;

Ruiz et al., 2015; Li et al., 2018). Sediment-dwelling macrofauna are continuously exposed to sediment-bound contaminants (Dean, 2008) so assessing toxicity using sediment as the delivery matrix over extended periods that represent a substantial part of an organism's life is essential. Polychaetes are dominant benthic invertebrates and are contaminant vectors as well as being ecologically and commercially important (Watson et al., 2017). *Alitta* (*Nereis*) *virens* was selected for its ecological relevance (Lewis and Watson, 2012) as it inhabits coastal muddy sand throughout the northern hemisphere. It is also a dominant species by biomass and size replacing *Hediste diversicolor* in high salinity areas (Kristensen, 1994). In addition, Lewis and Galloway (2008) showed that worms from contaminated sites have elevated DNA damage, although they did not identify the causal agent.

Ecotoxicological studies must mimic the exposure conditions for benthic organisms. The sediment-spiking approach has limitations (U.S.EPA, 2005), but its importance in the environmental pollution field is recognised (Fernandes, 1997) and commonly used (e.g. Simpson et al., 2004; Hutchins et al., 2009). For the first time we evaluate copper and zinc's chronic genotoxicity to benthic systems via a 9 month sediment-spiking exposure. We assess this by quantifying growth rates and DNA damage (e.g. single and double strand breaks) in exposed worms whilst monitoring the presumed bioavailable fraction in sediment, porewater and tissue concentrations. Together, these deliver critical information for reviewing SQGs (Sediment Quality Guidelines) as well as providing important data for monitoring the long-term effects on macrofaunal species and the potential tolerance of benthic polychaete species to metal exposure and consequent DNA damage. Finally, coastal sediments have suffered from severe industrial pollution for generations. Using long term concentration data from multiple sites collected from the UK Environment Agency (EA), we also highlight the ability of these metals to continue to impact benthic systems.

Materials and methods

Mesocosm study: sediment and organism collection, experimental setup

Sediment (upper 10 cm) from Chichester Harbour (50 48’43.23"N, 0 52’30.78"W) was collected and stored at 4°C in the dark until spiking. As worm weight is important in bioaccumulation processes (Poirier et al., 2006) cultured *Alitta virens* of 1-2 g were used. These were purchased from Dragon Baits Ltd and stored for 48 hours (unfed) in a flow-through system with a small amount of sediment before being randomly selected for each treatment. The target metal concentrations for spiking were based on bioavailable concentrations measured by Pini et al. (2015) at a number of sites in the UK spanning the full range of contamination and are shown in Table 1. 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 used with nine exposure boxes per treatment. Each polyethylene box (300 x 200 x 120 mm) was drilled with four 1 cm diameter holes covered with mesh and seawater-conditioned for a week. Five kilogrammes of sediment per box was spiked and mixed individually at 4°C. Sediments were spiked with copper chloride dihydrate ($CuCl_2.2H_2O$) or zinc chloride (ZnCl) or combined solutions and with seawater as a control. A 5:1 ratio of sediment/seawater was used with each box mixed for one minute with a paint mixer attached to a power drill 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 with dead worms replaced within 48 hours. Weekly measurements were taken for temperature, pH, salinity and dissolved oxygen (Table S1). 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). Sediment and porewater samples were collected at the start (22nd October 2012, month 0) to give initial metal concentrations. One box of each treatment from each tank was then sacrificially sampled every three months (22nd of January, April and July 2013). 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.

Mesocosm study: sediment, porewater and worm tissue processing

At each mesocosm sampling point sediment and porewater (0, 3, 6 and 9 months); and worms (3, 6 and 9 months) were sampled for metal analysis. Subsamples of <63 µm sediment (taken from a 5cm diameter x 10 cm deep core) and porewater (extracted using a pore-extractor device [Nayar et al., 2006]) for each box were stored at -20°C until analysis. Worms were removed and counted and left overnight at 4°C for gut depuration. Each worm (including those from field sites) was weighed before collecting coelomic fluid and tissue (2-3 cm of the anterior section), which were snap frozen in liquid nitrogen and stored at -80°C.

The ecological risks of metals in sediments and the potential for bioaccumulation depend on sediment characteristics and bioavailability (Amiard et al., 2007). Biodynamic modelling can be used to predict metal accumulation (e.g. Casado-Martinez et al., 2010), but this is usually with dietary and dissolved pathways. Total metal is useful for many geochemical applications, but the speciation of metals is more relevant in terms of what is readily available (the mobile fractions) for uptake by sediment-dwelling organisms (Luoma and

Rainbow, 2008). The sum of the 3-stage BCR (Bureau of Reference) extraction scheme (Pueyo et al., 2001) has been stated to be the presumed bioavailable (termed 'bioavailable' from now on) (Zimmerman and Weindorf, 2010). However, to complement bioavailability a full assessment requires porewater concentrations too (King et al., 2004). 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). The BCR procedure assesses the distribution of metals in three fractions: (1) exchangeable; (2) reducible and (3) oxidizable and summed for the bioavailable concentration. 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.

Mesocosm study: mortality, growth and DNA damage

Mortality rate was defined as the number of dead worms in each box at each sampling point. To calculate growth (percentage weight gained), the biomass of all worms from each box was recorded at the experiment's start and after each sampling time and then divided by the number surviving. The alkaline version of the comet assay was used to measure genotoxicity by detecting single (strand breaks and incomplete excision repair sites) and double strand breaks (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-minute UV light exposure as a positive control) with results presented in % DNA damage the Comet IV software.

Long term dataset analysis

Trace element sediment data for the southern UK region from the EA and the Marine Environment Monitoring and Assessment National (MERMAN) databases were used. Data entries from each were merged producing one database of 335 coastal and offshore UK sites covering multiple trace element concentrations in sediment (dry weight). A number of sites were selected for copper and zinc that had been monitored >10 years and represent low, medium and high contamination levels (Table S7). Although the extraction procedure used for these metals changed in the late 1990s (hydrofluoric acid replacing hot nitric acid /aqua regia), comparative analyses on samples and certified reference materials by Cook et al. (1997) gave equivalent results, thus allowing us to include recent samples.

Statistical analysis

Data were analysed using Minitab v17 and, if required, transformed if parametric assumptions were not met. Sediment, porewater and tissue metal concentrations for each time point were analysed using General Linear Models (GLMs) with tank and treatment as fixed factors followed by a Tukey HSD pairwise test of means. Differences between percentage mortality and percentage DNA damage (both arcsine transformed) were also analysed. The long term data were analysed using bootstrapped median regression. Quantile (including median) regression presents several advantages for environmental data:

it is robust to outliers; avoids parametric distribution assumptions; estimates rates of change in all parts of the response variable distribution and is invariant to monotonic transformations (Koenker and Bassett, 1978; Cade and Noon, 2003).

Results and discussion

Metal bioavailability

A full analysis (see Table S2) of the mesocosm sediment (based on UK Accreditation Service - methodology, ISO/IEC 17025:2005) confirms a poorly sorted sediment with high levels of silt/clay (55% < 63 µm; Md =48 µm) and low levels of metal and organic pollution except for TBT (tributyltin as cation) which exceeded the SQG (Simpson et al., 2013) and was higher than similar areas with greater perceived impacts (Langston et al., 2005). For copper and zinc, both the UK Accreditation Service aqua regia (Table S2) extraction and the BCR extraction of the control treatment at time 0 (Cu: 8 mg kg^{-1}; zinc: 23 mg kg^{-1}, Table 1) confirm that the background concentrations of copper and zinc were low and compared to other marine sites (Bryan and Langston, 1992; McQuillan et al., 2014) the sediment has very low contamination. Despite the elevated TBT seen in the sediment collected for the mesocosm study, it does not change the comparisons between control and other treatments. Although a known genotoxic substance (e.g. Hagger et al., 2002) with potential for mediating effects with the metals, it is unlikely that TBT's presence, was contributing substantially to baseline damage in the control as field sites with low TBT concentrations (Poole Harbour: 30 µg kg^{-1} [Langston et al., 2005]; Tamar Estuary: 6 µg kg^{-1}, [EA database]) have elevated DNA damage (see Figure 1).

Target concentrations of the spiked sediment reflect global coastal contamination (e.g. Bryan and Langston, 1992; Caplat et al., 2005; Pan and Wang 2012). Whilst the measured mean bioavailable concentrations in the sediment for treatments across the nine months approached the target values (Table 1), all were lower with some such as HZ and the zinc concentration within the HCZ treatment being less than half the target 1160 mg kg^{-1}. The subsequent decreases from initial high values and reductions in concentrations over the nine months for nearly all treatments highlight metal loss from the system. Longer equilibrium times as shown by Simpson et al. (2004) and increased mixing during the spiking process could have reduced these losses, although continuous water flow; permanent sediment submersion; ageing of the sediment and elevated bioturbation due to the high worm densities (Remaili et al., 2016) are likely to be the major drivers as well as possible adsorption to polyethylene boxes. Regardless of the losses, the bioavailable sediment concentrations over the nine months match real-world contamination levels (see field sites in Table 1) and the distinct risk levels of SQGs. SQG values (mg kg^{-1} dry sediment) from Simpson et al. (2013) were selected to compare with our data: SQG (Sediment Quality Guideline): 65 for Cu and 200 for Zn; and SQGH (Sediment Quality Guideline High value): 270 for Cu and 410 for Zn. Accounting for bioavailable concentrations which represent 83% and 80% of total concentrations for copper and zinc, respectively as calculated for the BCR-701 sediment by Sutherland (2010), comparisons show that the concentrations of both metals in the Control treatment, Langstone and Poole Harbour sediments were below SQGs, indicating minimal risk for toxic effects. In addition, the concentrations at 6 and 9 months for LC, LZ and zinc for LCZ and MCZ were also below SQGs. For the other sampling dates for these treatments and every date for MC, MZ and both metals for the LCZ and MCZ treatments, all had bioavailable metal concentrations within the transition zone (Batley et al., 2005), i.e. between the SQG and SQGH. Only in HC, HZ and HCZ did both metal concentrations for all time points exceed the SQGH. The significant differences between treatments shown in Tables 1 and S3 highlight clear groupings of contamination levels that are globally relevant. The control and low treatments (at least for the 6 and 9 month exposure) reflect minimal contamination seen in Solent harbours (Pini et al., 2015) and other systems (e.g. Bryan and Langston, 1992; Haynes et al., 1995; Naidu et al., 2012) and, according to SQGs, will pose minimal risk to benthos. The other low treatment time points are representative of sites such as Poole Harbour and others (e.g. Bryan and Langston, 1992; Larner et al., 2007; Pan and Wang, 2012) that have elevated, but low levels of contamination falling just within the transition zone. Medium treatments replicate higher contamination from areas with substantial past or ongoing inputs with greater potential for toxic effects as they are further within the transition zone (e.g. Bryan and Langston, 1992; Pan and Wang, 2012; Briant et al., 2013). Finally, the high concentrations represent highly polluted areas: the Fal Estuary and others (e.g. Bryan and Langston, 1992; Miller et al., 2000; Wang, 2002; Pan and Wang, 2012), with concentrations well above SQGHs that will have significant adverse effects.

Evaluating the bioaccumulation of metals also requires porewater concentrations as it is a key benthic exposure route (Chapman et al., 2002). As the sediment bioavailable fraction directly influences porewater levels, at least for copper (Pini et al., 2015) it is not surprising that concentrations varied significantly between treatments (Tables 1 and S4). Generally, the porewater concentrations of metals in the LC, LZ, MC, and MZ treatments and copper within the LCZ and MCZ treatments were not significantly different from each other and similar to the control, as well as corresponding to the concentrations found in all field sites and elsewhere (e.g. Simpson et al., 2002). Only in the high treatments for 0, 3 and sometimes 6 months were both metals significantly higher than the other treatments and exceed those from the most contaminated field site (Fal estuary), in some cases by several fold. In contrast, porewater concentrations of zinc across all combined treatments were generally much higher for most of the exposure period and likely to be driven by the spiking process (U.S.EPA, 2005). Porewater was also significantly strongly correlated with overlying water concentrations (Pini et al., 2015) suggesting that some metal losses could have been incorporated back into the porewater, possibly via high *A. virens* burrow irrigation (Kristensen and Kostka, 2005).

No specific threshold levels have been produced for porewater, however, concentrations can be compared to Ecotoxicological Assessment Criteria (EAC) for dissolved pollutants. A simple comparison reveals that treatment and site concentrations are close to or exceed the upper thresholds for copper (0.1–1.0 μg l^{-1}) proposed by OSPAR (Matthiessen et al., 1999) representing a potential ecotoxicological risk. With a 0.5–5.0 μg l^{-1} EAC for zinc, only HZ and the combined treatments (at least during the early exposure months) represent a potential risk.

Tissue bioaccumulation

Tissue concentrations generally followed the spiked sediment concentrations with the specific time point analysis (3, 6 and 9 months) for the copper-only treatments mostly separating them in to two distinct groups: the control and LC; and the MC and HC treatments (Tables 1 and S5). In contrast, the copper tissue concentrations from the combined treatments were much more variable between worms and boxes. Combined with the low sample numbers for the high concentration, this leads to a lack of clear groupings, although treatments were still elevated against the control. Consistently low concentrations from the field-collected worms support data of Pini et al. (2015); that *A. virens* can regulate copper leading to low tissue concentrations even with high sediment bioavailable concentrations. However, these reduced-uptake /enhanced-detoxification processes seem to have been interrupted within the medium and high treatments resulting in high tissue concentrations. Nevertheless, these elevated concentrations still match those found in the related polychaete *H. diversicolor* from many sites (Mouneyrac et al., 2003; Amiard et al., 2007; Rainbow et al., 2009) and other species (Garcês and Costa, 2009, Giangrande et al., 2017). They also support an alternative explanation: the high tissue concentrations are due to an increase in the number and density of detoxificatory Cu-containing granules as shown for *H. diversicolor* (Mourneyrac et al., 2003). As these are found close to the epicuticle these would have been included within the measured tissue concentration, so further work is required to see if this storage approach to detoxificiation is induced in *A. virens* after significant exposure as suggested by McQuillan (2014) for *H. diversicolor*.

Zinc tissue concentrations were similar to other polychaetes (e.g. Berthet et al., 2003; Amiard et al., 2007; Garcês and Costa, 2009; Rainbow et al., 2009). However, unlike copper, zinc concentrations closely tracked field-collected worms and those of Pini et al. (2015). Even though heads were removed which house the zinc-containing jaws (Bryan and Gibbs, 1979) reducing the overall concentrations recorded, these data do suggest that *A. virens* is performing some zinc regulation when exposed to high concentrations in the sediment with this regulation also seen in other species (Berthet et al., 2003; Amiard et al., 1987).

Although less evident in the combined treatments, tissue concentrations for both metals reduced over time. A number of hypotheses require further investigation including: tissue concentrations tracking the bioavailable sediment concentrations; delayed regulation via detoxification processes to remove the metals accumulated; surviving worms had lower tissue concentrations.

Effects of exposure

Both metals and in combination had significant impacts on survivorship over and above background mortality, except for month 9 where the background survival (in the control) had dropped to 63% (Table 2). The likely explanation for this lack of a significant effect at 9 months is post-spawning mortality as reproductive behaviours (males swimming, sperm release) were observed between April and July, obfuscating treatment mortality. The precocious gametogenesis and spawning is likely to be driven by high ration levels as rapid reproductive maturation can be achieved in a few months in commercial culture (P. Cowin, Pers. Comm).

Pairwise comparisons for treatments at 3 and 6 months show few and inconsistent groupings. Although the relatively low starting numbers per box gives reduced sensitivity for the effects on mortality, generally mortality was highest in the HC and HCZ treatments, which were distinct from the others. Copper and zinc interactions have been reported as additive or synergistic (Fukunaga et al., 2011). The comparable mortality for HC and HCZ and the lower mortality for the HZ treatment (at month 3) would indicate that it is the copper that was the primary agent in inducing mortality. This is supported by the similar tissue levels for HC and HCZ treatments (Table 1) and could be explained by inter-metal competition when sharing a mode of action (Walker et al., 2006).

Figure 1 and Table S6 show highly significant effects of treatment for the percentage tail DNA damage. Levels of damage over the three sampling periods in the control (mean per box) ranged from 1.7-4.2% as background compared to the highest (46%) from a box from the HCZ treatment in month 3. Across all three sampling points levels of damage were not significantly different between the Control and LZ treatments. For month 6 these were significantly different from all other treatments, but for the other two sampling periods they were also not significantly different from LC and MC treatments, with MZ for month 3 and HC for month 9 sharing this grouping. All other treatment/time combinations fall within a variety of subgroups and all had significantly higher levels of DNA damage, with some (e.g. HCZ treatment at 3 months) being significantly different from all other treatments. Pearson's correlations using the 9-month data confirm significant positive relationships of DNA damage and bioavailable sediment concentration of both metals (copper: $r = 0.775$; $p= 0.005$; zinc: $r = 0.598$; $p= 0.04$). Figure 1 also confirms that equivalent levels of DNA damage were present in worms from the field sites (means range from 7.4% [Langstone Harbour] to 17.6% for the Fal Estuary).

DNA damage resulting from toxicant exposure has been reported in many invertebrates (see review by Martins and Costa [2015]) but numerous marine studies using sediment have not identified the causal agent. For the first time our data show that zinc and copper via sediment exposure produce genotoxic effects at sediment concentrations routinely found in coastal regions (Bryan and Langston, 1992; Haynes, et al. 1995; Miller et al., 2000; Wang, 2002; Caplat et al., 2005; Larner et al., 2007; Naidu et al., 2012; Pan and Wang, 2012; Briant et al., 2013). Specifically, the month 6 analysis shows increased levels of damage for all treatments compared to the LZ and Control treatments supported by the level of damage measured in worms from the field sites and other polluted locations (Lewis and Galloway, 2008). As the bioavailable sediment concentrations in the LC, LCZ treatments and sites such as Poole Harbour and the Tamar estuary are representative of low levels of contamination and, according to the SQGs, should pose a minimal/limited toxic risk to benthos it is highly concerning that they induce DNA damage. Even for medium treatments that are comparable with coastal regions with higher contamination, the SQGs suggest only a *potential* for toxic effects. In contrast, both metals and in combination routinely induced up to a 500% increase in DNA damage at environmentally relevant concentrations indicating that a review of copper and zinc SQGs in light of these genotoxic effects is required.

Reviews by Chen and White (2004) and Martins and Costa (2015) highlight numerous studies using 'naturally' contaminated sediments that have not identified the putative genotoxic chemical. Whilst associated water-borne exposure trials and analytical chemistry can help identify chemicals involved, the diversity of pollutants routinely found within coastal sediments makes it challenging to tease apart the most important contributors to genotoxicity. Our data indicate that many of these studies could, therefore, have substantially underestimated copper and zinc's contribution to the DNA damage recorded. Ultimately, this will lead to an erroneous assessment of the genotoxicant risk attributable to these metals, impacting consequent management decisions for mitigation.

Copper and zinc are two of the most studied metals in terms of toxicity and for genotoxicity. Metals generate ROS (Reactive Oxygen Species) by interfering with the electron transport chain, or convert scarcely genotoxic ROS (e.g. H_2O_2) to the strong DNA oxidant hydroxyl radical, •OH (Cadet et al., 2010). Metal ions are well known inducers of oxidative stress (Lushchak, 2011) overwhelming an organism' antioxidant defences leading to DNA, lipid and protein damage (Stohs and Bagchi, 1995) if the total rate of uptake exceeds the combined rates of detoxification and excretion (Rainbow and Luoma, 2011). Metals can also exert pro-oncogenic effects by competing with Zn-finger domain repair enzymes (Hartwig et al., 2002). Studies have also linked chromosomal aberrations with survival and developmental effects in invertebrates (Hagger et al., 2002; Jha et al., 2000) and sediments contaminated with mutagens are known to cause a wide range of adverse effects including: DNA adducts and strand breaks (Reichert et al., 1998; Lee and Steinert, 2003); chromosomal aberrations (Hose and Brown, 1998); and cancer (Reichert et al., 1998). Despite the significant increases in DNA damage seen for both metals and in combination, biomass increases were still observed across treatments with no separate monthly analyses showing any significant treatment differences (Table 2). This is supported by the lack of significant correlations (using a Pearson's correlation, 9 month data only) between percentage biomass change and bioavailable sediment metal concentration for copper ($r = 0.264$; $p= 0.406$) and zinc ($r = -0.199$; $p= 0.558$). Growth in some treatments was substantial with worms gaining over 600% in weight in just 3 months, but the two lowest biomass increases were in worms from the HC and HCZ treatments and this is supported by a reduction in foraging and out-of-burrow activity (Pini, 2014) in worms from these treatments. Copper has been shown to cause sub-lethal behavioural effects in polychaetes including hypo-activity and abnormal crawling (Bonnard et al., 2009). Nevertheless, the differential treatment mortality may have enabled the surviving worms in the high concentration treatments to capture more food (Miron et al., 1991) (via reduced inter-individual competition) thus removing concentration treatment effects for biomass. As the mesocosm design used in this study did not separate individual worms, future work would benefit from individual exposures to prevent inter-individual interactions.

Our experimental data support other polychaete studies of metal exposure inducing DNA damage (e.g. Cong et al., 2011; 2014), but our long term exposure approach has shown for the first time that polychaetes can survive (and grow) whilst still acquiring significant DNA damage. The alkaline comet assay has been used for 30 years in biomonitoring and genotoxicity testing and has been recommended to regulatory agencies (Martins and Costa, 2015; Speit et al., 2015). Despite this, some have questioned the comet assay's relevance as it also measures transient (repairable) DNA breaks (Jha, 2008). Whilst the repair capabilities of polychaete free cells are not understood (Lewis and Galloway, 2008), coelomocytes are no longer actively dividing (Hoeger, unpublished data cited in Lewis and Galloway [2008]), which may diminish their repair capacities leading to the accumulation of damage.

Our data are the first to show experimentally this long-term tolerance, but it is supported by the worms from our field sites and those of Lewis and Galloway (2008). Metal tolerance can be heritable for polychaetes (e.g. Bryan and Hummerstone, 1973) and earthworms (e.g. Killie et al., 2013) so it is possible the field populations also acquired tolerance to DNA damage as an adaptive strategy. However, this does not explain *A. virens* developing rapid tolerance within the mesocosms (i.e. over weeks and months) by continuing to grow. Increases in P53, a key effector molecule, activates transcription of genes that mediate DNA repair preventing conversion of damage to mutations (Harris and Levine, 2005). However, exposure of ionic copper did not significantly affect *p53* transcription levels in HepG2 cells, whereas P53 protein levels did increase (Song et al., 2009) supporting the suggestion that protein stabilisation and post-transcriptional modifications rather than changes in gene transcription are responsible. Recently, genome-wide DNA methylation has also been shown to be involved in arsenic tolerance of earthworms (Kille et al., 2013) with epigenetic responses having an ever-increasing role in the interactions of species with stressors (e.g.

Vandegehuchte and Janssen, 2014; Clark et al., 2018). Both transcriptomic and epigenetic mechanisms would, therefore, be obvious areas for future work on *A. virens* and genotoxic tolerance.

Future impacts

The EA-sampled sites presented in Figures 2 and 3 are all inshore except for Solent Channel included as a 'baseline' comparison (see Table S7 for details) with minimal contamination. Temporal variation was present for both metals and all sites, but the trend lines and confidence intervals confirm that Langstone Harbour and Chichester Harbour represent low; Furzey Island and Middlebere Lake in Poole Harbour and Plymouth Sound represent medium; and the Fal and Tamar estuaries represent high contamination. These reflect the mesocosm values (Table 1) and the global range (Bryan and Langston, 1992; Haynes, et al. 1995; Miller et al., 2000; Wang, 2002; Caplat et al., 2005; Larner et al., 2007; Naidu et al., 2012; Pan and Wang, 2012; Briant et al., 2013; Pini et al, 2015). When compared with SQG lines (65 mg kg^{-1} for Cu and 200 mg kg^{-1} for Zn) from Simpson et al. (2013), it is clear the benthic communities at multiple sites (e.g. Fal and Tamar estuaries, Plymouth Sound) have been exposed to copper and zinc contamination with the potential to induce effects for decades. We can also now apply DNA damage thresholds (DNA.DT) using the mean concentrations of the lowest mesocosm treatment that was statistically different from the control (57 mg kg^{-1} for Cu and 141 mg kg^{-1} for Zn). However, to be comparable these values have been converted to total metal concentrations based on the relationship between bioavailable and total metal concentration (Sutherland, 2010) and so become 68 mg kg^{-1} for Cu and 175 mg kg^{-1} for Zn. Using these values, Poole and Chichester Harbour sediments, in addition to the three sites above are close to these thresholds for copper, exceeding them in some years showing that these benthic communities have been chronically exposed across multiple generations to copper and zinc contamination which induces genotoxic damage in *A. virens*. Considering ragworms are thought to be pollution-tolerant members of the macrofauanl community the risks of chronic copper and zinc exposure inducing DNA damage for the rest of the benthos are considerable. Whilst our data have confirmed that *A. virens* can grow in spite of extensive and persistent DNA damage, there are significant metabolic costs associated with induced metal resistance (Pook et al., 2009). These costs may impact on a single species' fitness that could have substantial implications for the benthic community's resilience and function within internationally important estuary/soft-sediment systems.

It is often assumed that natural attenuation caused by declining industrial inputs and driven by legislation will see sediment concentrations of pollutants fall (Rainbow et al., 2011). For zinc all the sampled sites showed clear declines, however, this process is still extremely slow, which leads to a continuous and persistent mutagenic hazard for the benthic community. What is more concerning is that copper concentrations are barely changing (*p* values not significant) or increasing at most sites. For example, extrapolated values for Fursey Island will have seen concentrations exceed the SQG and the DNA damage thresholds by 2015. Since the ban on the use of TBT-anti-fouling paints, copper-based products now dominate the market. In addition, copper-based nanoparticles are increasingly being used in industrial applications (Gao et al., 2013). We are unable to identify metal sources, but boating activity, in addition to nanoparticles (Thit et al., 2015) would be areas to investigate. Considering Johnson et al. (2017) places copper and zinc as first and third, respectively, in their toxicity ranking, urgent action is needed to address *current* inputs to reverse the already widespread, but increasing mutagenic threat of coastal sediments.

Supporting Information

Table S1: Mesocosm physiochemical water quality data

Table S2: Mesocosm sediment data

Table S3: Bioavailable sediment concentrations

Table S4: Porewater concentrations

Table S5: Tissue concentrations

Table S6: DNA damage

Table S7: Long term data collection information

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Table 2. Survivourship and biomass change of worms from mesocosm treatments

	Survivorship				Growth			
	Month 3	**Month 6**	**Month 9**	**Mean**	**Month 3**	**Month 6**	**Month 9**	**Mean**
Treatment	$F_{9,18}$ =4., p= 0.004	$F_{9,18}$ =7, p< 0.001	$F_{9,18}$ =5, p=0.066	-	$F_{9,18}$ =0.8, p=0.598	$F_{9,18}$ =1, p=0.305	$F_{9,18}$ =1.4, p=0.172	-
Tank	$F_{2,18}$ =3, p=0.075	$F_{2,18}$ =0.05, p=0.95	$F_{2,18}$ =3, p=0.7	-	$F_{2,18}$ =0.85, p=0.44	$F_{2,18}$ =0.7, p=0.507	$F_{2,18}$ =0.4, p=0.04	-
Control	92 ± 4 [a]	100 ± 0 [a]	63 ± 7	85 ± 20	240 ± 18	245 ± 42	581 ± 23	356 ± 113
LC	71 ± 11 [a, b]	92 ± 8 [a, b]	54 ± 8	72 ± 19	243 ± 39	283 ± 14	347 ± 111	291± 30
LZ	75 ± 14 [a, b]	96 ± 4 [a, b]	42 ± 17	71 ± 27	275 ± 48	331 ± 51	562 ± 103	389 ± 88
LCZ	71 ± 11 [a, b]	71 ± 4 [a, b, c]	46 ± 8	63 ± 14	271 ± 21	331 ± 49	621 ± 56	407 ± 108
MC	88 ± 0 [a, b]	88 ± 7 [a, b]	46 ± 11	74 ± 24	230 ± 30	260 ± 30	687 ± 146	392 ± 148
MZ	83 ± 11 [a, b]	88 ± 7 [a, b]	42 ± 15	71 ± 25	280 ± 29	313 ± 19	614 ± 208	403 ± 106
MCZ	83 ± 17 [a]	71 ± 8 [a, b, c]	25 ± 7	60 ± 31	276 ± 24	352 ± 66	762 ± 252	464 ± 151
HC	29 ± 11 [b, c]	50 ± 7 [b, c]	33 ± 18	38 ± 11	231 ± 78	171 ± 67	267 ± 54	223 ± 28
HZ	71 ± 11 [a, b]	80 ± 15 [a, b]	30 ± 11	60 ± 27	350 ± 47	326 ± 72	747 ± 223	474 ± 136
HCZ	21 ± 8 [c]	13 ± 7 [c]	4 ± 4	13 ± 8	214 ± 54	215 ± 57	273 ± -	234 ± 19

Footnote. Mean percentage (± SEM) survivorship per box (N=3 per sampling point) of mesocosm treatments at month 3, 6 and 9 and as a mean of all months combined. Mean percentage (± SEM) weight gained (per 3, 6 and 9 month of worms per box (N=3 per sampling point, except for HCZ 6 month and HC 9 month which were 2 boxes and HCZ 9 month which was 1 box). General Linear Models (GLMs) of arcsine transformed percentages were used for tanks and 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) treatments for each month separately. Treatments (compare vertically per month only) that share the same letters are not significantly different from each other when analysed using Tukey HSD pairwise comparisons.

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 ^{a}	-	-	-	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 ± 60.6
	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: 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.

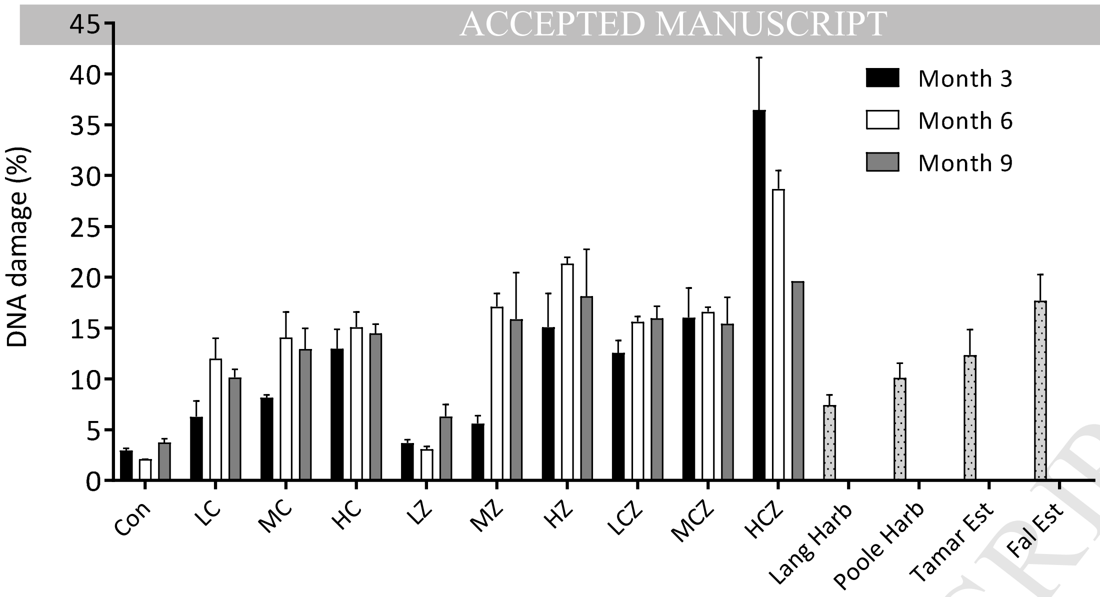

Figure 1. Mean percentage tail DNA damage (± SEM) per box for each treatment and time point and for worms collected from field sites. Mesocosm: n=3 boxes per sampling point, except for HCZ 6 month and HC 9 month which were 2 boxes and 1 box for HCZ 9 months. Number of worms sampled per box varies from 1 to 5 (mean of 2.4) Field sites were sampled between July-September 2013 with 6 worms sampled from each site, except Langstone Harbour with 5.

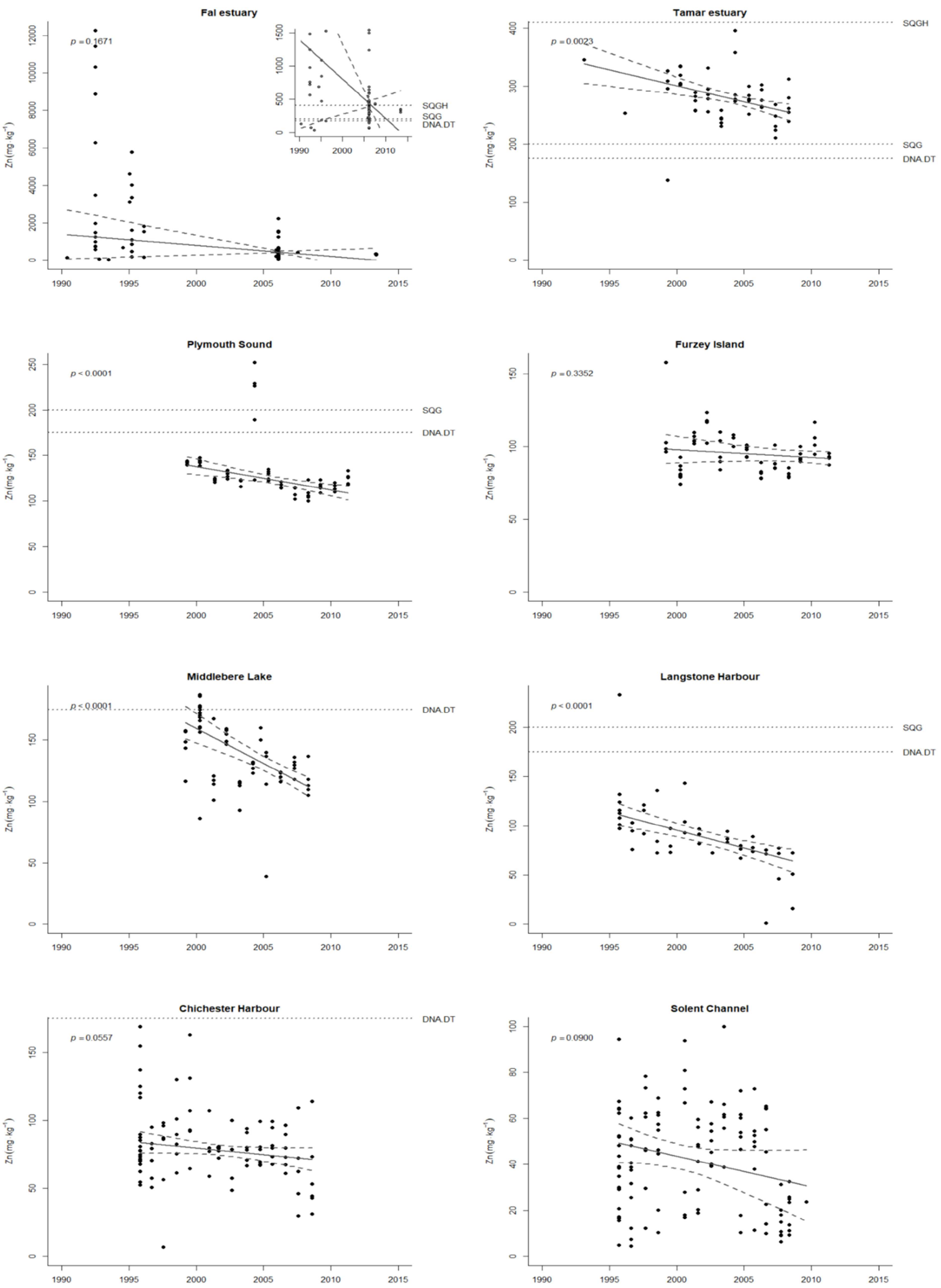

Figure 2. Temporal changes in sediment zinc concentrations (mg kg^{-1} dry weight of <63 µm fraction) from south coast of England sites. Inserted graphs focus on part of the concentration scale, excluding extreme values. Trend lines and confidence intervals generated with median regression models with an ANOVA confirming statistically significant temporal changes. SQG: indicates potential risk for toxic effects above concentration (200 mg kg^{-1}). SQGH: indicates toxic effects above concentration (410 mg kg^{-1}). DNA.DT: damage threshold represents lowest concentration adjusted for total metal concentration (175 mg kg^{-1}) from mesocosm treatment that induced statistically significant increases in DNA damage compared to the control.

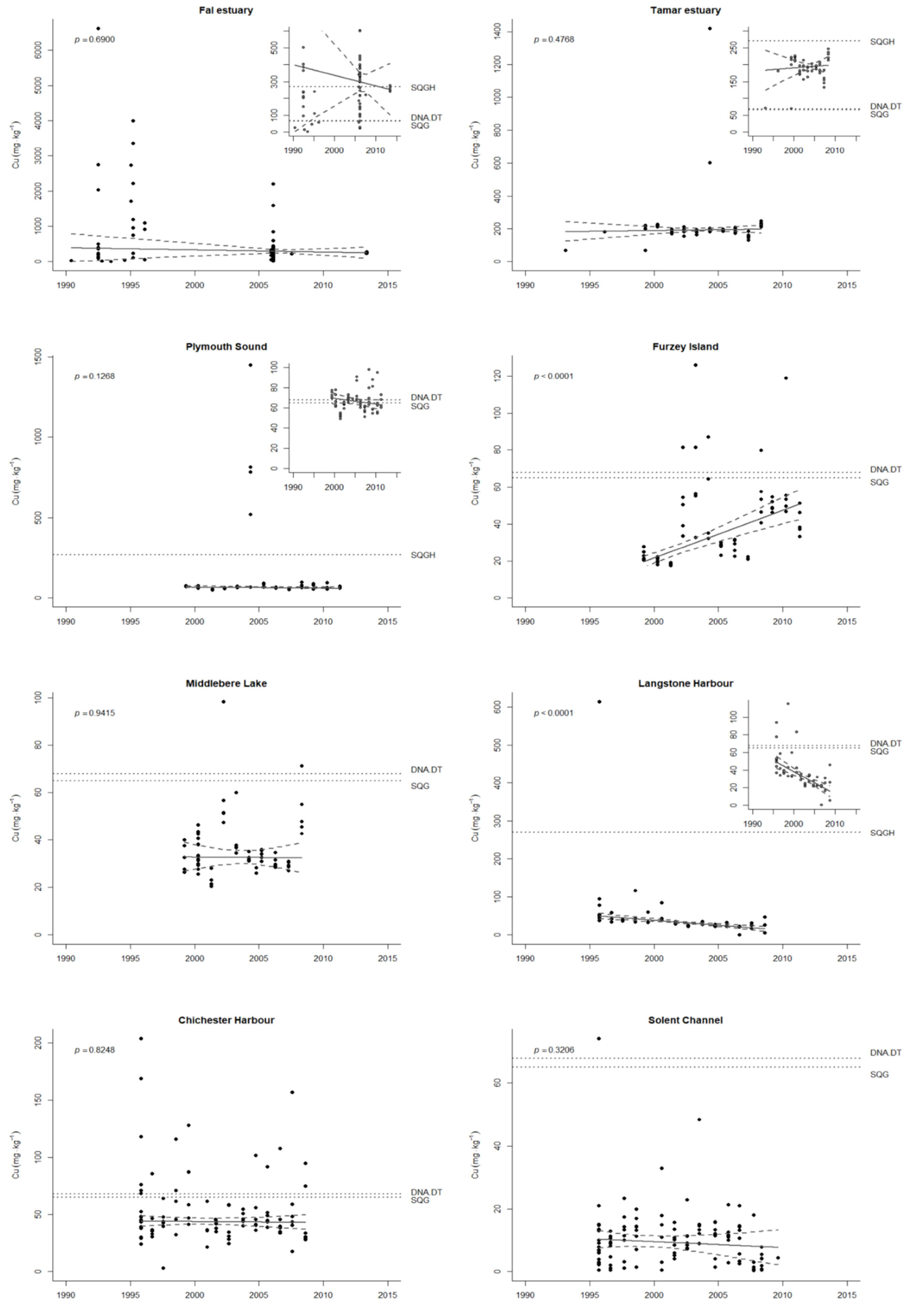

Figure 3. Temporal changes in sediment copper concentrations (mg kg^{-1} dry weight of <63 µm fraction) from south coast of England sites. Inserted graphs focus on part of the concentration scale, excluding extreme values. Trend lines and confidence intervals generated with median regression models with an ANOVA confirming statistically significant temporal changes. SQG: indicates potential risk for toxic effects above concentration (65 mg kg^{-1}). SQGH: indicates toxic effects above concentration (270 mg kg^{-1}). DNA.DT: damage threshold represents lowest concentration adjusted for total metal concentration (68 mg kg^{-1}) from mesocosm treatment that induced statistically significant increases in DNA damage compared to the control.

Highlights for Watson et al. (Environmental Pollution)

Effects of long-term (9 month) sediment exposure to copper and zinc were assessed.

Comet assay shows metals are genotoxic to the polychaete *Alitta virens*

Environmentally relevant sediment concentrations are genotoxic.

A. virens exhibits tolerance to elevated DNA damage

Coastal sites show stable or increasing sediment concentrations