Ocean acidification buffers the physiological responses of the king ragworm *Alitta virens* to the common pollutant copper.

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Abstract

Ocean acidification (OA) has the potential to alter the bioavailability of pH sensitive metals contaminating coastal sediments, particularly copper, by changing their speciation in seawater. Hence OA may drive increased toxicity of these metals to coastal biota. Here, we demonstrate complex interactions between OA and copper on the physiology and toxicity responses of the sediment dwelling polychaete *Alitta virens*. Worm coelomic fluid pCO_2 was not increased by exposure to OA conditions (pH_{NBS} 7.77, pCO_2 530 µatm) for 14 days, suggesting either a physiological or behavioural responses to control coelomic fluid pCO_2. Exposure to 0.25 µM nominal copper caused a decrease in coelomic fluid pCO_2 by 43.3% and bicarbonate ions by 44.6% but paradoxically this copper-induced effect was reduced under near-future OA conditions. Hence OA appeared to 'buffer' the copper-induced acid-base disturbance. DNA damage was significantly increased in worms exposed to copper under ambient pCO_2 conditions, rising by 11.1% compared to the worms in the no copper control, but there was no effect of OA conditions on the level of DNA damage induced by copper when exposed in combination. These interactions differ from the increased copper toxicity under OA conditions reported for several other invertebrate species. Hence this new evidence adds to the developing paradigm that species' physiology is key in determining the interactions of these two stressors rather than it purely being driven by the changes in metal chemistry under lower seawater pH.

Key words: - Polychaete; DNA-damage; oxidative stress; acid-base physiology

1. Introduction

Ocean acidification (OA) is now widely regarded as one of the major threats to marine organisms globally (Doney et al., 2009; Dupont and Portner, 2013; Gattuso et al., 2015). Increased atmospheric carbon dioxide concentrations have led to an average global ocean pH decrease of 0.1 units with a further decrease of 0.3 - 0.43 units predicted by the end of this century (Bao et al., 2012; Fabry et al., 2008). Coastal ecosystems are more complex and variable than open oceans, being governed by interactions between processes on land, in the open ocean and the atmosphere (Aufdenkampe et al., 2011). Multi-decadal trends in coastal pH reveal fluctuations of about 0.5 pH units over tidal, daily and seasonal timescales, with OA already influencing this trend by an additional 0.1 unit decline (Duarte et al., 2013) and this variability predicted to intensify as atmospheric CO_2 rises (Kwiatkowski and Orr, 2018). There is now a huge body of evidence that support the paradigm that the levels of OA predicted for the end of the century will have negative impacts on a wide range of species and physiological processes (Cao et al., 2018; Dupont et al., 2010; Kroeker et al., 2010; Wang et al., 2018; Wittmann and Portner, 2013).

Whilst initial studies focused on the negative impact of OA on calcification (Orr et al., 2005; Riebesell et al., 2000), OA has also been found to affect a wide range of other physiological and behavioural processes, including an organisms' ability to acid-base regulate and an increased energetic demands of homeostasis (Lannig et al., 2010; Miles et al., 2007), hence OA can also negatively impact many non-calcifying species. Marine animals acutely subjected to seawater with elevated pCO_2 experience a corresponding extracellular acidosis (Portner, 2008) and whilst many fish and crustaceans are able to regulate these acid-base perturbations by the elevation of extracellular bicarbonate ions (HCO_3^-) other invertebrates are less able to acid-base regulate and experience acidosis under OA conditions. Despite their ecological importance, only a few studies to fate have looked at the physiological impacts of OA on non-calcifying, sediment-dwelling polychaetes. Early studies on the king ragworm *Alitta (Nereis) virens* found no impact of OA on mortality or burrowing behaviour (Widdicombe and Needham, 2007), however subsequent studies over longer exposures revealed OA effects do manifest over time, reducing growth, bioturbation and bioirrigation behaviour in *A. virens* that, in turn, affect nutrient generation (Godbold and Solan, 2013).

Physiological end points may be more sensitive to OA. In the harbour ragworm *Hediste diversicolor* increased oxidative stress, measured as lipid peroxidation and elevated antioxidant enzyme activity (SOD) was observed when exposed to reduced seawater pH_{NBS} of 7.5 compared to ambient pH conditions (8.1) (Freitas et al., 2017).

Increasing OA is not the only stressor that marine organisms are currently being exposed to, with multiple anthropogenic threats affecting marine environments globally. Of particular relevance for coastal species is the potential for OA to alter the bioavailability and toxicity of certain pollutants (Roberts et al., 2013). Metals, in particular copper, continue to be some of the most wide-spread environmental contaminants, found at elevated concentrations in the majority of estuarine and coastal environments (compared to open ocean) as a result of local mining (past or present), road run-off, effluent discharges use in antifouling paints and nanoparticles, with evidence that concentrations in sediments are currently increasing (Watson et al., 2018). Copper can be found in coastal waters at concentrations ranging from low levels of 0.004 µM (Jones and Bolam, 2007) to much higher levels of 1.61 µM (Bryan and Gibbs, 1983). A comprehensive review by Bryan and Langston (1992) reported copper levels for 19 estuaries around the U.K reporting sediment levels for copper of 7 mg kg^{-1} to 648 mg kg^{-1} of dry weight sediment with extreme values of 2389 mg kg^{-1} for Restronguet Creek, Cornwall. Pore water is the key exposure route for organisms living and feeding within sediment (Chapman et al., 2002). More recent work by Pini et al., (2015) measured pore water concentrations across 7 sites along the English Channel, reporting concentrations for copper of 0.68 µg L-1 to 1.85 µg L^{-1}. Sediment characteristics, sediment organic content and the complexation of copper with organic matter present in seawater are all known to also play a role in determining the bioavailability of copper and zinc to sediment dwelling polychaetes (Van den Berg, 2000; Pini et al., 2015).

The speciation in seawater of many metals is pH-sensitive; hence the decrease in seawater pH and subsequent changes in hydroxide, carbonate and bicarbonate ion concentrations due to OA will change the speciation of many of the metal ions commonly present in coastal seawater (Byrne, 2002; Stockdale et al., 2016). Copper (II) ions form strong complexes with carbonate and a change in pH will lead to an increase in the more toxic copper free ions

(Millero et al., 2009; Stockdale et al., 2016) such that an increase of 48 - 115% in free copper ions due to OA, coupled with the increase in sea surface temperature is predicted for the end of the century (Richards et al., 2011; Stockdale et al., 2016). Hence OA is expected to alter the bioavailability of pH sensitive metals, such as copper, to marine biota and therefore potentially alter their toxicity responses. A number of studies have now investigated the potential for OA to alter the toxicity effects of copper in marine invertebrates, with many supporting the hypothesis that the toxicity of copper to biota is relatively higher when exposed under OA conditions (e.g. Lewis et al., 2013; Lewis et al., 2016; Siddiqui and Bielmyer-Fraser, 2015). However, these altered toxicity responses under OA conditions reported across studies vary in magnitude according to both species and life history stage, and are not consistent across the different endpoints measured, suggesting that the observed altered toxicity is not simply being driven by the change in metal speciation but that physiology also plays a role (Campbell et al., 2014; Gopalakrishnan et al., 2007; Lewis et al., 2016; Scanes et al., 2018). Since most contaminants, including copper, accumulate in sediments and concentrations of heavy metals in sediments usually exceed those of the overlying water by between 3 to 5 orders of magnitude (Bryan and Langston, 1992) sediment dwelling organisms are often exposed to the highest levels of any contaminant. Therefore, it is important to look at the potential for OA-metal interactions in sediment dwelling organisms such as polychaetes, which often dwell in polluted sediments (Lewis and Galloway, 2008).

The king ragworm, *Alitta* (formally *Nereis*) *virens,* is an ecologically and commercially important sediment dwelling polychaete (Watson et al., 2017) found in coastal waters and estuaries (Kristensen et al., 1985). Here, using a suite of representative physiological (acid-base balance) and toxicological (DNA damage and oxidative stress) endpoints, we test the hypothesis that copper toxicity will increase under OA seawater conditions relative to those experienced under ambient (i.e. current) seawater pCO_2 conditions in polychaetes, as has been observed in other taxa (e.g. molluscs and echinoderms) by examining the responses of the polychaete *A. virens* to combined OA and copper exposures.

2. Methods

2.1 Animal Collection and Maintenance

Immature (assessed by colour and transparency) adult *Alitta virens* specimens (0.3 – 1.2 g wet weight) were collected from Starcross, Devon, England (50°37'36.5"N 3°26'47.2"W) during February 2016 by carefully digging them from the intertidal mud (mid-shore) with a fork. Starcross has been reported as having estuarine water copper levels in the range ~ 0.5 – 2.5 µg L^{-1} (i.e. ~0.009 - 0.04 µM; Langston et al., 2003). Worms were maintained in a holding tank in natural sediment from the collection site at 15 °C for 48 hours before the experiment started. Sediment grain size for this site was within the 125-250 µm range. The worms were then transferred into individual 2 L tanks, each with a small tube as an artificial burrow. Each tank contained well aerated artificial seawater and was kept at a salinity of 34 ppt. The salinity was monitored daily using a salinity probe (SevenGo Duo, pH/conductivity meter SG23). The tanks were kept covered for the duration of the exposure to simulate the darkness the animal would experience when burrowing.

2.2 Seawater Manipulation

Artificial seawater (Tropic Marine©) was used to fill the individual tanks. Seawater pH_{NBS} values of 8.1 (with resulting pCO_2 of 453 µatm) and 7.7 (pCO_2 1305 µatm) were targeted, representing current and near future OA treatments respectively according to the IPCC WGI AR5 RCP 8.5 scenario (Meinshausen et al., 2011; Stocker, 2013). Full seawater chemistry is provided in Table 1. Oxygen saturation states were measured every other day for each treatment and were maintained as fully saturated across all treatments by bubbling air into each individual tank. Seawater pH_{NBS} in the OA conditions was maintained at 7.7 using an Aalborg Mass Flow Controller GFC set at the correct ratio of air to CO_2 and bubbled separately into each individual tank. Seawater pH (SevenGo Duo, pH/conductivity meter SG23), temperature and salinity (SevenGo Duo, pH/conductivity meter SG23) were measured daily in the holding tanks and the individual exposure tanks.

Water samples (36 for DIC, 14 for copper) were taken throughout the two week experiment. Samples were taken every third day, to cover both just before and just after water changes (where re-dosing occurred) to best represent the exposure condition experienced by the

worms over this period. Water samples for DIC analysis were preserved (0.04 % of final volume) with 4 % mercuric chloride for storage prior to analysis (Dickson, 2007) whilst samples for metals analysis were added to acid-washed 50 ml tubes and acidified using 50 µl of concentrated hydrochloric acid. Seawater DIC analysis was carried out using a bespoke system based on that described by Friederich et al. (2002) and using Dickson seawater `standards, as described in detail in Lewis et al. (2013). Total alkalinity (TA) and pCO_2 were calculated from the measured values of pH_{NBS} and DIC using CO2sys, applying the constants from Mehrbach et al., (1973) and the KSO_4 dissociation constants from Dickson (1990). The concentrations of copper in the seawater samples were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using an Agilent 7900 Spectrometer utilising a collision cell with helium as the collision gas to minimise polyatomic interferences. Copper standards, matrix matched to the samples, at concentrations of 1, 10 and 100 mg/l respectively, were used for instrument calibrations. Analysis employed in-line addition of scandium as an internal standard and calibration of the ICP-MS was validated by the use of a quality control standard of 10 mg/l.

2.3 Experimental design

Ten worms per treatment (maintained in individual tanks and for OA treatments each with a mixed gas supply) were transferred to each of the following four treatments; (1) ambient conditions (pH_{NBS} 8.1) with no added copper, (2) ambient conditions (pH_{NBS} 8.1) with 0.25 µM copper sulphate added, (3) OA conditions (pH_{NBS} 7.7, the expected pH under RCP 8.5, IPCC AR5) with no added copper, (4) OA conditions (pH_{NBS} 7.7) with 0.25 µM copper sulphate added. Individuals were kept at 15 °C for 14 days in their own tank. A concentration on 0.25 µM of copper sulphate was used as initial experiments using lower concentrations of copper found no toxicity response in this species. Therefore, a concentration equivalent to a polluted site, such as Restronguet Creek (Bryan and Gibbs, 1983) was used to induce a sub-lethal response to enable relative responses across pCO_2 treatments to be compared. Partial water changes and re-dosing of exposure water with the treatment nominal copper concentration (pre equilibrated to the treatment pCO_2) were performed every three days to maintain the copper concentration.

Following the 14 day exposure each individual worm was analysed for all endpoints measured. Worms were removed from their tanks and samples of coelomic fluid were collected from each individual worm using an 18 gauge needle and 1 ml syringe carefully inserted into the coelomic cavity of the organism, working from the anterior region to the posterior region. Coelomic fluid was immediately analysed for acid-base physiology (below) and an aliquot taken for use in the Comet assay. Worms were then snap-frozen in liquid nitrogen for later use in the oxidative stress assays. Prior to use, frozen worms were defrosted then homogenised in PBS buffer using a hand-held homogeniser, centrifuged at 10,000 g for 20 minutes then the supernatant frozen until use at -20 °C.

2.4 Acid-base physiology

The pH_{NBS} of *A. virens* (10 worms per treatment) coelomic fluid samples (taken as above) was measured immediately after sampling at 15 °C in microcentrifuge tubes using a Metrohm 826 pH mobile pH electrode and meter calibrated using NBS buffers. From this fluid, 50 µl was stored in a micro capillary tubed sealed with paraffin oil and the Critoseal® sealant and were analysed for TCO_2 the same day as collection using a Mettler Toledo 965D Carbon Dioxide Analyser. Fifty microlitres of a 10 mM $NaHCO_3$ standard was used between each sample for calibration purposes. Acid-base parameters were then calculated using a modified version of the Henderson-Hasselbalch equation, as described in Lewis et al., 2016 paper (Lewis et al., 2016). This used previously calculated constants from Truchot, 1976, based on the crab, *Carcinus maenas* (Truchot, 1976).

2.5 DNA Damage

DNA damage was measured as single strand breaks using the comet assay, according to the methods described by Lewis & Galloway (2008), under alkaline conditions at 5°C. From the coelomic fluid collected as described above, 100 µl from each individual (10 worms per treatment) was combined with 100 µl of phosphate buffer and centrifuged. Briefly, the supernatant was removed and 180 µl of low melting point agarose was added, this was then pipetted out onto a frosted slide, previously coated in high melting point agarose, and left to cool. The slides were placed in Lysis solution for 1 hour at 4°C and then into an

electrophoresis tank. Here the slides were covered with electrophoresis solution for 40 minutes before the current (25 V) was switched on for 30 minutes. Finally, the slides were rinsed in neutralising buffer before being stained with SYBRSafe (1 µL in 10 mL TBE buffer) and viewed using a fluorescence microscope (excitation: 502 nm, emission 530 nm). One hundred cells per replicate worm were quantified for DNA damage using COMET IV Software (Perceptive Instruments Ltd.), which measures the percentage of DNA present in the comet tail for each cell as the measure of DNA damage.

2.6 Oxidative Stress Endpoints

Superoxide dismutase (SOD) is an enzyme which is essential in the defence against oxidative damage (McCord et al., 1971). The SOD assay generates O_2- and uses nitroblue tetrazolium (NBT) which changes colour, from clear to purple, when it comes in contact with a free radical. SOD inhibits this colour change hence levels can be quantified by determining the level of inhibition of this colour change in a sample compared to a standard (Beaucham and Fridovic, 1971). Initially 5 µl of homogenised samples (10 worms per treatment) or standards were added to 96-well plates along with 30 µl of buffer A (2.28 g/500 ml Na_2CO_3 and 1.18 g/ 500 ml $NaHCO_3$) and 195 µl substrate solution B (0.1 mM xanthine, 0.1 mM EDTA, 0.05 mg BSA and 0.025 mM NBT). Free radicals were created using xanthine oxidase (4.95 units/ml in a 1:80 dilution in buffer A), where 10 µl was added to the microplate immediately prior to reading at a wavelength of 573 nm.

Lipid peroxidation was determined using the thiobarbituric acid reactive substances (TBARS) assay (Camejo, 1998) which quantifies malondialdehyde, a secondary product of lipid peroxidation, via its reaction with thiobarbituric acid (Lewis et al., 2016). In microcentrifuge tubes, 100 µl of homogenised samples (10 worms per treatment) or standards (see below) were added along with 300 µl of PBS + EDTA (372.24 mg EDTA in 1L PBS), 150 µl thiobarbituric acid (1.95g in 150ml NaOH), 100 µl trichloroacetic acid (50 g/100 ml DI water) and 20 µl butylated hydroxytoluene (22 mg/100 ml ethanol). All microcentrifuge tubes were vortexed and incubated at 60^{o}C for 60 minutes, then centrifuged for 7 minutes at 13000 rmp. In a 96-well plate either 200 µl of standards or 100 µl of sample + 100 µl PBS + EDTA were added in

triplicate to the corresponding wells and absorbance at 530 nm measured using a spectrophotometer. Results were compared to a standard curve prepared using 1,1,3,3-tetraethoxypropane (a stabilized form of MDA) and normalised to protein content using standardised Bradford protocol (Bradford, 1976).

2.7 Statistical analysis

All data was analysed for normality using the Shapiro-Wilk test. DNA damage (%) was first normalised using the arcsine transformation. Normal data (DNA damage, pCO_2, bicarbonate and pH) was analysed using a 2-way analysis of variance (ANOVA) general linear model with 'pH', 'copper' and 'pH x copper' as fixed factors. Non-normal data (TBARS and SOD) was analysed using Scheirer-Ray-Hare, a non-parametric method. Tukey's post-hoc test was carried out on all data. All statistical analysis was performed using SPSS software.

3. Results

3.1 Seawater chemistry

The carbonate chemistry of the seawater from the four treatments together with the measured total copper levels for each of the exposures are summarised in Table 1. Whilst there was some loss of copper to the experimental system during the exposures despite the regular re-dosing, a one-way t-test confirmed that there was no significant difference in measured seawater copper concentrations betwee the ambient (pH_{NBS} 8.1) copper and OA (pH_{NBS} 7.7) copper treatments (independent-sample t test; t=-0.601, p=0.951). The two treatments with added copper had significantly higher copper concentrations than those with no added copper (independent-sample t test; for pH 8.1 t=1.389, p=0.004; for pH 7.7 t=1.814, p=0.039). The seawater for all treatments was fully saturated with oxygen throughout the 14 days (monitored daily).

3.2 Acid-base physiology

Coelomic fluid pCO_2 in *Alitta virens* under ambient seawater pH_{NBS} of 8.1 was 2.03 ± 0.34 mmHg. Exposure to OA conditions, pH_{NBS} 7.7, for 14 days did not cause any increase in the worms' coelomic fluid pCO_2, with values of 2.03 ± 0.21 mmHg measured in worms exposed to this treatment (two-way ANOVA for pH, F = 1.757, P = 0.193). The presence of copper induced a significant decrease in pCO_2 in both the ambient and OA treatments (two-way ANOVA for copper F = 6.694, P = 0.014, Figure 1a). Under ambient conditions, pH_{NBS} 8.1, pCO_2 levels fell to 1.15 ± 0.078 mmHg, a 43% decrease. A smaller decrease of 14% was seen in OA conditions (pH_{NBS} 7.7) where coelomic fluid pCO_2 decreased to 1.75 ± 0.181 mmHg. There was no significant interaction factor (two-way ANOVA for pH*copper F = 1.847, P = 0.183; Figure 1a).

Coelomic fluid bicarbonate levels showed a similar pattern to the pCO_2 levels (Figure 1b). Under ambient seawater pH/pCO_2 conditions bicarbonate levels were 5.74 ± 1.07 mmol l^{-1}. OA, pH_{NBS} 7.7, caused a significant increase in coelomic fluid bicarbonate levels to 6.77 ± 0.79 mmol l^{-1} (two-way ANOVA for pH F = 5.681, P = 0.023). The addition of copper resulted in a decrease of bicarbonate levels in both seawater treatments. At an ambient pH_{NBS} of 8.1, bicarbonate levels decreased to 3.18 ± 0.23 mmol l^{-1}. A decrease was also seen at pH_{NBS} of 7.7, although it was not as large. Here, bicarbonate levels fell to 5.49 ± 0.36 mmol l^{-1}. The results show that there was a significant effect from the addition of copper (two-way ANOVA for copper F = 7.509, P = 0.009). There was no significant interaction term between OA and copper (two-way ANOVA for pH*copper F = 0.882, P = 0.371, Figure 1b).

There was no significant effect of exposure to OA (two-way ANOVA for OA F = 3.159, P = 0.084) on *A. virens* coelomic fluid pH_{NBS}, which was measured as 7.78 ± 0.05 and 7.84 ± 0.23 under ambient seawater *p*CO2/pH and OA conditions respectively (Figure 1c). Exposure to copper also had no effect on coelomic fluid pH (two-way ANOVA for copper F= 0.003, P = 0.957), and there was no interaction between OA and copper (two-way ANOVA for pH*copper F = 0.001, P = 0.979). These acid-base physiology data can be visualised together as a Davenport Diagram, as in Figure 2 (Davenport, 1974). Plotting the data in this way highlights the difference in acid-base status of *A. virens* in the ambient pCO_2 plus copper treatment compared to the other three treatments.

3.3 DNA Damage

Exposure to 0.25 µM copper under both seawater pH/pCO_2 treatments led to a significant increase in DNA damage in *A. virens* coelomocytes (Figure 3a; two-way ANOVA for copper, F = 31.106, P < 0.001), with % DNA damage increasing from 18.7 ± 1.49 % (ambient pH/pCO_2 no copper) to 29.8 ± 1.9% (ambient pH/pCO_2 with copper) and 34.0 ± 3.2% (OA with copper) respectively. Worms in the OA treatment had similar levels of DNA damage to the controls of 17.7 ± 2.79 %., with no significant effect of pH on DNA damage measured (two-way ANOVA for pH, F = 0.519, P = 0.476). However, there was no significant difference in DNA damage between the two copper treatments and no significant interaction term between OA and copper (two-way ANOVA for pH*copper, F = 1.187, P = 0.283, Figure 3a).

3.4 Oxidative Stress

Activity of the anti-oxidant enzyme SOD significantly increased under exposure to OA conditions, from 0.137 ± 0.03 units per mg of protein under ambient pH/pCO_2 to 0.361 ± 0.06 units per mg of protein under OA (Figure 2b, Scheirer-Ray-Hare for pH, H = 1.617, P = 0.008). There was no significant effect of the addition of 0.25 µM of copper under either seawater pH/pCO_2 treatments (Scheirer-Ray-Hare for copper, H= 0.382, P = 0.171), with SOD activity of 0.176 ± 0.06 units per mg of protein (ambient pH/pCO_2 with copper) and 0.186 ± 0.03 units per mg of protein (OA with copper) respectively, and no significant interaction term between pH and copper on SOD activity (Scheirer-Ray-Hare for pH*copper, H = 0.629, P = 0.082, Figure 2c).

There were no significant effects of either copper or OA on the levels of lipid peroxidation in *A. virens* (Figure 2c; Scheirer-Ray-Hare for pH, H = 0.191, P = 0.660; for copper, H = 0.003, P = 0.960; for pH*copper, H = 0.704, P = 0.400). A slight increase in lipid peroxidation from 5.03 ± 1.57 to 6.12 ± 2.37 nM per mg of protein was observed with the addition of copper for the ambient pH/pCO_2 scenarios. This slight trend was reversed under OA conditions, however, where the levels of lipid peroxidation decreased slightly when copper was added (but not significantly) from 6.12 ± 2.37 nM per mg of protein (OA no copper) to 4.01 ± 0.78 nM per mg of protein (OA with copper).

4. Discussion

Our data reveals that in the ecologically and commercially important polychaete, the king ragworm *Alitta virens,* OA conditions do not cause an increase in the toxicity effects induced by exposure to copper relative to those experienced under ambient (present day) pCO_2/pH seawater conditions. This is the opposite effect to that observed for a number of other coastal invertebrate species, where a relative increase in copper toxicity under OA has been reported (e.g. Campbell et al., 2014; Freitas et al., 2016; Roberts et al., 2013; Siddiqui and Bielmyer-Fraser, 2015). Here, *A. virens* was surprisingly robust to both short-term OA exposure and relatively high copper contamination. No significant increase in the internal pCO_2 of the worms was observed in response to the elevated pCO_2 levels in their surrounding seawater under the OA exposure. This aligns with previous studies using CO_2 vents as proxies for OA that reveal non-calcifying polychaetes (e.g. *Syllis prolifera* often do well under the lower pH conditions nearest the vents (Cigliano et al., 2010). Whilst exposure to nominal 0.25 µM copper (representative of a highly contaminated site) induced elevated levels of DNA damage in the worm's coelomocytes, this was not significantly altered when experienced under OA conditions. In fact the opposite effect to that hypothesised was observed, with OA appearing to buffer the impacts of copper toxicity on *A. virens*, with a significant reduction in the levels of copper-induced lipid peroxidation and acid-base disturbance measured in the adult worms when experienced under OA conditions.

The addition of 0.25 µM copper under ambient seawater pCO_2/pH conditions induced a 1.6 fold increase in DNA damage in the coelomocytes of *A. virens*. Genotoxic responses of *A. virens* to copper have been previously observed in long-term sediment exposures, where 9 month exposures (to nominal copper concentrations of 70 - 575 mg kg^{-1}) resulted in significant increases in DNA damage accumulating (Watson et al., 2018). Despite high levels of DNA-damage accumulating worms maintained positive growth over this period, although some mortality was observed (Watson et al., 2018). Thriving populations of *A. virens* have been found living in polluted sediments with coelomocyte DNA damage levels averaging ~35% (Lewis and Galloway, 2008) and the response of *A. virens* to known genotoxins has been demonstrated to be about 50% lower than for two other polychaete species tested. Whilst copper exposure often leads to increased lipid peroxidation (e.g. Brown et al., 2004; Maria

and Bebianno, 2011) and changes in activity of the anti-oxidant enzyme SOD (Xu et al., 2018), here we found no significant changes in lipid peroxidation or SOD activity in *A. virens* in response to copper under ambient seawater pH/pCO_2 conditions. This again is in contrast to the responses previously reported for the mussel *Mytilus edulis* and the sea urchin *Paracentrotus lividus* (Lewis et al., 2016), further supporting the idea that this *A. virens* is relatively robust to copper contamination. Our data aligns with the suggestion that *A. virens* can regulate copper uptake, leading to low tissue concentrations even when exposed to high bioavailable concentrations (Pini et al., 2015; Watson et al., 2018).

We found no significant acid-base disturbance in *A. virens* during exposure to OA conditions. The influence of OA on the acid-base physiology of marine fauna is well documented (Portner et al., 2004; Widdicombe and Spicer, 2008) whereby animals subjected to seawater with elevated pCO_2 experience a corresponding increase in internal pCO_2 in the blood or haemolymph driving an extracellular acidosis. Whilst most fish and crustaceans are able to regulate these acid-base perturbations by elevating their extracellular bicarbonate ions (HCO_3^-) (Spicer et al., 2007) many other invertebrates, such as mussels and some urchin species, are less able to acid-base regulate (Collard et al., 2013; Gazeau et al., 2013) and experience acidosis under elevated seawater pCO_2. This lack of increase in coelomic fluid pCO_2 under OA conditions is counter-intuitive, given that *A. virens* are a simple, soft bodied organism with gas exchange occurring, via diffusion, across the general integument (Gomme, 1984). We used artificial burrows (gas permeable plastic tubes) in our experiments as habitat for the worms which may influence the pCO_2 conditions immediately surrounding the worms. Polychaetes are known to irrigate their burrows via active or passive irrigation which may alter under OA conditions and also play a role in the pCO_2 levels that build-up within the tubes. Hence a combination of microhabitat conditions and behavioural responses may act to buffer the worms from the changes in the seawater pCO_2 but this requires further work to elucidate.

The addition of 0.25 µM copper, however, did significantly alter the acid-base balance of the worms, causing a significant decrease in their coelomic fluid pCO_2 and bicarbonate levels. No corresponding change to the coelomic fluid pH was observed suggesting that the reduction in

coelomic fluid bicarbonate levels balanced out the reduced pCO_2 effect, preventing alkalosis. A blood alkalosis in response to copper exposure has been previously reported in the sea urchin *P. lividus* (Lewis et al., 2016) and in Rainbow Trout *(Oncorhynchus mykiss)* (Wang et al., 1998) whilst studies in crabs have reported an acidosis of the haemolymph in response to copper exposure (Boitel and Truchot, 1989; Weeks et al., 1993). The mechanisms underpinning these differing responses have not been elucidated.

Rather than OA increasing the relative toxicity of copper to *A. virens* as was hypothesised, OA conditions have altered the physiological and toxicological response of the worms to copper, reducing the copper toxicity effects on lipid peroxidation and acid-base disturbance. This is evident looking at the Davenport diagram (Figure 2), where the copper only treatment is quite distinct from the other three treatments, whilst the 'copper and OA' worms have over-lapping acid-base status to the worms were no copper was added exposed to elevated copper. A protective effect of hypercapnia on copper toxicity has also been suggested by the work of Larsen et al., (1997) in the cod *Gadus morhua*. This is contrary to what would be predicted if toxicity were driven by the availability of the copper (II) ion (Cu^{2+}) ion alone, since metal speciation models predict an increase in the more toxic free copper (II) ion with a decrease in seawater pH (Millero et al., 2009).

Other studies looking at interactions between OA and the toxicity of pH-sensitive metals report a range of contrasting responses, from strong increased toxicity under OA (Campbell et al., 2014; Freitas et al., 2016; Lewis et al., 2016; Roberts et al., 2013; Scanes et al., 2018) to no change in toxicity under OA (Dorey et al., 2018; Lewis et al., 2013; Scanes et al., 2018) and now reduced toxicity under OA. This raises the question as to what biological mechanisms underpin these different responses. Our data further supports the developing paradigm that the acid-base physiology of a species/ life history stage plays an important role in determining any OA-copper interaction. In *A. virens* here there was no OA effect on the internal pCO_2 or pH of the worms, therefore speciation of copper within the worms would theoretically be the same under the OA and the ambient pH/pCO_2 treatments. In the sea urchin *P. lividus,* a relatively good acid-base regulator, a small increase in copper toxicity under OA was observed (Lewis et al., 2016), whereas the poor acid-base regulator *M. edulis* (common mussel) OA

conditions induced a much larger relative increase in copper toxicity alongside an acidosis of the haemolymph (Lewis et al., 2016).

Hence an organisms' ability to acid-base regulate and the pH of the extracellular fluid may be key to determining both the direction and magnitude of any interaction between OA and copper toxicity. Differences in the uptake and accumulation of copper may also play a role in species differences, with OA known to also influence trace metal accumulation (Breitbarth et al., 2010; Lacoue-Labarthe et al., 2009; Lacoue-Labarthe et al., 2011). The idea that physiology can determine the interaction between copper and another environmental stressor, in this case salinity, has previously been suggested. (Grosell et al., 2007) reviewed data for copper toxicity responses of marine fish and invertebrates under varying salinities and found four orders of magnitude differences between species in their sensitivity to copper in seawater. The authors argued that the majority of these differences could be attributed to differences in physiology (in this case turnover rates of the Na+ ion) rather than water chemistry (Grosell et al., 2007). Given the variety of species with differing physiologies that will be experiencing coastal pollution as OA progresses it is vital to gain a better understanding of how these factors interact to determine species tolerance to combined anthropogenic stressors as climate change progresses.

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Table 1: Seawater carbonate chemistry and copper levels for all experimental treatments over the exposure period. Data as mean ± standard error. Temperature, salinity, and pH were measured daily in each replicate tank. DIC (n = 9) and copper concentration (n = 7) were measured every three days. The other carbonate parameters were calculated using CO2sys.

Treatment	Temperature (ºC)	pH_{NBS}	Salinity	Copper (µM)	TA (µmol/kg)	pCO_2 (µatm)	HCO_3^- (µmol/kg)	CO_3^{2-} (µmol/kg)	ΩCa	ΩAr
8.1 + Cu	14.03 ± 0.05	8.19 ± 0.01	34.07 ± 0.03	0.046 ± 0.0177	2790.4 ± 47.3	453.3 ± 7.9	2325.3 ± 40.5	194.9 ± 3.4	4.7 ± 0.1	3.0 ± 0.1
8.1	13.91 ± 0.05	8.19 ± 0.01	34.05 ± 0.03	0.016 ± 0.016	2801.9 ± 60.9	453.3 ± 10.1	2336.9 ± 52.2	194.9 ± 4.4	4.7 ± 0.1	3.0 ± 0.1
7.7 + Cu	14.08 ± 0.04	7.77 ± 0.01	34.13 ± 0.04	0.061 ± 0.0196	2756.5 ± 44.9	1310.9 ± 21.6	2556.3 ± 42.2	81.7 ± 1.3	2.0 ± 0.0	1.3 ± 0.0
7.7	14.12 ± 0.05	7.77 ± 0.01	34.20 ± 0.05	0.019 ± 0.0018	2743.8 ± 50.9	1305.9 ± 24.5	2546.5 ± 47.8	81.7 ± 1.5	2.0 ± 0.0	1.3 ± 0.0

Figures

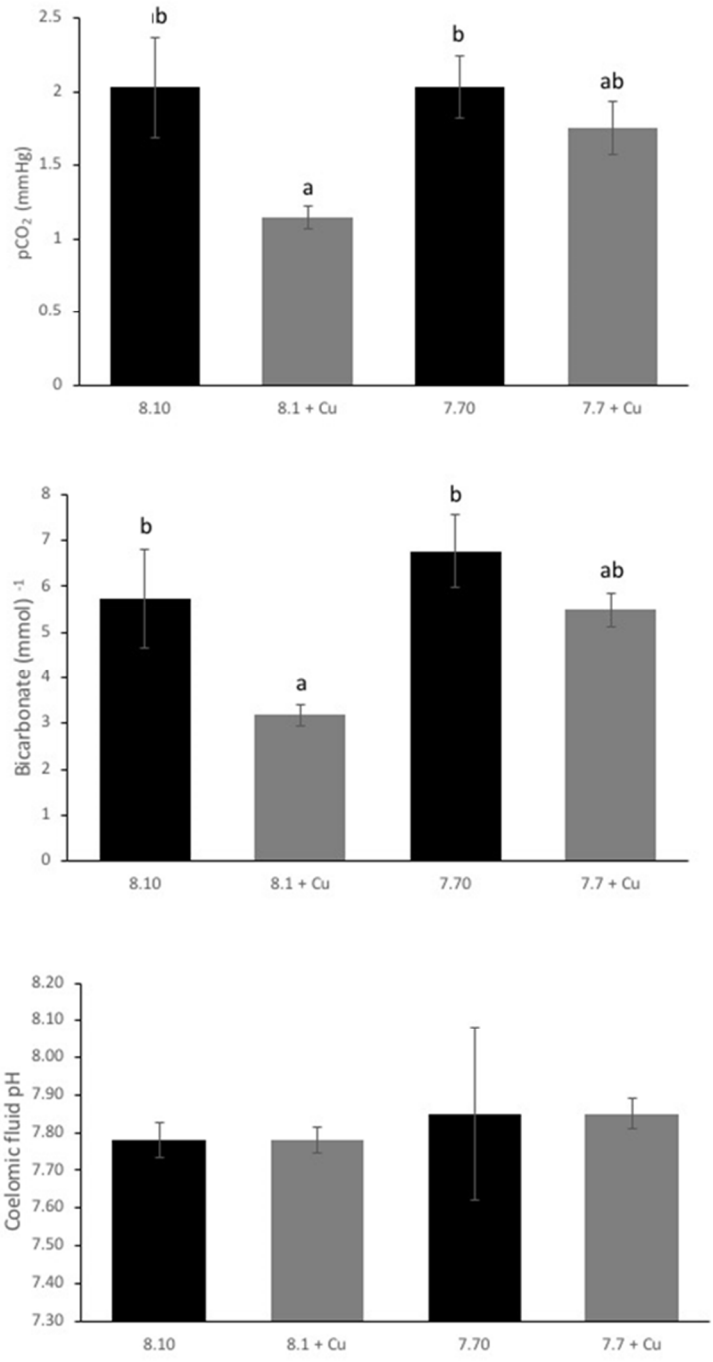

Figure 1: Acid-base parameters in the coelomic fluid of *Alitta virens* following a 14 day exposure with and without 0.25 µM copper under ambient and near future OA pCO_2/pH scenarios. Mean (±SE). (A) Coelomic fluid pCO_2 (n=40), (B) Coelomic fluid bicarbonate levels (n=40), (C) Coelomic fluid pH_{NBS} (n=40). Differing letters denote significant differences between treatments (Tukey's post-hoc: $p<0.05$).

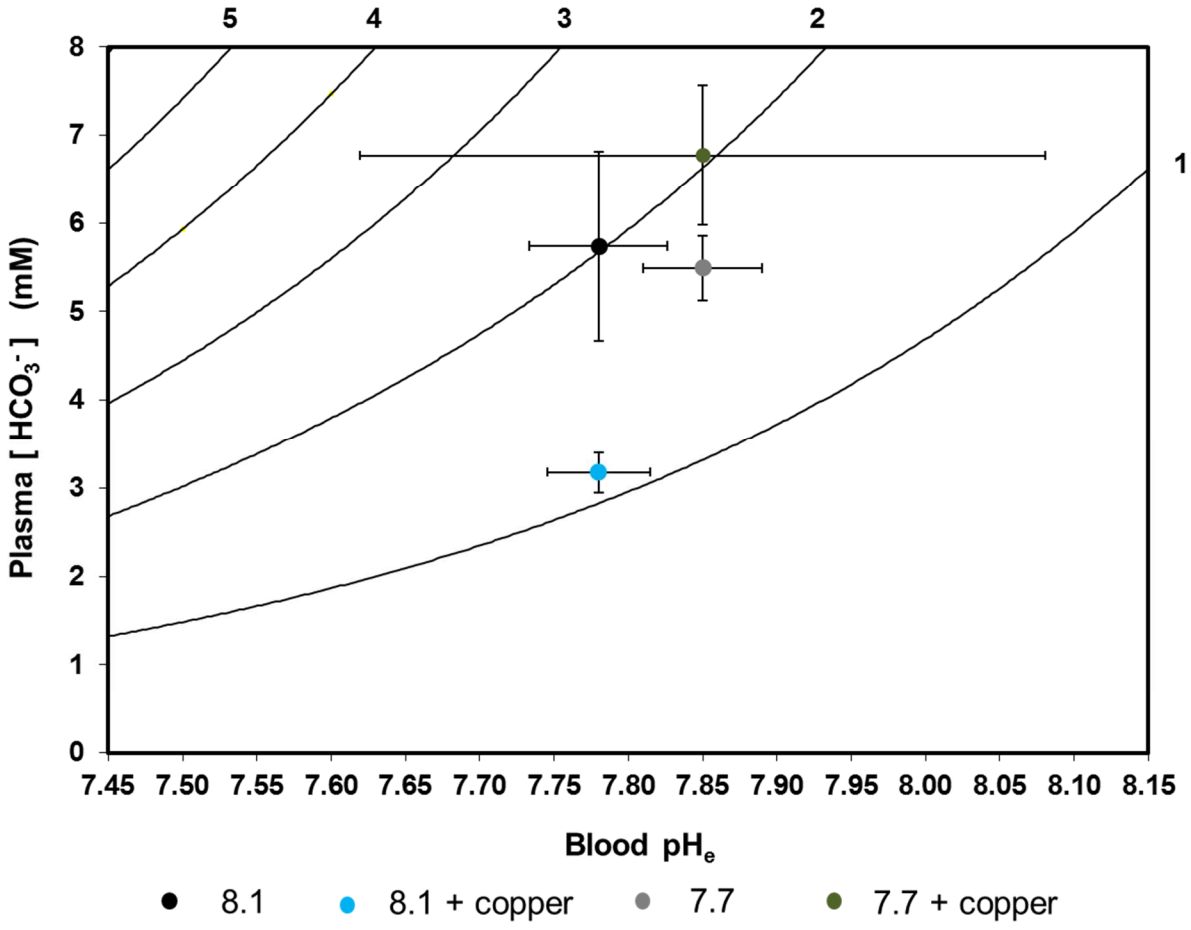

Figure 2: Davenport diagram visualising the relationship between pH, pCO_2 and bicarbonate ion concentrations in the coelomic fluid of *Alitta virens* at 15°C & 35ppt under the four different treatment conditions, demonstrating the effect of copper on haemolymph HCO_3^- concentrations under ambient conditions. Lines represent isopleths of equal pCO_2 (mmHg).

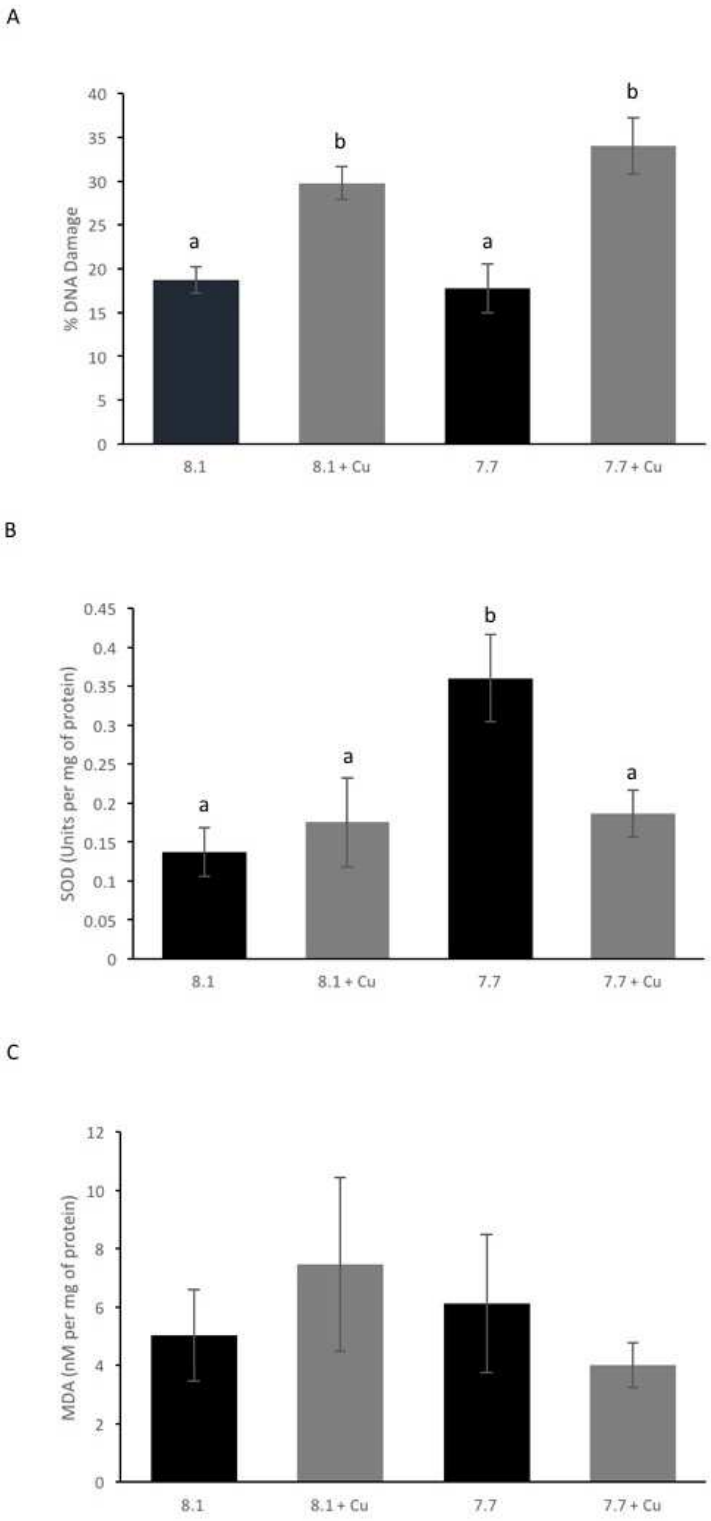

Figure 3: Oxidative stress indicators in *Alitta virens* following a 14 day exposure with and without 0.25 µM copper under ambient and near-future OA pCO_2/pH scenarios (data as mean ±SE). (A) DNA damage measured as percentage single strand breaks (n=40), (B) Superoxide dismutase activity (SOD) in units per mg of protein (n=40), (C) Lipid peroxidation (n=40). Differing letters denote significant differences between treatments (Tukey's post-hoc: $p<0.05$).