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Ocean Acidification Increases Copper Toxicity to the Early Life History Stages of the Polychaete *Arenicola marina* in Artificial Seawater

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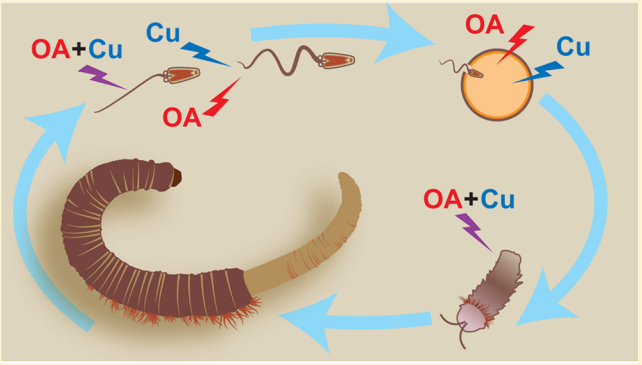
Supporting Information

ABSTRACT: The speciation and therefore bioavailability of the common pollutant copper is predicted to increase within the pH range anticipated under near-future ocean acidification (OA), hence the potential exists for copper toxicity to marine organisms to also increase. We investigated the impact of OA (seawater pH values of 7.77 (pCO_2 1400 μatm) and 7.47 (pCO_2 3000 μatm)) upon copper toxicity responses in early life history stages of the polychaete *Arenicola marina* and found both synergistic and additive toxicity effects of combined exposures depending on life history stage. The toxicity of copper on sperm DNA damage and early larval survivorship was synergistically increased under OA conditions. Larval survival was reduced by 24% when exposed to both OA and copper combined compared to single OA or copper exposures. Sperm motility was negatively affected by both OA and copper singularly with additive toxicity effects of the two stressors when combined. Fertilization success was also negatively affected by both OA and copper individually, but no additive effects when exposed as combined stressors were present for this stage. These findings add to the growing body of evidence that OA will act to increase the toxicity of copper to marine organisms, which has clear implications for coastal benthic ecosystems suffering chronic metal pollution as pCO_2 levels rise and drive a reduction in seawater pH.



INTRODUCTION

Ocean acidification (OA), the change in pH and carbonate chemistry of the world's oceans as a result of increasing atmospheric concentrations of carbon dioxide (CO_2),[1] is now broadly considered to represent a major threat to global marine biodiversity.[2−4] Recent projections forming part of the Representative Concentration Pathways (RCP) see atmospheric pCO_2 surpassing 1000 μatm shortly into the next century (RCP 8.5).[5] Average ocean surface pH has already dropped by 0.1 units since the industrial revolution, indicating a rise of 30% in seawater H^+ concentration,[6] with climate models projecting a further decrease by as much as 0.43 units by the end of this century to a global average pH of around 7.73.[7] A wide body of evidence now suggests that this change in ocean chemistry has the potential to negatively influence the health and fitness of a wide range of marine invertebrate species and life history stages.[3,8−10]

OA is not happening in isolation and marine habitats face a wide array of current and evolving threats from multiple anthropogenic stressors. Pollution of the marine environment is a continued and growing global issue with metals in particular known to be persistent ubiquitous contaminants of a wide range of habitats.[11,12] Copper remains a common contaminant in coastal waters,[12] with concentrations ranging from 0.004 μM[13] to in excess of 1.6 μM in highly contaminated areas[14] with the 76/464/EEC directive environmental quality standard (EQS) set at 0.08 μM. While copper is a naturally occurring trace element essential for some biological functions, elevated levels can be toxic to a range of marine organisms.[15,16] Metals, such as copper, exert toxicity via the production of reactive oxygen species (ROS). At elevated levels these can overwhelm an organism's antioxidant defenses and induce oxidative damage of cellular components such as proteins, lipids, and DNA[17,18] or directly through the covalent binding of free ionic copper to macromolecules.

The behavior, speciation and therefore bioavailability of many heavy metals in seawater is strongly dependent on seawater chemistry, with a number of metals known to be sensitive to speciation changes within the pH range projected for near-future OA.[19,20] OA may also alter the behavior of metals bound to sediments, influencing metal fluxes from contaminated sediments.[21] OA is predicted to increase the toxic free ion concentration of copper (Cu^{2+}) in coastal waters by as much

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Table 1. Measured and Calculated (CO_2SYS[39]) Seawater Parameters for Experimental Treatments[a]

	measured parameters				calculated parameters			
pH treatment	T (°C)	S[b]	pH (NBS scale)	DIC (μmol kg^{-1})	TA (μmol kg^{-1})	pCO_2 (μatm)	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})
8.28[A,B]	12 ± 0.1	35	8.29	2657.2	2976.0	359.5	2399.6	242.9
8.28[C]	12 ± 0.1	35	8.28	2847.9	3175.6	394.9	2576.8	254.9
8.28[D]	12 ± 0.1	35	8.26	2862.8	3176.9	417.5	2600.1	245.5
7.77[A]	12 ± 0.1	35	7.76	3071.5	3129.0	1486.7	2925.7	74.2
7.77[B]	12 ± 0.1	35	7.77	2918.3	2978.5	1394.5	2808.1	85.7
7.77[C]	12 ± 0.1	35	7.76	3060.1	3117.5	1481.2	2912.6	86.8
7.77[D]	12 ± 0.1	35	7.77	3089.2	3151.1	1459.7	2939.6	89.8
7.47[A]	12 ± 0.1	35	7.48	3147.5	3092.7	3309.0	2971.5	40.5
7.47[B]	12 ± 0.1	35	7.46	2986.6	2927.2	2873.1	2826.7	42.2

[a]A: sperm motility analysis, B: sperm comet assay, C: day 1 fertilizations, D: day 2 fertilizations and larval survival assays. [b]Salinity was adjusted to 35 by blending commercial salt mixture with distilled water.

as 115% in the next 100 years.[19] In addition, the inorganic speciation of copper is dominated by complexation to the CO_3^{2-} ion which will reduce under OA conditions.[20] Hence it might be predicted that the toxicity of copper to marine organisms will increase under near-future OA.[22] Only a few studies to date have investigated this potential for altered bioavailability and/or toxicity of metals under near-future projections for seawater pH. Bioaccumulation of a number of trace metals was found to be altered in the eggs of the squid *Loligo vulgaris* and the cuttlefish *Sepia officinalis* under elevated pCO_2, such that accumulation of some metals increased while others decreased.[23,24] Roberts et al.[21] identified a 2.7 fold increase in toxicity, measured as DNA damage, induced by exposures to field-collected contaminated sediment at elevated pCO_2 (750 μatm, pH 7.90) in the amphipod *Corophium volutator*. Elevated pCO_2 has also been shown to increase the sensitivity of larvae to copper in the polychaete *Pomatoceros lamarckii*[25] and decrease larval production rates in the copepod *Tisbe battagliai*.[26]

The early life history stages of marine invertebrates are generally much more sensitive to environmental stressors than their adult forms,[27] hence are likely to form the bottlenecks in any population experiencing environmental stress. As such they are regularly used for regulatory toxicity testing, with the U.S. Environmental Protection Agency's standardized water toxicity tests including a sea urchin and sand dollar fertilization assay.[28] Fertilization and sperm motility are known to be sensitive to copper, with responses ranging from a slight increase in sperm swimming speeds under very low doses[29] to reduced sperm swimming speeds and fertilization success at elevated copper concentrations.[30] For example sea urchin fertilization appears to be generally quite sensitive to copper exposure with reported EC_{50}s of between 1.9 and 59 μg L^{-1},[31] whereas other species such as the polychaete *Nereis virens* appear less sensitive, with no observable reduction in fertilization success below 500 μg L^{-1}.[32]

The polychaete *Arenicola marina* is an ecologically important benthic species inhabiting intertidal sediments across Northern Europe with important roles as a sediment engineer and prey species for wading birds and fish. Its responses to environmental stressors are therefore of key importance to coastal ecosystems, yet they remain understudied with respect to OA. *A. marina* reproduce by the female spawning eggs into her burrow while sperm is shed onto the surface of the sand on an incoming tide, with fertilization and early larval development thought to occur within the female burrow.[33] As such these stages are likely to be exposed to any coastal metal contamination present which often accumulates in sediment and pore waters. Here we investigate the response of the early life history stages of *A. marina* to combined exposure to OA conditions and copper in order to test the hypothesis that copper toxicity will be enhanced at reduced seawater pH.

MATERIALS AND METHODS

Animal Collection and Maintenance. Animals were collected from Mothecombe beach, Devon, UK (50°31″23 N, −3°94″58 W) during November 2013 and assessed for sex and maturity. This site is considered to be relatively "clean" and free of any significant contamination (Environment Agency, 2007). Males and females were maintained in separate static 16 L tanks in a temperature controlled room (12 ± 0.5 °C) for a minimum of 7 days prior to experimental procedures. Tanks were filled with natural sediment collected from Mothecombe and well aerated artificial seawater (ASW, Tropic Marin) made up to a salinity of 35 psu. Salinity was monitored using a Mettler Toledo SG7 SevenGo pro conductivity meter to an accuracy of ±0.1 psu.

Gamete Collection. Spawning was induced in males by injection of 1 mL 8,11,14-eicosatrienoic acid (Sigma Co.) into the coelomic cavity.[34] Spawning followed approximately 1 h after injection with sperm collected "dry" as it was extruded from the nephromixia to prevent premature activation and stored in microcentrifuge tubes on ice until use within 4 h. Females were injected with 1 mL prostomial homogenate (equivalent to one per individual) on the day prior to an experiment and kept overnight in individual crystallizing dishes containing 180 mL of ASW filtered to 1 μm (FASW). Eggs were collected the following morning and stored in microcentrifuge tubes in fresh FASW on ice until use within 6 h of collection. Microlitres of dry sperm were diluted with known volumes of FASW and replicate sperm counts were performed using a Neubauer Hemocytometer. Egg counts were performed on three 1 μL microvolumes and an average concentration of settled oocytes calculated for each female under a compound microscope.

Water Chemistry. Artificial seawater was aerated for 2−4 h prior to an experiment and constituted the ambient seawater pH treatment (pH 8.28, ∼400 μatm pCO_2, described in Table 1). Seawater pH values of 7.77 and 7.47 were targetted to represent near and medium-term ocean acidification treatments (as projected according to scenario RCP 8.5 and the 2013 IPCC WGI AR5,[35] full seawater chemistry is provided in Table 1). For the short-term sperm exposures, FASW was acidified to the desired pH using a computerized control system (NBS scale, AquaMedic, Germany) which regulated pH via a solenoid valve and triggered the injection of pure gaseous CO_2 once seawater pH exceeded preprogrammed levels (+0.05) in conjunction with constant vigorous aeration. Seawater pH was monitored with an

additional Metrohm (827 pH lab) pH_{NBS} electrode and NBS buffers to ensure water was collected at ±0.02 the desired pH. The Tropic Marin© artificial seawater used in these exposures has high alkalinity compared to average natural seawater (see Table 1). As a result of this extra buffering capacity the pCO_2 levels required to reach the IPCC relevant pH values for OA (2013 IPCC WGI AR5[35]) were higher than would be required for natural seawater, resulting in these experiments having higher pCO_2 levels than the RCP 8.5 predicted values (1400 μatm and 3000 μatm, respectively). Seawater pH values from the RCP 8.5 scenarios were targeted rather than pCO_2 since pH is the main driver of speciation change in copper which was the focus of this study.

The appropriate volume of copper sulfate stock solution (20 mM) was added to seawater pH treatments to give nominal copper concentrations representing natural background levels in coastal systems (0.2 μM[13]), a polluted site (2 μM[36]) and also a higher concentration (20 μM) found in acute preliminary exposures to induce similar levels of DNA damage as measured in the sperm and somatic cells of polychaetes, including *A. marina*, from chronically polluted field populations.[37] This higher concentration was used for mechanistic insight and does not relate to a field-relevant concentration. Seawater samples were collected from experimental treatments for dissolved inorganic carbon (DIC) analysis which was carried out using a custom built system described by Friederich et al.[38] and following the methodology detailed in Lewis et al.[25] This analytical system allowed the measurement of seawater DIC with a precision of ±3 μM. DIC measurements were checked against certified reference materials (produced in the Scripps Oceanographic laboratory by Andrew Dickson) to ensure the accuracy of this system. Total alkalinity and pCO_2 were calculated using CO2SYS,[39] using the parameters selected by Findlay et al.,[40] the DIC, pH and salinity measurements using the NBS scale, the K_1 and K_2 values determined by Mehrbach[41] (values refitted by Dickson and Millero[42]) and K_{SO4} as determined by Dickson.[43]

Sperm Motility Analysis. Sperm motility was assessed at the full range of seawater pH treatments and copper concentrations by computer assisted sperm analysis (CASA). Two mL of experimental seawater followed by 13 μL of sperm was added to 5 mL vials and repeated for each male (n = 5). Vials were gently agitated to mix the sperm-seawater solution and incubated at 12 ± 0.1 °C for 10 min. Three microlitre volumes of sperm−seawater solutions were immediately transferred to Leja 20 mm standard counting chambers. Motility analysis was carried out using a Microptic Sperm Class Analyzer (Microm, UK) fitted with a Nikon Eclipse 50i negative phase contrast microscope (100× magnification and a Peltier cooled stage) which was operated at 12 ± 0.1 °C. Images were captured at a rate of 100 frames per second and individual sperm were tracked for 0.5 s. A minimum of 500 sperm were tracked in each sample with data on percent motility and a number of CASA derived motility parameters calculated. Threshold values of 10 μms^{-1} curvilinear velocity (VCL) and 3.2 μms^{-1} average path velocity (VAP) were utilized to remove nonmotile sperm from the subsequent analysis and determine percentage sperm motility (i.e., the percentage of sperm with motility parameters recorded above threshold values of the total number analyzed). The recommendations of Mortimer et al.[44] on the use of CASA systems in research were followed where applicable to marine invertebrate sperm, that is, sperm loading times and the avoidance of wall effects, in order to standardize the readings taken.

Comet Assay. The comet assay was utilized to measure DNA strand breaks in sperm exposed to seawater pH and copper treatments using methodology previously described by Lewis and Galloway[45] with minor modifications. A 10 μL aliquot of undiluted sperm from each male (n = 8) was added to individual vials containing 1.85 mL of treatment seawater. Sperm-seawater solutions were incubated at 12 ± 0.1 °C for 1 h before being centrifuged for 4 min at 7826 g. The excess fluid was removed and the cell concentrate gently mixed with 1% low melting point agarose heated to 37 °C and dropped onto slides previously coated in 1% high melting point agarose. Briefly, cells were subjected to 2 h of lysis in alkaline conditions at 5 °C, followed by 45 min of denaturation in electrophoresis buffer (0.3 M NaOH and 1 mM EDTA), 30 min of electrophoresis at 25 V and a final neutralization step. Slides were stained with SYBR Safe DNA Gel Stain and examined using fluorescence microscopy (excitation: 502 nm; emission: 530 nm). One hundred cells per slide were analyzed using Comet Assay IV (Perceptive Instruments Ltd.) to quantify the percentage of DNA in the comet tail (resulting from DNA strand breaks) to approximate the percentage of DNA damage induced in each treatment.

Fertilizations. The fertilization experiments took place over 2 days with freshly spawned gametes collected on each day. The gametes from each male and female were completely crossed under each experimental treatment (day 1, n = 3 females and n = 3 males; day 2, n = 3 females; and n = 2 males). We selected our near-future pH scenario (pH 7.77, 1400 μatm pCO_2) and highest copper concentration (20 μM) as the experimental treatments. The volume equivalent to 1000 oocytes from each female was added to individual wells of a 12 well plastic tissue culturing plate containing 5 mL of treatment seawater. Sperm was diluted with seawater of the corresponding treatment to a final concentration of 1×10^4 sperm mL^{-1} (measured in the field as the sperm concentration for a spawning population by Williams et al.[46]) and added to the corresponding wells; this was repeated individually for each cross. Each well was gently agitated to maximize sperm-oocyte encounters before being covered and incubated at 12 ± 0.1 °C. After 10 min the eggs were washed three times in treatment seawater to remove excess sperm and prevent any further fertilization before incubation for a further 10 h. Wells were then fixed in 5% paraformaldehyde in seawater and fertilization success and developmental stage) recorded from samples of 50 oocytes per well. Fertilization success was calculated as the percentage of viable postfertilization stages (determined as having intact fertilization membranes and no discolouration) recorded from the total number of oocytes scored.

Larval Survival. To assess the combined effects of OA and copper on larval survivorship in *A. marina*, sperm from two males was pooled and diluted to a concentration of 1×10^4 sperm mL^{-1} before being added to individual beakers (n = 3) each containing ambient FASW and 20 000 eggs from one female (three females used in total). Beakers were covered and incubated at 12 ± 0.1 °C for 1 h to allow fertilization under ambient conditions and checked for successful fertilization. A volume equivalent to 7000 fertilized eggs was added to cylindrical 6 cm diameter plastic pots containing 180 mL of treatment seawater. We selected our near-future pH scenario (pH 7.77, 1400 μatm pCO_2) and highest copper concentration (20 μM) as the experimental treatments. Pots (n = 3) were incubated at 12 ± 0.1 °C and either aerated to maintain ambient conditions or received a mixed gas input through a 21 g hypodermic needle and a GFC mass flow controller to maintain a seawater pH of approximately 7.77 units.

Daily pH measurements were taken of randomly selected pots from each treatment using a Metrohm (827 pH lab) pH_{NBS} electrode and NBS buffers and flow rate adjusted accordingly. Five days postfertilization 300 embryos or larvae from each pot were scored as to developmental stage and viability. Larval survival was calculated as the percentage of viable larvae from the postfertilization stages scored.

Statistical Analysis. Linear mixed-effects modeling explored the influence of seawater pH and copper on average sperm VCL including male identity as a random term. The residuals of linear mixed-effects models on arcsine transformed percent motility data were not normally distributed so a generalized linear mixed-effects modeling approach with a binomial error family and logit link was adopted. Proportion sperm motility data was weighted by the number of sperm analyzed in each sample and male identity was included as a random term. The percentage DNA damage, fertilization success and larval survival data were arcsine transformed prior to analyses and linear mixed-effects modeling explored the influence of seawater pH and copper on each response variable. Where appropriate male identity, female identity, pair identity, and/or day were included as random effects when considered biologically important. Missing data was omitted from analyses. Any nonsignificant terms were dropped from models to give the appropriate minimum adequate model (MAM). All parametric models were checked to ensure that residuals were normally distributed. All statistical analyses were conducted using R version 3.02 and the nlme, lme4 and ggplot2 packages.[47]

RESULTS

Sperm Motility. The swimming speeds of motile sperm ranged from 10.1 to 332.7 μms^{-1} across experimental treatments. Individually copper and reduced seawater pH were found to reduce sperm swimming speeds, measured as average VCL, compared to those under ambient conditions (Figure 1A, see Supporting Information (SI) Table SI-1 for the full output from the linear mixed-effects model). This was true for the two highest nominal copper concentrations; 2 μM ($t = -3.839$, df = 49, $p < 0.001$) and 20 μM ($t = -3.817$, df = 49, $p < 0.001$), and at a reduced pH of either 7.77 (1400 μatm pCO_2, $t = -5.213$, df = 49, $p < 0.001$) or 7.47 (3000 μatm pCO_2, $t = -4.832$, df = 49, $p < 0.001$). In combination, motility was reduced by up to 46% from average speeds of 141.2 μms^{-1} in ambient conditions to as little as 78.3 μms^{-1} as a result of negative additive effects of the two stressors combined. Statistical analysis did not identify an interaction term (Likelihood ratio test, $p = 0.579$) suggesting additive toxicity. Percent motility response ranged from a 1% enhancement to a 10% reduction across treatments from an average of 98% motile sperm under ambient conditions (Figure 1B). We found a slight but significant motility enhancement under near-future OA but there were negative interactions between this pH and each concentration of copper (Table 2). Nominal copper concentrations of 2 and 20 μM reduced percentage motility by up to 4%. Medium-term OA reduced the percentage of motile sperm by on average 3% from ambient present-day conditions.

Sperm DNA Damage. There was an increase in spermatozoan DNA damage in the presence of copper across all pH treatments from an average of 14% damage in our controls to 26% in copper treatments (Figure 2, the full output from the linear mixed-effects model is presented in SI Table SI-2). Seawater pH and copper interactively induced sperm DNA damage and subsequent analysis identified that this interaction took place between copper and our lowest seawater pH ($t = 4.380$, df = 32, $p < 0.001$). Medium-term OA significantly influenced sperm DNA damage ($t = 2.418$, df = 32, $p = 0.022$).

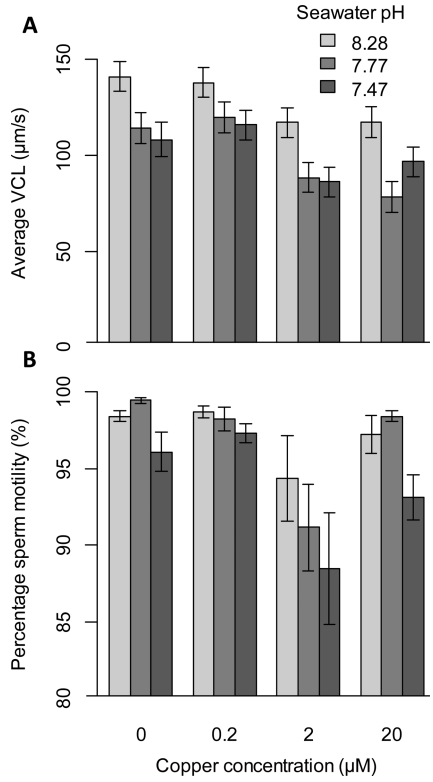

Figure 1. Effects of seawater pH and copper on (A) sperm swimming velocity and (B) percentage sperm motility in *A. marina* ($n = 5$). Data displayed as fitted group means (A) and group means (B) ± 1 se. Significance levels not included.

Table 2. Results of Generalized Linear Mixed-Effects Modelling of the Influence of Seawater pH and Copper on the Percentage of Motile Sperm[a]

	estimate	std. error	Z	p
intercept	4.157	0.195	21.303	**<0.001**
(A) pH				
7.77	1.050	0.290	3.636	**<0.001**
7.47	−0.820	0.185	−4.429	**<0.001**
(B) Copper (μM)				
0.2	0.243	0.218	1.115	0.265
2	−1.280	0.169	−7.590	**<0.001**
20	−0.566	0.187	−3.019	**0.003**
(C) pH: Copper Interactions (pH Units: μM)				
7.77:0.2	−1.356	0.357	−3.795	**<0.001**
7.47:0.2	0.120	0.268	0.447	0.655
7.77:2	−1.554	0.307	−5.055	**<0.001**
7.47:2	0.005	0.209	0.025	0.980
7.77:20	−2.145	0.319	−6.730	**<0.001**
7.47:20	−0.111	0.229	−0.484	0.629

[a]Male identity was included as a random term and the analysis was weighted by the number of sperm analysed from each sample (significant p values ($p \leq 0.05$) are highlighted in bold and all results are in comparison to reference "control" conditions (pH 8.28, 0 μM copper)).

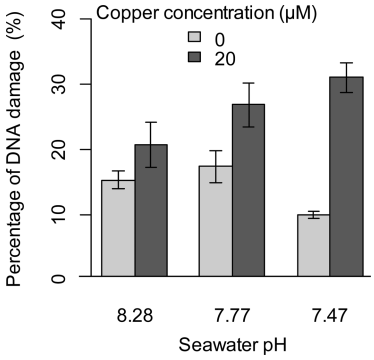

Figure 2. Effects of seawater pH and copper on the percentage of sperm DNA damage in *A. marina* (n = 8). Data displayed as group means ±1 SE. Significance levels not included.

Gamete Fertilization Success. We observed an average 96% fertilization success in our ambient pH and no copper controls. Both copper exposure (t = −10.239, df = 33, p < 0.001) and seawater pH reduction (t = −6.146, df = 33, p < 0.001) significantly reduced fertilization success (full linear mixed-effects model output is presented in SI Table SI-3). There was no further reduction in fertilization success under combined copper and OA exposures, with a near identical percentage success in our two copper treatments irrespective of seawater pH (Figure 3A).

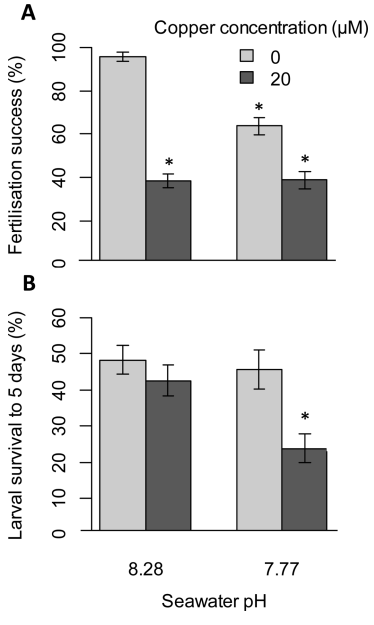

Figure 3. Effects of seawater pH reduction and copper on (A) fertilization success (n = 15 crosses) and (B) larval survivorship 5 days postfertilization (n = 3) in *A. marina*. Data displayed as group means ±1 SE (* indicates a significant difference from success or survival under ambient seawater pH and no copper ($p \leq 0.05$)).

Statistical analysis confirmed this to be a statistically significant positive interaction between the two stressors (t = 5.037, df = 33, p < 0.001) rather than any additive or synergistic toxicity.

Larval Survival. Larval survivorship 5 days after fertilization had dropped to 48% in the control treatment of ambient pH and no added copper (Figure 3B). Individually, exposure to copper (t = −1.324, df = 6, p = 0.234) or reduced seawater pH (t = −0.610, df = 6, p = 0.564) had no significant influence on larval survival (full linear mixed-effects model output is presented in SI Table SI-4). However, a strong significant interaction was found between OA and copper on larval survival, with a 24% reduction in larval survival under combined exposures compared to them as individual stressors after 5 days (t = −2.871, df = 6, p = 0.028).

DISCUSSION

This series of combined OA relevant seawater pH−copper exposure experiments have clearly demonstrated the potential for OA to alter the toxicity of the common contaminant copper to early stages of the polychaete *Arenicola marina*. The influence of OA on copper toxicity varied according to life history stage. A strong negative synergism between OA and copper (i.e., greater effects in combination than the sum of effects of the individual stressors) was measured for sperm DNA damage and early larval survival, with significantly greater toxicity responses measured under reduced seawater pH (either 7.77 (1400 μatm pCO_2) or 7.47 (3000 μatm pCO_2)) for these end points compared to ambient pH levels. Sperm swimming velocities were negatively influenced by both near-future seawater pH and copper concentrations that mimic a heavily polluted coastal site, with additive toxicity effects of the two stressors when exposed in combination. These findings align with previous studies showing a similar influence of near-future ocean acidification on metal-induced DNA damage,[21] larval mortalities[25] and larval production[26] in a number of marine invertebrate species.

Sperm swimming speed and the number of motile sperm are important parameters determining fertilization success for any broadcast spawning marine invertebrate, particularly when sperm concentrations are low.[48,49] We found that swimming speeds were strongly affected by both OA and copper exposures individually, and these stressors acted additively in combination leading to substantial speed reductions. The percentage of motile sperm (i.e., sperm which have been activated and initiated motility) appears more robust than swimming velocity to near-future pH in this species and was slightly but significantly enhanced in our near-future pH treatment. This disagrees with the majority of studies to date that have directly examined the impacts of reduced seawater pH/elevated pCO_2 on percentage sperm motility and identified reductions under near-future OA.[50−55] The 19% OA-induced decrease in sperm swimming speeds we observed in *A. marina* was much greater than that measured by Lewis et al.[25] in the tubeworm *Pomatoceros lamarckii* for similar pH levels, with *A. marina* also having much faster sperm in general. They also exceed the swimming speed reductions reported in a sea urchin and a further polychaete species exposed to near-future OA[53,55] suggesting *A. marina* sperm might be particularly sensitive to reduced pH/elevated pCO_2. Our results add to increasing evidence that sperm responses to reduced seawater pH are highly species-specific and can range from decreased sperm velocities[53,55] to robust swimming responses[56] and even motility enhancement.[57] While other studies have also shown strong between male differences within a species[52,55] our analysis took this intermale variation into account in order to identify the influence of our seawater pH treatments across males.

Fertilization success decreased by 32% under near-future OA from our control treatment, most likely resulting from fewer gamete collisions as a consequence of reduced sperm swimming speeds. This is comparable to a 20% reduction in successful fertilization measured in the sea urchin *Helicidaris erythrogramma* under similar pH conditions[53] and makes *A. marina* one of the more sensitive marine invertebrate species to OA-induced disruption of fertilization success. Numerous studies have

investigated the impacts of OA on fertilization success as a single stressor across a wide range of invertebrate species, with conflicting results reported both within and between species.[58−61] Some studies have reported significant reductions in fertilization success under near-future OA conditions,[53,62,63] while others have reported fertilization to be robust to elevated seawater pCO_2[58,64−66] although there may be differences in fertilization response between individual mating pairs.[67] These differences may be down to true intraspecific differences in OA response, driven by the strong intermale and interspecies variation in sperm functional response to OA being observed.[52,55] However, experimental design has also been suggested as a possible source of these differences in fertilization response to OA[10,68] making interpretation difficult.

Our results are perhaps surprising for an intertidal polychaete, as an emerging paradigm within OA research suggests that any organism experiencing natural variability in pH conditions within their habitat will be more resilient to future OA.[69,70] This hypothesis would predict that the gametes of intertidal species would be less sensitive to OA reflecting adaptation to the fluctuating pH conditions they may experience.[58,70] In *A. marina* fertilization takes place within intertidal female burrows on an incoming tide as sperm are washed into the burrows. Information on seawater pH and carbonate chemistry conditions for the intertidal zone in general is poor, however there is evidence that this environment is highly variable in terms of pH and pCO_2.[71,72] Conditions within an *A. marina* burrow at the time of fertilization have not been measured but would be expected to show strong tidal and seasonal fluctuations, being influenced by the chemistry of the incoming tide and the respiration activity of the animals within the burrow. We cannot currently make any accurate predictions of the pH conditions fertilization for this species would normally take place under. These stronger sperm responses in the OA only treatments may of course be influenced by the higher pCO_2 needed to reach our seawater pH values in our exposure system given the high alkalinity of the artificial seawater. The pCO_2 values of 1400 μatm and 3000 μatm are higher than the IPCC predicted values (2013 IPCC WGI AR5[35]) and so these results must be taken in that context. The role of seawater pH versus pCO_2 in determining sperm swimming responses, or many OA responses for that matter, have not been clearly resolved. The focus of these experiments was the interaction of a CO_2-induced reduction of seawater pH and copper on toxicity responses from a mechanistic viewpoint, and these have been clearly demonstrated.

Copper acted to strongly reduce fertilization success irrespective of seawater pH. Copper toxicity to gametes during fertilization has been reported at a range of environmentally relevant concentrations (as well as much higher concentrations) for a number of marine invertebrate species.[73−75] Echinoderms appear to be a more sensitive group of invertebrates, with EC_{50}s reported of between 1.9−59 $\mu g\ L^{-1}$,[31] while polychaetes appear to be more tolerant and are often found living in contaminated habitats.[36] Caldwell et al.[30] report an EC_{50} value of 2.2 μM in the polychaete *Nereis virens,* while Watson et al.[32]report conflicting sensitivity for *N. virens* observing no reduction in fertilization success below 500 $\mu g\ L^{-1}$ copper.[32] Hollows et al.[49] identified sperm concentration-specific reductions to fertilization success at very low copper concentrations ($\geq$0.16 μM) in another polychaete species, *Galeolaria caespitosa*. The reductions we observed are likely to be a result of direct copper toxicity upon processes such as sperm-egg binding or the sperm acrosome reaction which are crucial to fertilization in a number of marine species,[76] or via direct effects upon the oocyte membrane. Surprisingly, given our other results, there was no additive toxicity effect of combined OA and copper on fertilization success. We used a relatively high concentration of copper in these experiments to enhance any interaction between OA and copper that may exist from a mechanistic viewpoint; however this may actually have masked more subtle interactions between the two stressors such as those observed in the sperm swimming speed data where additive effects were observed at lower copper concentrations.

More interesting was the strong interaction between OA and copper upon sperm DNA damage observed in our short-term sperm exposures. A similar synergism was observed for somatic cells in the amphipod *Corophium volutator* exposed to metal contaminated sediment under a range of OA conditions[21] providing tentative evidence that this pattern may be consistent across cell types and marine invertebrate species. For the same nominal copper exposure we observed a 10% increase in sperm DNA damage under reduced seawater pH suggestive of increased oxidative stress under combined exposure scenarios. The most parsimonious explanation for this is the predicted increase in the toxic free ion concentration of copper under OA[77] enhancing ROS production.[18] Intriguingly this contrasts with a recent study using isolated mantle cells of the hard clam *Mercenaria mercenaria* which found that, while reduced seawater pH increased copper uptake into these cells, ROS generation was attenuated under OA.[78] The authors suggested this may be due to an up-regulation of antioxidant proteins, which may explain the different response observed here since sperm are generally believed to be lacking in both antioxidant defenses and DNA repair enzymes.[79] Sperm may also be more susceptible to oxidative damage due to the abundance of polyunsaturated fatty acids acting as substrates for ROS.[78] Our exposures were very short-term in vitro exposures of 1 h with no prior paternal exposure. Sperm are perceived as having very limited DNA repair capability[80] and thus oxidative DNA damage is likely to accumulate over time. Relatively high copper concentrations were used in our exposures to mimic the accumulated damage over longer chronic exposures measured previously in the sperm of polychaetes from polluted habitats.[37] Sperm DNA damage has been shown to have consequences for population fitness, with embryos fathered by sperm with induced DNA damage suffering a higher incidence of severe developmental abnormalities.[45]

This strong synergistic toxicity between reduced seawater pH and copper was also present in the early larval survivorship of A. *marina*. Data identifying the point the early larvae of *A. marina* are washed out of the female burrow and enter the water column is not available, but laboratory studies suggest they spend 3−4 days in the water column before returning to the benthic environment just prior to settlement. Larvae are often considered to be the most sensitive life history stage to environmental stressors, particularly in free spawning marine invertebrates with biphasic life histories. While *A. marina* larvae were relatively robust to high concentrations of copper and near-future OA as single stressors, combined exposure caused a significant drop in survivorship to half that under ambient conditions after 5 days. This finding parallels earlier work in the intertidal polychaete *P. lamarckii* at an environmentally realistic copper concentration (0.002 μM).[25] This synergistic toxicity may also simply be a result of enhanced copper bioavailability at reduced seawater pH, however the biological processes underpinning toxicity responses in larvae will be much more complex than for sperm due to the potential for repair and detoxification processes and

energetic trade-offs. Oxidative damage to cellular components (lipids, DNA and proteins) may have overwhelmed larval antioxidant defenses and the increased energetic costs of expensive DNA repair[81] and cellular detoxification may have exceeded larval energy budgets. Alternatively the energetic costs of coping with two stressors simultaneously may have overwhelmed larvae. According to the compensation hypothesis animals under stress will make energetic trade-offs between different physiological energy requiring processes in order to meet elevated energy demands under stress.[81] Larval development under OA may be energetically expensive due to potential increases in ion regulatory processes,[82] OA compensatory mechanisms,[83] costs to maintaining cellular homeostasis[84] and/or oxidative stress[85] and energy may have been diverted away from physiological maintenance processes. This in addition to the costs associated with copper detoxification and the repair of cellular oxidative damage may have exceeded an energetic "tipping point" dramatically influencing larval survival when exposed to both stressors in combination. *A. marina* have lecithotrophic (i.e., nonfeeding) larvae, hence have a limited energy reserve with which to reach the settlement stage where feeding commences. This means that as larvae they are not reliant on food supplies, possibly buffering them from single stressors such as OA in isolation.[86] This may, however, make them more sensitive to the additive energetic demands of multiple stressors compared to a planktotrophic larvae in an environment with high food availability (or laboratory experiment where animals are fed ad libitum), which may be able to compensate the additional energetic costs with increased food intake (as shown by Thomsen et al.[87]). Decreases in early larval survival are likely to negatively affect settlement and juvenile recruitment[88] with potential consequences for population persistence and size maintenance.

Our results demonstrate the potential for the projected reduction in seawater pH under OA to significantly alter the susceptibility of early life history stages of *A. marina* to the common coastal pollutant copper. Short-term "shock" exposure experiments cannot simply be scaled-up for century-scale responses of organisms to OA, but they do provide us with an understanding of potential mechanisms of impact upon populations that warrant further investigation and that are relevant for looking at how OA might alter contaminant toxicities. Since coastal contamination is widespread this interaction has significant implications both for current predictions of OA impacts upon coastal marine invertebrates and for ecological risk management of marine habitats. There may be other existing and emerging contaminants whose toxicity are influenced by seawater pH and whose effects need to be considered within the context of OA if environmental risk assessments are to work effectively to protect our marine ecosystems over the coming century.

■ ASSOCIATED CONTENT

Supporting Information

Supporting Information for this study is provided and includes the results tables of linear mixed-effects modeling of the influence of seawater pH and copper upon sperm swimming velocity and DNA damage, fertilization success and larval survival. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

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