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Toxicities of copper oxide nanomaterial and copper sulphate in early life stage zebrafish: Effects of pH and intermittent pulse exposure

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ABSTRACT

Effort has been made to standardise regulatory ecotoxicity tests for engineered nanomaterials (ENMs), but the environmental realism of altered water quality and/or pulse exposure to these pollutants should be considered. This study aimed to investigate the relative toxicity to early life-stage zebrafish of CuO ENMs at acid pH and then under pulse exposure conditions, all compared to $CuSO_4$. At all pH values, $CuSO_4$ was more toxic to zebrafish than CuO ENMs. Additions of H^+ were protective of $CuSO_4$ toxicity, with median lethal concentrations LC_{50} (with 95% confidence intervals) of: 0.36 (0.33–0.40), 0.22 (0.20–0.24) and 0.27 (0.25–0.29) mg L^{-1} at pH 5, pH 6 and pH 7, respectively. In contrast, the toxicity of CuO ENMs increased with acidity; LC_{50} values were: 6.6 (4.5–8.5), 19.4 (11.6–27.2) and >100 mg L^{-1} at pH 5, pH 6 and pH 7, respectively. The increased toxicity of the CuO ENMs in acid water corresponded with greater dissolution of dissolved Cu from the particles at low pH, suggesting free Cu^{2+} ion delivery to the zebrafish was responsible for the pH-effect. In continuous 96 h exposures to the substances at the LC_{10} values and at pH 6, both $CuSO_4$ and CuO ENMs caused Cu accumulation, inhibition of Na^+/K^+-ATPase and depletions in total glutathione in zebrafish. However, two 24 h pulses of $CuSO_4$ or CuO ENMs at the same peak concentration caused similar effects to the continuous 96 h exposure, despite the shorter exposure durations of the former; suggesting that the pulses were more hazardous than the continuous exposure. In conclusion, the current water quality correction for pH with respect to Cu toxicity to freshwater fish should not be applied to the nano form. Crucially, CuO ENMs are more toxic in pulse than continuous exposure and new corrections for both water pH and the Cu exposure profile are needed for environmental risk assessment.

1. Introduction

In regulatory toxicology, single species toxicity tests are widely used to provide the data for the environmental hazard assessment of contaminants. These tests are also used for the hazard identification of engineered nanomaterials (ENMs, Crane et al., 2008Crane et al., 2008), albeit with some modifications to improve their utility for ENMs (Handy et al., 2012). However, regulatory toxicity tests have long been criticised for a lack of environmental realism (Cairns, 1983; McCahon and Pascoe, 1990; Artigas et al., 2012; McCarty et al., 2018). One of these concerns is that regulatory tests often use steady-state concentrations of the test substance, and a prescribed pH, dissolved oxygen, etc., which do not reflect the temporal dynamics and complexity of real pollution events (McCahon and Pascoe, 1990; Handy, 1994; Vignati et al., 2007). For example, freshwaters are chemically and temporally heterogeneous due to daily fluctuations in temperature, oxygen, pH, rainfall, run-off from urban or agricultural land, and the inputs of pollutants (review, Handy, 1994; Edwards and Moore, 2014). Field measurements show variable profiles of contaminants in aquatic ecosystems (Edwards and Moore, 2014) and even the consented discharge of effluents will change according to the anthropogenic activities producing the wastes. Consequently, aquatic organisms are more likely to be exposed to single or repeated pulses of pollutants (Handy, 1994). For metals, such as Cu that is widely found in industrial effluents, the challenge has been to identify the relative hazard of intermittent and complex pulse exposure compared to the results of continuous exposure toxicity tests.

The effects of dissolved Cu on freshwater fishes have been extensively studied for several decades (see reviews by Handy et al., 1989; Grosell, 2012). This research has mainly used continuous exposures to define the lethal concentrations to fish (LC_{50} range from 10 to 150 μg L^{-1} in soft water, Handy, 2003) and the mechanistic aspects of Cu toxicity; including the water quality variables that alter toxicity. The mechanism of acute toxicity of dissolved Cu in the water column involve inhibition of the Na^+/K^+-ATPase in the fish gill, with subsequent disturbances to osmoregulation (Laurén and McDonald, 1985

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; Shaw et al., 2012). Water chemistry has been shown to offer some protection to fishes. For example, lower pH values are generally protective of fish due to competition between Cu and H^+ for binding at the gills (Cusimano et al., 1986). Exposure modelling for dissolved Cu, such as the biotic ligand model, has therefore been able to identify the bioavailable fraction of metal that is hazardous to fish (Santore et al., 2001). This has been reflected in water quality criteria for Cu derived from site-specific freshwater parameters such as water hardness, pH and the presence of dissolved organic matter (e.g., in China, Zhang et al., 2017a; and as used by the US Environmental Protection Agency, 2007).

Whether or not dissolved Cu is more toxic as a pulse exposure or as a continuous exposure is dependent on the number of pulses, the peak concentration of each pulse, the average concentration or dose within each pulse profile, and the organisms used (Handy, 1994). Pulse exposure might show similar toxicity compared to continuous exposure when the sum of the pulse profile(s) happens to be similar to the continuous exposure dose, but can also show toxicity greater than that expected of the continuous exposure equivalent (Handy, 1994). For example, Bearr et al. (2006) reported the same mortality level in fathead minnow (*Pimephales promelas*) exposed to two 24 h pulses of dissolved Cu with a peak concentration of 40 μg L^{-1} within a 7 d period, compared to exposure to 40 μg Cu L^{-1} continuously for the entire 7 d duration. Thus the toxicity could be attributed to the pulse profile, not the peak concentrations that were the same in both types of exposure. Changes in physiology such as altered ventilation of the gills to decrease contaminant uptake, or increased activity of detoxification mechanisms, can mitigate the effects of continuous exposures. For example, it has been shown that 'copper-acclimated' rainbow trout (*Oncorhynchus mykiss*) clear plasma-Cu more effectively than non-acclimated fish (Grosell et al., 2001). However, physiological adjustment may not be initiated in response to short or infrequent pulse exposures. Crucially, the amount of time in clean water between pulses can also inform on how much Cu can be cleared from the body burden; and when this time is short a cumulative effect on the body burden is observed (Handy, 1992). Environmental standards derived from continuous exposures to Cu are therefore difficult to apply to dynamic pollution events.

For ENMs, there is an emerging literature on Cu toxicity from continuous exposure studies with fish, but intermittent, pulse exposures have not been explored. In the continuous exposure studies, copper ENMs have also been shown to affect sodium homeostasis in fish (zebrafish, Griffitt et al., 2007; trout, Shaw et al., 2012). Many of the sub-lethal effects of Cu ENMs in trout (e.g., haematology, oxidative stress, organ pathologies) have been shown to be similar to the metal salt except the aetiology of pathology of the ENMs manifested later during exposure or at higher relative concentrations (Shaw et al., 2012; Al-Bairuty et al., 2013). These similarities in the patterns of toxicity suggest that Cu ENMs release a dissolved fraction of metal that is toxic to the fish. Indeed, some dissolution of Cu from Cu NPs occurs in freshwater and this is increased when water pH is lowered (Al-Bairuty et al., 2016).

Alterations of water pH may also cause physiological stress in fish. Real pollution events involving pulse exposure to metals can be accompanied by dynamic changes of pH. For example, after rainfall or snowmelt on sulphide bearing rock (Dold, 2014; Hindar and Nordstrom, 2015), runoff from roads (Zhang et al., 2016) and on soil in areas of heavy industry (Alexander et al., 2017). It is not known which variable causes the most hazard to fish; i.e., the pulse of the toxic substance, or the exposure to acid pH. However, a reduction in water pH has, much like Cu exposure, been shown to perturb sodium balance in fish (e.g. at pH 4.0 in rainbow trout; Freda and McDonald, 1988). Such information is also not easily derived from data obtained in routine regulatory toxicity tests (e.g., OECD TG210 with fish), because the test protocols prescribe a constant water chemistry during the tests. A pulse exposure protocol would therefore be a useful add-on for regulatory testing (e.g., as used for organics by Zhang et al., 2017b) and with consideration of other critical variables such as pH.

This study aimed to explore the effects of water pH and intermittent pulse exposures on CuO ENM toxicity to early life stage zebrafish (*Danio rerio*). Zebrafish were chosen because they have utility as a test organism for ENMs (e.g., Nel et al., 2012; Lin et al., 2013; Shaw et al., 2016) and are suitable for the practical aspects of managing pulse exposures in the laboratory. Zebrafish are also acid-tolerant and survive well in acid water as low as pH 4.0 (Lewis and Kwong, 2018) by upregulating cells involved in proton secretion (Horng et al., 2009) and the tight-junction proteins involved in decreasing epithelial Na^+ permeability (Kwong and Perry, 2013). The study design also included a metal salt control ($CuSO_4$) to enable direct comparison with particle effects. A first series of experiments explored the effects of pH alone on the acute toxicity of CuO ENMs in media adjusted to pH 5, pH 6 and pH 7. Then a second series of experiments explored the effects of pulse exposure to CuO ENMs. For these latter experiments, a suitable acidic pH was selected (pH 6) and peak concentrations of CuO ENMs that were intended to be sub-lethal (96 h LC_{10} derived from acute toxicity tests). Three pulse exposure scenarios were conducted in experiments that lasted 96 h with CuO ENMs or $CuSO_4$: (1) a single pulse of exposure (0–24 h) early in the development of the fish embryo and then the remaining time in clean water. This pulse was conducted to determine if exposure of zebrafish embryo to a pulse caused any effects that persisted as the animals hatched into clean water. (2) A double pulse exposure with time in clean water between the pulses (i.e., pulses at 24–48 h and 72–96 h); and finally, (3) a continuous exposure control lasting the entire 96 h.

2. Methods

2.1. Zebrafish

Brood stock zebrafish were maintained in conditioned Plymouth tap water. The latter had been left aerating in large drums to dechlorinate before use (water chemistry in mM, means ± SD, n = 3: Ca^{2+}, 1.12 ± 0.05; K^+, 0.10 ± 0.01; Mg^{2+}, 0.14 ± 0.01; Na^+, 0.93 ± 0.04; pH 7.3; conductivity 168.3 μS/cm). The background Cu concentration in Plymouth tap water was 4.7 ± 0.1 μg L^{-1}. Stock fish were held in a recirculating water system at 27 ± 1 °C and under a photoperiod of 12 h light and 12 h dark, in a dedicated approved aquarium facility at Plymouth University. Fish were fed daily on newly hatched brine shrimp *Artemia* spp. and TetraMin Tropical flake food (Tetra, UK). To obtain embryos, pairs of zebrafish (one male and one female) were netted from stock tanks, gently transferred to 1 L breeding tanks fitted with a partition to separate the fish (Tecniplast, Italy) and left overnight. The following morning, the water in the breeding tanks was refreshed, the partitions removed, and the fish allowed to spawn. After 1 h, the adult fish were transferred back to stock tanks and all embryos from multiple spawning pairs were pooled into a single 2 L beaker to randomise the subsequent distribution of embryos from all fish between treatment groups. Embryos were rinsed with dechlorinated and filtered (0.2 μm VWR Complete Filtration Unit, VWR, UK) Plymouth tap water and non-fertilised eggs and debris were removed by hand with a Pasteur pipette. The Animal Welfare Ethical Review Board at Plymouth University approved all experiments with early-life stage zebrafish and in compliance with Directive 2010/63/EU and its exemptions.

2.2. Copper oxide ENMs, $CuSO_4$ and particle characterisation

A 1 mg mL^{-1} stock of Cu (as $CuSO_4$) was prepared in ultrapure water (18.2 MΩ, ELGA, UK) in an acid-washed volumetric flask and

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used as required. Copper oxide (CuO) ENMs were obtained as a dry powder from PlasmaChem GmbH (Berlin, Germany) and were supplied as part of the Sustainable Nanotechnologies Project (SUN) funded by the EU 7th Framework Programme. Bicho et al. (2017) report additional characterisation of the material and the manufacturer's information. Stock suspensions of CuO ENMs were prepared fresh when needed to avoid extended periods of ENM dissolution before dosing of the exposure vessels. Primary stock suspensions of CuO ENMs were prepared in 10 mL acid-washed volumetric flasks at 1 g L^{-1} in ultrapure water and dispersed by sonication (1 h, 50/60 Hz, 35 Watts, FB15048, Fisherbrand). The primary particle sizes of CuO ENMs were confirmed by electron microscopy using suspensions prepared at 1 mg mL^{-1} and dispersed as described above. Briefly, the dispersions were dispensed on to copper grids and images collected on a JEOL JEM 1400 transmission electron microscope (JEOL, South Korea). The primary particle diameters of $n = 100$ ENMs were 18.2 ± 5.6 nm (mean ± SD, $n = 100$, Fig. 1A) calculated using ImageJ (http://imagej.nih.gov/ij/).

The hydrodynamic diameters and agglomeration state of the dispersions, and the settling behaviours of the ENMs, were measured over 24 h and in triplicate beakers per treatment. Particle behaviours were explored at pH 5, pH 6 and pH 7 using 100 mg L^{-1} of ENMs (equivalent to 79.88 mg Cu metal L^{-1}) prepared by a × 10 dilution of the primary stock above in 60 mL of conditioned pH-adjusted (0.2 N HCl) Plymouth tap water. The behaviours of the ENMs were also analysed in ultrapure water for reference. The concentration of CuO ENMs was chosen to match the top concentration used in the acute exposures (see section 2.4) and was also high enough to ensure sufficient particles were in suspension for robust measurement of hydrodynamic diameters and agglomeration state of the dispersions with Nanoparticle Tracking Analysis (NTA, NanoSight LM10, Malvern Instruments, Malvern, UK). Measurements were made over 24 h to match the duration between media renewal in exposures with zebrafish. For measurements of both settling behaviour and agglomeration state of the ENMs, 1 mL samples were withdrawn from immediately beneath the surface of the media to avoid disturbing the unstirred dispersions and to ensure measurements were made only on ENMs remaining in suspension. For NTA, water samples were taken at 0 and 24 h, only. The background concentrations of natural particles in filtered Plymouth tap water were $0.14 \pm 0.07 \times 10^8$ particles mL^{-1}. No particles were detected in the ultrapure water. To measure the rate of sedimentation, an extended sampling series was followed with 1 mL samples taken to measure total Cu concentrations remaining in suspension at 0, 1, 3, 6 and 24 h. Concentrations of Cu in the samples were measured in acidified samples diluted in ultrapure water to a final concentration of 5% HNO_3 as described in section 2.4.

The dissolution of the CuO ENMs was measured by dialysis in conditioned Plymouth tap water at different pH values and in ultrapure water to aid the interpretation of the ecotoxicity experiments (see below). Due to logistical constraints, the dissolution analyses were performed at 20 °C (room temperature). The dissolution measurements were conducted in triplicate using previously acid-washed beakers that were rinsed in ultrapure water. Briefly, the pH of the tap water was adjusted to pH 5, 6 and 7 with 0.2 M H_2SO_4. Dialysis tubing was cut (n = 3/ treatment) to 2.5 cm × 15 cm lengths and was washed overnight in tap water according to the manufacturer's instructions (12 kDa cut-off, Sigma, UK). Then, 3 mL of a stock of CuO ENMs at 1 mg mL^{-1} in ultrapure water (dispersed as described above)

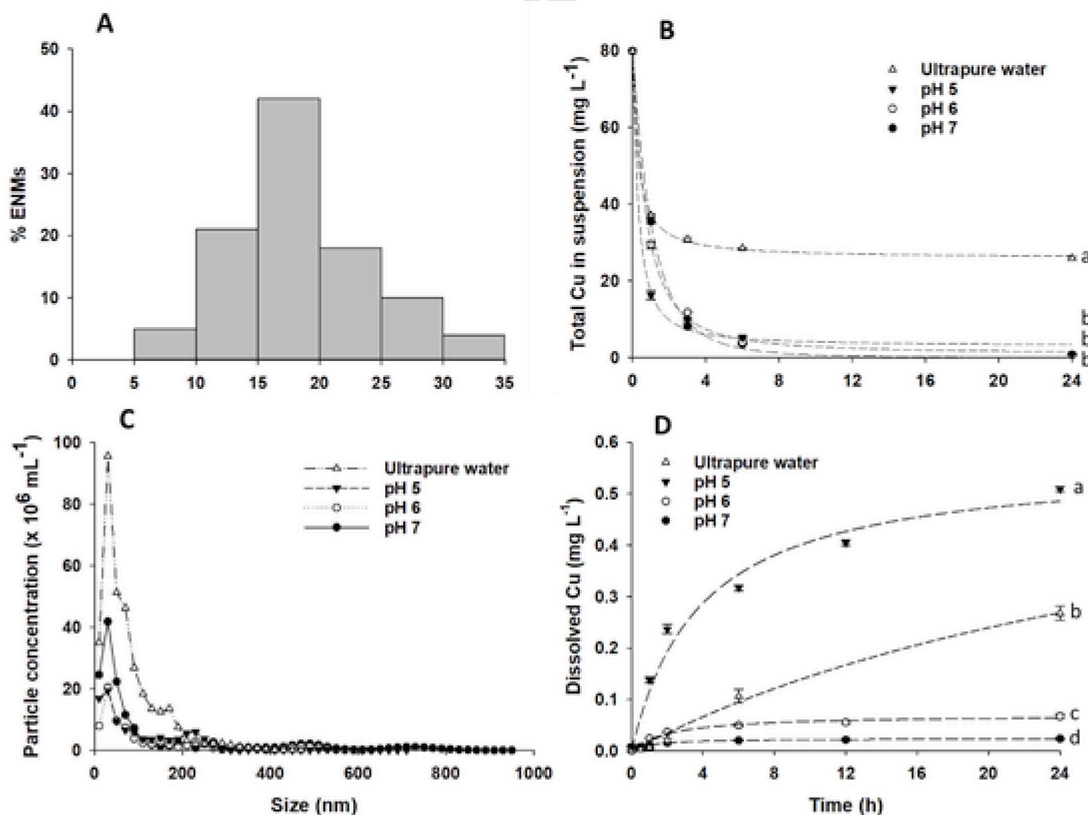

Fig. 1. Physicochemical behaviours of CuO ENMs in aqueous suspensions in ultrapure water and Plymouth tap water adjusted to pH 5, 6 and 7. (A) Primary particle size distribution of $n = 100$ CuO ENMs in freshly made ENM stock in ultrapure water calculated from electron micrographs (B) Concentrations of total Cu (i.e. both particulate and dissolved fractions); remaining in suspension. At 0 h, the nominal total [Cu] was 79.88 mg Cu L^{-1} for the CuO ENMs. Curves were fitted with a 3-phase exponential decay; (C) The hydrodynamic diameters of CuO ENMs in suspension 24 h after dosing measured with Nanoparticle Tracking Analysis; (D) The dissolution (mg Cu L^{-1} in the beaker) of 10 mg L^{-1} CuO ENMs, with curves were fitted with a rectangular hyperbole function. All data are means ± SEM, $n = 3$. Different lower case letters in (B) and (D) indicate significant differences between treatments in Cu concentrations at 24 h (one-way ANOVA, $p < 0.05$).

were pipetted into the dialysis bags. The end of the tubing was sealed and the dialysis bag then immersed in 297 mL of the exposure media (1 vol ultrapure water: 9 vol of Plymouth tap water). After equilibration on the bench in air, the ultrapure water was pH 5.73. Beakers were placed on to an orbital shaker and gently rocked for 24 h. At 0 (before adding dialysis bags), 0.5, 1, 2, 3, 6, 12 and 24 h, water samples (2.5 mL) were taken from outside the dialysis bags and immediately acidified with 0.5 mL of concentrated HNO_3. A water sample at 12 h was not collected from the ultrapure water treatment group. Samples were then diluted with ultrapure water (final concentration 5% HNO_3) and stored prior to metal analysis (see section 2.4). Samples of the ENMs from within the dialysis tubing were also collected for visual analysis via electron microscopy. There were no apparent changes in morphology of the CuO ENMs following pH treatments (Supplementary Fig. S1).

2.3. The effect of water pH on the acute toxicities of $CuSO_4$ or CuO ENMs

To inform on dosimetry, acute toxicity curves were generated at different pH values (see Supplementary Fig. S2). The 96 h median lethal concentrations (LC_{50}) of Cu as $CuSO_4$, or CuO ENMs, were calculated for zebrafish exposed in Plymouth tap water at pH 5, 6 and 7, and the estimated LC_{10} used for experiments. Cleaned embryos at approximately 2 h post fertilisation (hpf) were transferred to individual wells on a 48-well plate ($n = 1$ embryos well^{-1}) containing 900 μL of tap water adjusted to pH 5, 6 or 7. Plymouth tap water was pH-adjusted with 0.2 M H_2SO_4 in a 2 L beaker and then dispensed into wells. The volume of acid added to water was low and adjustment of 2 L Plymouth tap water to pH 5.0 required approximately 1 mL 0.2 M H_2SO_4. Each well was then spiked with 100 μL of ultrapure water containing either $CuSO_4$ or dispersed CuO ENMs at the required concentrations. The exposure series for each material included eleven concentrations of $CuSO_4$ or CuO ENMs at each pH and appropriate controls (controls received 100 μL of ultrapure water i.e. no added Cu). Addition of the materials did not alter the pH of the media. The concentrations used were based on observations from preliminary range-finding experiments and were 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.6 and 0.8 mg L^{-1} Cu as $CuSO_4$ and 1, 2, 5, 10, 20, 30, 40, 50, 60, 80 and 100 mg L^{-1} of CuO ENMs. Unless otherwise stated, data for the ENMs are shown as mass concentration of CuO per unit volume and not normalised to the concentration of Cu metal in the particle. This is because the dissolution rates of ENMs were relatively low and therefore CuO ENMs were the dominant form of Cu in water throughout ENM exposures, and this approach better reflects the study goal of understanding particle toxicity. At each concentration and at each pH, $n = 8$ embryos were exposed. Every 24 h during the 96 h exposure, the numbers of dead (those with no heartbeat) and hatched larvae were scored under a binocular microscope. To maintain the exposures and relevant pH, every 24 h each embryo or larva was carefully transferred to new plates with Pasteur pipettes; taking care to minimise the transfer of any old media. Each well was then re-dosed with $CuSO_4$ and CuO ENMs at appropriate concentrations and as described above. This entire procedure was repeated on three separate occasions and with three different batches of embryos for a total of $n = 24$ embryos for each pH and material concentration combination.

2.4. Effects of $CuSO_4$ and CuO ENMs at low dose LC_{10} pulse exposure at pH 6

The effects of pulse exposures to low concentrations (LC_{10}) of the CuO ENMs or the equivalent sublethal dose of $CuSO_4$ in zebrafish was investigated at pH 6 only. A comparison of effects at all the different pH values used in the previous experiment was not performed because the LC_{50} for CuO ENMs at pH 7 exceeded the highest dose used in the acute toxicity tests (100 mg L^{-1}) and therefore these data would have greater uncertainty in the LC_{10} estimation compared to other treatment groups. Preliminary data indicated that although no mortality or hatch inhibition was observed in control zebrafish at pH 5 and pH 6 compared to pH 7, the activity of Na^+/K^+-ATPase was significantly elevated and total glutathione (GSH) was significantly decreased in embryos in acid water at pH 5 (details of the assays are described below; Supplementary Fig. S3). Thus pH 6 was selected as the most relevant pH for the pulse experiment.

The total test period for sub-lethal exposures was 96 h. Embryos were exposed to $CuSO_4$ or CuO ENMs at the 96 h LC_{10} of each substance with re-dosing every 24 h where relevant. The different sub-lethal pulse exposures were as follows: (i) early exposure from 0 to 24 h followed by transfer to clean water until 96 h to determine any latent effect of the pulse as the embryos developed into juveniles; (ii) intermittent exposure to two pulses of CuO ENMs or $CuSO_4$, characterised by periods in clean water (no added Cu at 0–24 h and 48–72 h) interspersed with two periods of exposure to Cu (i.e., at 24–48 h and 72–96 h); and (3), continuous exposure to $CuSO_4$ or CuO ENMs from 0 to 96 h. Therefore, embryos receiving early or intermittent exposures were exposed to 0.25 and 0.5 of the total Cu dose received by embryos in the continuous exposure. Unexposed controls maintained for 96 h in clean media adjusted to pH 6 were also included in the experimental run. To guarantee sufficient tissue for biochemistry and metal analyses at 96 h, embryos were exposed in quadruplicate as groups of $n = 60$ embryos in each of $n = 4$ beakers per treatment containing 60 mL media (i.e., 240 animals/treatment). The preparation of the exposure media and the stock suspensions of the materials were prepared as described in section 2.2. The hatch rate and mortalities in each beaker were scored every 24 h and debris and dead zebrafish were removed prior to transferring the live zebrafish to facilitate re-dosing in previously unused clean cups every 24 h where the media was then refreshed.

At the end of the 96 h exposure, sub-samples of the embryos/larvae were collected for total Cu accumulation and biochemistry (pools of 10–15 embryos). Both $CuSO_4$ and CuO ENMs caused hatch inhibition in embryos (controls were >98% hatched at 96 hpf). To avoid incidentally carrying-over high concentrations of exposure media and chorion-associated Cu that may otherwise interfere with subsequent assays (Ong et al., 2014a), embryos were manually dechorionated with fine watchmaker's forceps (Fisher, UK), washed in 3 × clean exposure media and 1 × ultrapure water. Larvae were then sampled for total Cu concentrations (see below) or snap frozen in liquid N_2 and then stored at −80 °C for measurements of the activity of Na^+/K^+-ATPase and the concentration of GSH. A small sub-set of chorionated embryos ($n = 3$–5 per beaker) were retained to measure the total embryo associated Cu fraction including the chorion.

Total Cu concentrations in zebrafish, water samples from exposures taken immediately after dosing and again after 24 h, and water samples from the dissolution and settling measurements (see section 2.2), were determined by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Electron Corporation X-Series II quadruple) or inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian 725-ES, Agilent Technologies Inc.). Zebrafish were digested in 0.5 mL HNO_3 (68%, Primar-Plus Trace Analysis Grade, Fisher, UK) for 1 h at 60 °C and diluted to 3.5 mL with ultrapure water. This digestion protocol has previously given recoveries of Cu from Cu ENMs of >90% (Shaw et al., 2012). Samples were compared to matrix matched analytical standards (Fisher, UK) that were also included throughout the run to correct for instrument drift. Procedural blanks (HNO_3 only) prepared at the same time and handled in the same manner as the samples were also included in the run to account for extraneous sources of Cu. The limit of detection of the instrument run, calculated as the mean + 3 × SD of the Cu concentration in the procedural blanks was 0.21 μg L^{-1} and equated to 0.049 ng Cu larva^{-1} in a digest contain-

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ing $n = 15$ zebrafish. Concentrations of Cu in the animals are expressed as ng Cu $larva^{-1}$ due to the low mass of the animals.

The activities of Na^{+}/K^{+}-ATPase and concentrations of total GSH in zebrafish were measured in microplates according to methods extensively detailed by Boyle et al. (2014) and following an original method for Na^{+}/K^{+}-ATPase activity by McCormick (1993). The larvae were homogenized in an ice-cold isotonic sucrose buffer (in mM: 300 sucrose, 20 HEPES, 0.1 EDTA. pH 7.8) using a hand-held motor-driven pestle. The homogenate was then centrifuged at 13000 rpm for 2 min and the supernatant used in assays. Total glutathione was quantified in buffer with final assay concentrations of (in mM): 76.5 phosphate buffer (pH 7.5), 3.8 EDTA, 0.6 DNTB, 0.2 NADPH and 0.12 U mL^{-1} glutathione reductase. Activity of Na^{+}/K^{+}-ATPase was assayed with and without 0.5 mM ouabain in buffer with final assay concentrations of (in mM): 2.1 PEP, 0.53 ATP, 0.16 NADH, 37.25 HEPES, 47.25 NaCl, 2.63 $MgCl_2$, 10.5 KCl (pH 7.5), and with 3.75 U mL^{-1} pyruvate kinase and 3 U mL^{-1} lactate dehydrogenase. Data were normalised to the total protein concentration in the supernatant measured with the Pierce BCA Protein Assay Kit according to the manufacturer's instructions (ThermoFisher Scientific, UK).

2.5. Data handling and statistical analyses

Probit analyses to calculate LC_{10} and LC_{50} (mortality) and EC_{50} (hatch) values for the materials were performed with SPSS (*v.* 22, SPSS Inc., US). Calculated LC_{50} and EC_{50} values are presented with 95% confidence intervals (CI). All other statistical tests were performed with SigmaPlot (*v.* 13.0, Systat Software Inc.). Data are presented as means ± standard error (SEM) except where stated. All data were tested for normality (Shapiro-Wilk test) and equality of variances (Brown-Forsythe test) and if not normally distributed were either log_{10} transformed or arcsine transformed. No observed effect concentrations and lowest observed effect concentrations for Cu (as $CuSO_4$) and CuO ENMs were calculated from acute toxicity data with one-way ANOVA. The data of ENM dissolution were fitted with a rectangular hyperbole function. Data of ENM settling were fitted with three-phase exponential decay curves. Statistically significant differences between datasets were detected using one-way and two-way ANOVA with the Holm-Sidak test *a posteriori*. A p value of <0.05 was considered significant.

3. Results

3.1. Physicochemical behaviours of CuO ENMs in suspension

The pH of the media had little effect on the settling rates of the ENMs in beakers without animals present, but the ionic strength of the tap water media clearly did (Fig. 1B). In all media tested, > 50% of the ENMs were removed from suspension in 1 h, and the measured total Cu concentrations in the beakers was stable between 6 and 24 h. In Plymouth tap water adjusted to pH 5, pH 6 and pH 7, total Cu concentrations in suspension at 24 h were 0.91 ± 0.02, 0.71 ± 0.02, 0.73 ± 0.12 mg Cu L^{-1}, respectively. This compared to 25.87 ± 0.46 mg Cu L^{-1} remaining in suspension in ultrapure water.

Despite the rapid loss of the ENMs from suspension (Fig. 1B), ENMs were still present in the suspensions at 24 h (Fig. 1C). There was also little difference in either particle number concentration or hydrodynamic diameters of the agglomerates in the tap water adjusted to different pH values. The total concentration of particulates at 24 h was 0.96 ± 0.31, 0.75 ± 0.18 and 1.48 ± 0.64 × 10^8 particles mL^{-1}, at pH 5, pH 6 and pH 7, respectively. This was a considerably lower than the 3.37 ± 0.48 × 10^8 particles mL^{-1} in ultrapure water at 24 h, but greater than the background concentration of particulates in Plymouth tap water, 0.22 × 10^8 particles mL^{-1}. There was little difference in the hydrodynamic diameters of particles in suspension at 24 h in any media (Fig. 1 C). At all the pH values tested, and in ultrapure water, > 50% of particles/agglomerates in suspension were <90 nm and few larger agglomerates were in suspension.

The pH of Plymouth tap water had a pronounced effect on the rate and extent of dissolution of the CuO ENMs (Fig. 1D). As the pH of the water was decreased, the concentration of dissolved Cu in the beakers increased. The pattern of Cu release (ENM dissolution) over time was similar at all pH values and was characterised by an initial rapid release (greatest at pH 5) in the first 1 h, followed by a steady decrease in the rate of Cu release to a point of stable equilibrium in the closed volume system. During the first hour, rates of dissolution were 12.96 ± 0.55, 1.74 ± 0.02 and 0.50 ± 0.01 μg mg^{-1} CuO ENM h^{-1} at pH 5, pH 6 and pH 7, respectively. After 24 h, dissolved Cu concentrations in the external compartment of the beaker were: 6.27 ± 0.11%, 0.75 ± 0.10% and 0.21 ± 0.03% of the initial total Cu added to the dialysis bags at pH 5, 6 and 7, respectively. In comparison, 2.68 ± 0.14% of total Cu as CuO ENMs was in the dissolved form after 24 h in ultrapure water.

3.2. Acute toxicity and hatch inhibition of CuO ENMs

The CuO ENMs were much less toxic to early life-stage zebrafish compared to Cu in the form of $CuSO_4$ (96 h LC_{50} are shown in Fig. 2A, raw data are shown in Supplementary Fig. S2). At pH 7, 50% mortality of zebrafish was not observed at concentrations of CuO ENMs of ≤100 mg L^{-1}. This compared with a LC_{50} (95% CI) of 0.27 (0.25–0.29) mg L^{-1} for Cu as $CuSO_4$. At acid pH the toxicities of both substances, and especially the CuO ENMs, were changed. The LC_{50} of the CuO ENMs decreased to 19.5 (11.6–27.2) mg L^{-1} at pH 6, and to 6.6 (4.5–8.5) mg L^{-1} at pH 5. In contrast, the toxicity of $CuSO_4$ was more consistent across all pH tested, but was slightly less toxic at pH 5 (LC_{50} 0.36, 95% CI 0.33–0.40) than at pH 6 (LC_{50} 0.22, 95% CI 0.20–0.24) and pH 7. The increase in acute toxicity of the CuO ENMs at acid pH also corresponded to the predicted increase in the dissolved fraction of Cu which would be in exposures to the CuO ENMs (i.e., the Cu release determined by dialysis, data shown in Fig. 1D). With the exception of pH 7, the estimated concentrations of dissolved Cu in exposures to ENMs at the calculated LC_{50} values are shown as the hatched areas of the bars and show overlap with the calculated LC_{50} for $CuSO_4$ (Fig. 2A).

Calculated NOECs and LOECs were dependent on concentrations used in the acute toxicity tests and are shown here for completeness. Calculated NOECs were 5 and 40 mg L^{-1} CuO ENMs at pH 6 and pH 7, respectively. A NOEC was not calculable at pH 5. LOECs for CuO ENMs were 1, 10 and 50 mg L^{-1} at pH 5, pH 6 and pH 7, respectively. Calculated NOECs were 0.25, 0.1, 0.2 mg L^{-1} Cu as $CuSO_4$ at pH 5, pH 6 and pH 7, respectively. Calculated LOECs were 0.3, 0.15, 0.25 mg L^{-1} Cu as $CuSO_4$ at pH 5, pH 6 and pH 7, respectively.

Inhibition of embryo hatching also occurred at lower concentrations in exposures to $CuSO_4$ compared to CuO ENMs (Fig. 2B, Supplementary Fig. S2). For example, at pH 7 calculated EC_{50} were 0.12 and 11.05 mg L^{-1} of Cu as $CuSO_4$ and CuO ENMs, respectively. In exposures with $CuSO_4$, there was also no indication of a pH-dependent effect on hatch and EC_{50} values were similar at acid and neutral pH. In contrast, there was a clear effect of the ENMs on hatch inhibition, greater effect at acid pH, but there was no agreement between the estimated concentration of dissolved Cu in exposures to the CuO ENMs and hatch inhibition observed from $CuSO_4$ exposure i.e. no overlap of the hatched area, with the exception of exposures at pH 5.

3.3. Effects of $CuSO_4$ and CuO ENMs during low dose LC_{10} pulse exposure at pH 6

Investigation of the importance of pulse exposure to CuO ENMs or $CuSO_4$ in acid waters was performed at pH 6. Low (LC_{10}) concentrations of both substances were calculated from the nominal concentra-

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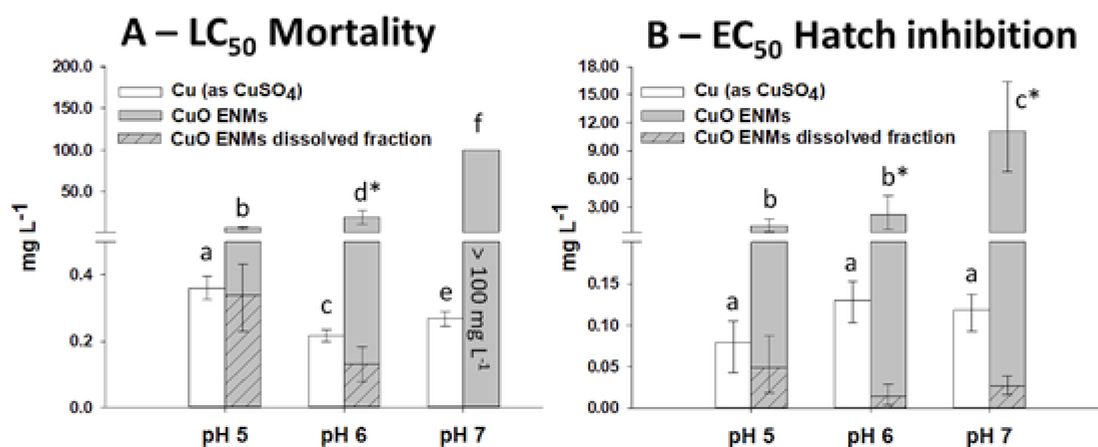

Fig. 2. Calculated median lethal concentrations (LC_{50}) of dissolved Cu (as $CuSO_4$) or CuO ENMs (A), and median effect concentrations (EC_{50}) of the materials on hatching (B) in early-life stage zebrafish. Zebrafish were exposed from 2 hpf for 96 h in pH-adjusted Plymouth tap water. The predicted dissolved Cu concentration in CuO ENMs exposures (predicted from dissolution analyses, see Fig. 1) at the calculated LC_{50} and EC_{50} are shown as the hatched area of the bar. Data are means ± 95% CI. The LC_{50} for the CuO ENMs at pH 7 was >100 mg L^{-1} i.e., the top concentration tested and therefore it was not possible to calculate a predicted dissolved fraction at the LC_{50}. Different lowercase letters indicate significant differences between treatment groups and exposure methods (i.e., without overlapping 95% CI). * indicates significant differences between data for $CuSO_4$ and dissolved Cu fraction in exposures with CuO ENMs. Raw data used to calculate these values are shown in Supplementary Fig. S2.

tions used in the acute toxicity exposures (i.e., Supplementary Fig. S2) and were 6.54 and 0.151 mg L^{-1} for CuO ENMs and Cu as $CuSO_4$, respectively. Measured total Cu concentrations in the vessels immediately after dosing were 3.83 ± 0.22 and 0.136 ± 0.014 mg L^{-1} (means ± SD, $n = 8$) for ENMs and $CuSO_4$ treatments, respectively. Unlike the CuO ENMs (see Fig. 1B for settling rates) the concentration of Cu in solution in the $CuSO_4$ treatment was stable during the period between media renewals and after 24 h, 90 ± 3% of the initial 0 h concentration remained in solution. The pH of the water increased from pH 6.0 to pH 6.41 ± 0.04 ($n = 12$ beakers; there were no significant differences between treatments).

Mortalities were observed in zebrafish exposed to low concentrations of $CuSO_4$ and CuO ENMs, and there was also a 12.1 ± 4.7% incidence of background mortality in controls (Table 1). However, there were no significant differences in the percentage of mortalities between treatment groups or between treatment groups and the control (one-way ANOVA, $p = 0.119$). Nevertheless, there were clear trends in the data to suggest greater mortality associated with exposure to the Cu materials. Hatching was significantly inhibited by exposure to the Cu substances at LC_{10} and this was expected (see section 3.2, one-way ANOVA, $p < 0.001$). Hatch was 98.6 ± 0.5% of surviving control zebrafish at 96 hpf but was significantly decreased after early, pulse and continuous exposures to both Cu materials. There were few significant differences between Cu treatments and exposures with one exception: early (0–24 hpf) exposure to $CuSO_4$ had a lesser impact on hatch compared to early exposure to CuO ENMs, and all other treatment/exposure regimen.

Table 1
Mortality and hatch of zebrafish at 96 hpf after exposure to $CuSO_4$ or CuO ENMs at LC_{10}.

Substance	Exposure	Mortality (%)	Hatch (%) [a]
–	Control	12.1 ± 4.7 [a]	98.6 ± 0.5 [a]
$CuSO_4$	Early 0–24 h	17.1 ± 1.7 [a]	39.2 ± 4.8 [b]
	Pulse 24–48, 72–96 h	27.9 ± 8.5 [a]	8.4 ± 1.0 [c]
	Continuous 0–96 h	47.9 ± 14.6 [a]	0 [c]
CuO ENMs	Early 0–24 h	46.7 ± 8.8 [a]	11.3 ± 2.0 [c]
	Pulse 24–48, 72–96 h	34.2 ± 11.7 [a]	4.5 ± 2.7 [c]
	Continuous 0–96 h	39.2 ± 13.0 [a]	4.2 ± 0.9 [c]

Data are means ± SEM ($n = 4$).
Different lower case letters indicate significant differences between treatments (one-way ANOVA, Holm-Sidak post hoc test, $p < 0.05$).
[a] Hatch is expressed as the percentage of the surviving zebrafish at 96 h.

The exposure was verified by measuring Cu concentrations in the embryos at 96 h. Continuous exposure to $CuSO_4$ or CuO ENMs for 96 h led to significant increases in Cu concentrations versus controls, only, in dechorionated embryos (one-way ANOVA, $p = 0.002$, Holm-Sidak test for both materials $p < 0.05$; Fig. 3). Neither early single pulse nor double pulse exposures led to measurable Cu increases compared to controls (all $p > 0.05$). For chorionated embryos, Cu concentrations were much greater than for dechorionated zebrafish i.e., tissue associated Cu at 96 h (Fig. 3B). There was also significantly greater total Cu from CuO ENMs associated with the chorion compared to that for the $CuSO_4$ treatment at their respective LC_{10} values (one-way ANOVA, $p < 0.001$). For example, measured Cu concentrations in chorionated embryos from continuous exposures were 20 ± 4 and 1706 ± 310 ng $larva^{-1}$ from exposure to $CuSO_4$ and CuO ENMs, respectively (Holm-Sidak test, $p < 0.001$). It was not possible to measure Cu concentrations in chorionated control embryos because they had all hatched by 96 h, with the exception of one animal.

At the LC_{10} concentrations used, both $CuSO_4$ and CuO ENMs caused significant depletion of total GSH of approximately 50% compared to control embryos after continuous exposure for 96 h (one-way ANOVA with Holm-Sidak test, both $p < 0.001$). Moreover, there was no significant difference between the Cu forms (Holm-Sidak test, $p = 0.178$). Within each substance, the magnitude of the depletion compared to controls was also the same irrespective of whether zebrafish embryos were exposed to two pulses of either CuO ENMs or $CuSO_4$ or in continuous exposures (all Holm-Sidak post hoc test $p > 0.05$). This was despite the double pulse exposed fish receiving half the total Cu dose as in the continuous exposures. Early single pulse exposure to both Cu materials for the first 24 h of development also resulted in significant depletion of GSH versus controls (both $p < 0.001$) and there was evidence of a nanoscale specific effect with early exposure to CuO ENMs causing a significantly greater GSH depletion after 96 h as compared to $CuSO_4$ ($p = 0.007$).

The effects of CuO ENMs and $CuSO_4$ on the activity of Na^{+}/K^{+}-ATPase mirrored the decreases in measured concentrations of total

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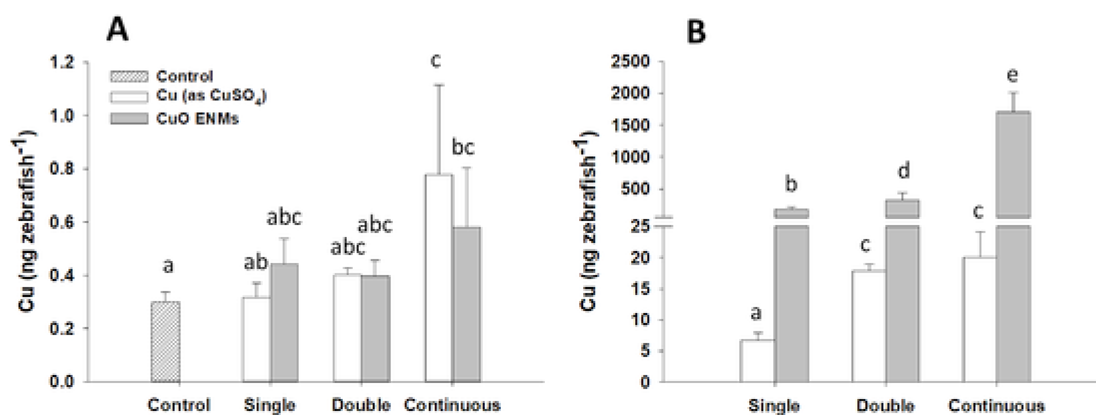

Fig. 3. Total Cu concentrations (ng $zebrafish^{-1}$) in dechorionated (A) and chorionated (B) zebrafish embryos at 96 h. Zebrafish were sampled at 96 h after single pulse (0–24 h), double pulse (24–48 and 72–96 h) and continuous (0–96 h) exposures to dissolved Cu (as $CuSO_4$) or CuO ENMs. Data are means ± SEM, $n = 3/4$. Different lowercase letters indicate significant differences between treatment groups and exposure methods (one-way ANOVA, $p < 0.05$). Note the differences in scale between panels (A) and (B).

GSH (one-way ANOVA, $p < 0.001$). Continuous exposures to either CuO ENMs or $CuSO_4$ caused inhibition of Na^+/K^+-ATPase compared to controls after 96 h (both Holm-Sidak, $p < 0.001$) with no significant differences between the substances ($p = 0.169$). Double pulse exposure also caused the same level of effect seen in embryos exposed continuously for 96 h (for comparisons within substances, $p > 0.05$). There was also a significant nanomaterial effect from early single pulse Cu exposure with greater Na^+/K^+-ATPase inhibition in embryos observed for CuO ENMs as compared to $CuSO_4$ ($p = 0.01$).

4. Discussion

In this study, the data showed that the toxicity of CuO ENMs to zebrafish was much lower than that of $CuSO_4$ at pH 7, and as expected $CuSO_4$ was also less toxic at pH 5 than at pH 7. In contrast, CuO ENMs were more hazardous at lower pH. In continuous 96 h exposures to the substances at pH 6, both $CuSO_4$ and CuO ENMs caused inhibition of Na^+/K^+-ATPase and depletions in total GSH in zebrafish. However, two 24 h pulses of $CuSO_4$ or CuO ENMs caused the same magnitudes of effect on Na^+/K^+-ATPase and total GSH, suggesting on an equivalent dose basis that the latter is more hazardous.

4.1. Toxicity of $CuSO_4$ and CuO ENMs at low pH

At pH 7, the CuO ENMs were considerably less toxic than the $CuSO_4$ (Fig. 2), with the median acute lethal toxicity concentration (LC_{50}) of CuO ENMs exceeding 100 mg L^{-1} which was above the highest concentration used in this experiment and a greater material concentration than is routinely used in regulatory toxicity tests (e.g., OECD TG 210). These data are in agreement with several other studies with different Cu ENMs in fish that have shown lower toxicity compared to dissolved Cu at neutral pH (Lin et al., 2013; Shaw et al., 2012; Vicario-Parés et al., 2014). At pH 5, the toxicity of $CuSO_4$ was lower than at pH 7 (Fig. 2) and the expected protective effect of low pH (Cusimano et al., 1986) is explained by competition between dissolved Cu and H^+ for binding at the gills leading to reduced Cu uptake (Santore et al., 2001).

In contrast to $CuSO_4$, the CuO ENMs showed increased toxicity at lower pH (Fig. 2) and this effect was almost entirely due to dissolution of dissolved Cu from the particles at low pH rather than any pH-dependent effects on the particle dispersion. The CuO ENMs used here had moderate stability in ultrapure water (Fig. 1B) likely due to some charge repulsion (zeta potential of +28.1 mV, Bicho et al. (2017)); but, in Plymouth tap water, the higher ionic strength of the media caused more particle settling than observed in ultrapure water (Fig. 1B). Peng et al. (2017) reported a pH of zero point of charge of 6.21 for uncoated CuO ENMs in 10 mM NaCl and with smaller agglomerates and greater suspension stability at pH 5 or lower. A similar pH-dependent effect of Plymouth tap water on suspension stability was not evident in the present study because the pH range tested was necessarily narrower here to avoid detrimental effects of H^+ toxicity to the zebrafish. However, lowering the water pH to 5 during dialysis experiments did increase the concentration of total Cu in suspension after 24 h when compared to pH 6 and 7 and this was almost certainly due to greater ENM dissolution observed for acid water, as observed previously with Cu-containing particles (Al-Bairuty et al., 2016). Under acidic conditions, the high $[H^+]$ reacts with the CuO ENM surface to liberate Cu^{2+} (Wang et al., 2013). There was negligible mortality in the presence of acid pH alone (Fig. S3). It is therefore the pH-dependent release of dissolved Cu from the CuO ENMs that is of most concern with respect to toxicity to the fish. The predicted dissolved Cu concentration from the dialysis experiments are close to the LC_{50} values for $CuSO_4$ at pH 5 and pH 6 (Fig. 2A); suggesting that the dissolution of dissolved Cu from the particles best explains the observed mortality. The mortality was not confounded by the effects of pH alone on zebrafish biochemistry (no inhibition at those pH values, Fig. S3). The predominant role of dissolved Cu in the toxicity of CuO ENMs has also been documented in fish cell lines (e.g., Galbis-Martinez et al., 2018). In contrast, where CuO ENMs have been engineered to not dissolve in media (e.g., by Fe-doping the ENMs), the toxicity to zebrafish embryos has been ameliorated (Naatz et al., 2017).

Exposure to $CuSO_4$ prevented the hatching success of the embryos, with a total Cu concentration of 0.4 mg L^{-1} or more causing 100% inhibition of hatching (Fig. S2), regardless of water pH. The low pH alone caused negligible interference with hatching success (Fig. S2), suggesting the effect was mainly attributed to $CuSO_4$ exposure. Hatching success was a sensitive endpoint for the metal salt with EC_{50} values of between 0.08 and 0.13 mg L^{-1} for $CuSO_4$, and similar to previous reports on zebrafish (52% hatching success at 0.06 mg L^{-1} of Cu as $CuSO_4$, Bai et al., 2010). The CuO ENMs also inhibited hatching success, but only at concentrations an order of magnitude higher than that of the equivalent $CuSO_4$ treatment (Fig. 2B). Notably, only a small fraction of the EC_{50} for hatching could be attributed to the estimated dissolved Cu concentration arising from particle dissolution, especially at pH 6 and 7 (Fig. 2B), suggesting mechanisms other than dissolved Cu toxicity. Failure of zebrafish hatching has been shown for several ENMs, including CuO (e.g., Bai et al., 2010; Lin et al., 2013; Ong et al., 2014b; Muller et al., 2015). However, the mechanisms involved are disputed. Increased locomotor activity, combined with a weakening of the chorion via a protease enzyme secreted into the perivitelline fluid, usually enables hatching success. In the presence of ENMs, inhibition of the protease activity needed for hatch (assumed zebrafish hatching enzyme, Zhe1) has been demonstrated (Ong et al., 2014b). How-

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ever, oxidative stress from CuO ENM exposure may preferentially reduce locomotor activity of the embryo so that the animal cannot break through the chorion, rather than altering the gene expression controlling the secretion of the protease (Zhang et al., 2018). The precise mechanism by which oxidative stress from CuO ENMs reduces motility in the embryo is less clear, but for example, could involve damage to the skeletal muscle fibres on the flank of this fish (observed in trout, Al-Bairuty et al., 2013) or respiratory distress (i.e., inability to exercise).

4.2. Effects of pulse exposures

The pulse exposures caused low levels of mortality, as expected for the LC_{10} concentrations of each form of Cu used, but sub-lethal effects were able to be measured in a majority of the animals. The focus was therefore on Cu accumulation and sub-lethal effects in the animals relative to the exposure dose in the pulses compared to the continuous exposure. The unhatched animals (i.e., not dechorionated) from the $CuSO_4$ treatment showed more apparent Cu accumulation in/on the embryos in the double pulse compared to the single pulse, in keeping with the dosimetry. This was not the case for the continuous exposure which was not different from the double pulse of $CuSO_4$ (Fig. 3B). However, the Cu concentrations in the unhatched animals should be interpreted with caution, because the dechorionated animals had orders of magnitude less total Cu (Fig. 3A) and this strongly suggests that most of the Cu was surface-bound. The binding of divalent cations to polyanionic residues (Shephard, 1989), such as those in the chorion and the mucoproteins in the perivitelline fluid is well-known and this severely limits the diffusion of soluble divalent metals to protect the embryo inside the egg (Peterson and Martin-Robichaud, 1986). In the dechorionated animals, the total Cu concentrations in the $CuSO_4$ treatment were not any different from the controls, except in the continuous exposure (Fig. 3A).

Despite the limited internal accumulation of Cu in the embryos, there were some depletions of total GSH and inhibition of the Na^+/K^+-ATPase activity in the $CuSO_4$ treatments compared to the unexposed controls (Fig. 4). This observation is consistent with the mechanisms of dissolved Cu toxicity to fish i.e. oxidative stress and ionoregulatory disturbances (Grosell, 2012; Handy, 2003). Glutathione is the first line of defence against oxidative stress in fish and is also a Cu chaperone (Handy, 2003). The modest decrease in total GSH here (i.e., not depleted, Fig. 4) has also been seen in trout (e.g., Shaw et al., 2012), and in such circumstances there is no evidence of gross pathology due to oxidative damage *in vivo* (Al-Bairuty et al., 2013). Regardless of the mechanistic details, the double pulse caused more depletion of total GSH and inhibition of Na^+/K^+-ATPase activity than the single pulse of $CuSO_4$, suggesting that multiple pulses may be more hazardous, as shown previously for metals (Handy, 1994), partly because the double pulse inevitably has double the exposure dose. Notably, the double pulse had the same effect on total GSH and Na^+/K^+-ATPase activity as the continuous exposure. Since the peak concentrations in the pulse and continuous exposures were similar, then the notion that the 'dose' equals concentration multiplied by exposure time can be applied (Handy, 1994). The two 24 h pulses had the same net effect on biochemistry, at half the dose of the continuous exposure, therefore the double pulse can be regarded as more hazardous. Nevertheless, the biochemistry (Fig. 4) did not correlate well with the accumulated Cu concentration in the individual animals from the pulses, suggesting other secondary effects of $CuSO_4$ exposure were responsible for the biochemical perturbations. For example, sodium ion depletion from the embryo (Alsop and Wood, 2011) leading to inhibition of Na^+/K^+-ATPase activity.

The pattern of Cu accumulation and sub-lethal effects in the animals following pulse exposures to CuO ENMs broadly followed that of $CuSO_4$ with two important exceptions. Firstly, the relative Cu accumulation in the intact animals with a chorion (i.e., mostly surface adsorption to the chorion) was higher than that of the equivalent $CuSO_4$ treatment (Fig. 3B). The attachment of CuO ENMs to the chorion of zebrafish embryos has been demonstrated by microscopy (Böhme et al., 2017). Copper oxide ENMs have also recently been shown not to accumulate in adult zebrafish (Vicario-Parés et al., 2018). Secondly, the present study also showed greater sub-lethal effects of early exposure to CuO ENMs than $CuSO_4$. This implies that while the CuO ENM had low bioavailability compared to the metal salt, it may present a greater hazard to fish embryos when delivered as a pulse. Indeed, the single pulse of CuO ENMs caused more depletion of total GSH and inhibition of the Na^+/K^+-ATPase activity than the equivalent pulse of $CuSO_4$ after 96 h (Fig. 4). The depletion of total GSH in conjunction with decreased activity of Na^+/K^+-ATPase, might suggest a loss of antioxidant capacity, or rather some oxidative injury to the protein structure of the enzyme, was partly driving failure of the Na^+ pump. A broadly similar observation was made on toxicity in *Daphnia magna* in which greater toxicity in pulse exposure to CuO ENMs than dissolved Cu was attributed to higher retention of ENMs in the gut (Sørensen et al., 2016).

4.3. Conclusions and regulatory perspective

The CuO ENMs were acutely toxic to zebrafish embryos and dissolution of dissolved Cu from the particles was a key factor in the particle toxicity. Overall, the CuO ENMs were less toxic than $CuSO_4$, but crucially and unlike the metal salt, the nano form became more haz-

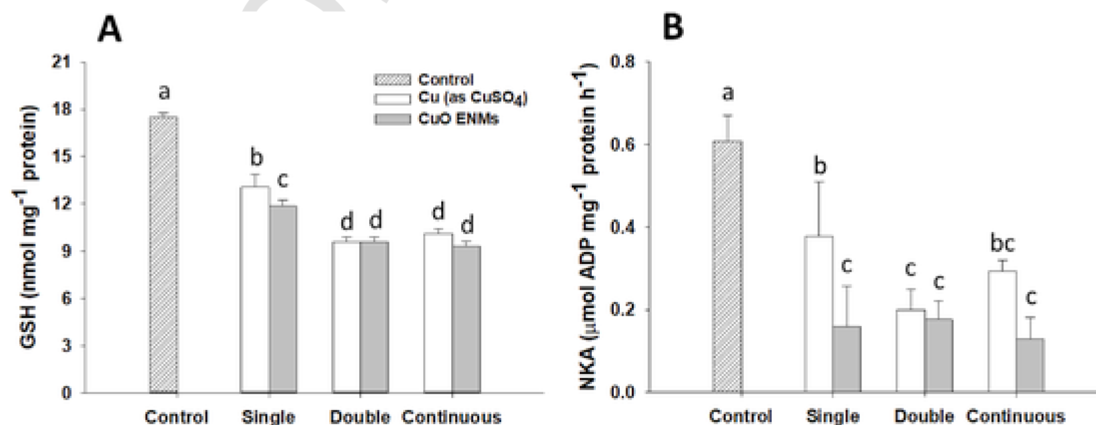

Fig. 4. Concentrations of total glutathione (GSH, A) and activities of Na^+/K^+-ATPase (NKA, B) in zebrafish. Zebrafish were sampled at 96 h after single pulse (0–24 h), double pulse (24–48 and 72–96 h) and continuous (0–96 h) exposures to dissolved Cu (as $CuSO_4$) or CuO ENMs. Data are means ± SEM, $n = 4$. Different lowercase letters indicate significant differences between treatment groups and exposure methods (one-way ANOVA, $p < 0.05$).

ardous at low pH. In the pulse exposure studies at pH 6, the embryos exposed continuously to either $CuSO_4$ or CuO ENMs showed a trend toward more Cu accumulation than either of the pulse exposures, in keeping with the exposure dose informing on total Cu accumulation. However, there were sub-lethal toxic effects associated with the pulses that were more likely explained by secondary oxidative stress than as a consequence of the total Cu concentration inside the animals. Importantly, the double pulse was as toxic as the continuous exposure, suggesting that repeated intermittent exposure to CuO ENMs present an additional hazard that would not be detected in routine continuous exposure test methods with zebrafish. From a regulatory perspective and the application of models such as the BLM (Santore et al., 2001), the assumption that H^+ is protective of metal toxicity may not apply to ENMs. Thus current catchment specific corrections of water quality criteria for pH will not protect from Cu toxicity in the nano form. The demonstration that pulses of ENMs might be as toxic as a continuous exposure should also be considered with respect to permits for consented discharges. More understanding of the temporal profiles of ENMs in natural water is also needed to inform the risk assessment.

Author contribution statement

Boyle: Conceptualisation, methodology, validation, formal analysis, investigation, data curation, writing – original draft and editing, visualisation, data curation, project administration. **Clark**: validation, formal analysis, investigation, writing – contribution to original draft. **Handy**: Conceptualisation, methodology, data curation, writing – original draft, review and editing, supervision, project administration, funding acquisition.

Declaration of competing interests

The authors have no interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109985.

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