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Sublethal effects of copper sulphate compared to copper nanoparticles in rainbow trout (*Oncorhynchus mykiss*) at low pH: physiology and metal accumulation

Genan A. Al-Bairuty[a], David Boyle[a], Theodore B. Henry[a,b,c], Richard D. Handy[a,*]

[a] Ecotoxicology Research and Innovation Centre, School of Biological Sciences, Plymouth University, Plymouth, UK
[b] School of Life Sciences, Heriot-Watt University, Edinburgh, UK
[c] Department of Forestry Wildlife and Fisheries, and Center for Environmental Biotechnology, The University of Tennessee, Knoxville, TN, USA

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ABSTRACT

A few studies have investigated the interaction between copper toxicity and water pH in fishes, but little is known about the effects of acidic pH on the toxicity of copper nanoparticles (Cu-NPs). This study aimed to describe the sub-lethal toxic effects of Cu-NPs compared to $CuSO_4$ at neutral and acidic water pH values in juvenile rainbow trout. Fish were exposed in triplicate (3 tanks/treatment) to control (no added Cu), or 20 $\mu g\,l^{-1}$ of either Cu as $CuSO_4$ or Cu-NPs, at pH 7 and 5 in a semi-static aqueous exposure regime for up to 7 days. Acidification of the water altered the mean primary particle size (at pH 7, 60 ± 2 nm and pH 5, 55 ± 1 nm) and dialysis experiments to measure dissolution showed an increased release of dissolved Cu from Cu-NPs at pH 5 compared to pH 7. Copper accumulation was observed in the gills of trout exposed to $CuSO_4$ and Cu-NPs at pH 7 and 5, with a greater accumulation from the $CuSO_4$ treatment than Cu-NPs at each pH. The liver also showed Cu accumulation with both Cu treatments at pH 7 only, whereas, the spleen and kidney did not show measurable accumulation of Cu at any of the water pH values. Exposure to acid water caused changes in the ionoregulatory physiology of control fish and also altered the observed effects of Cu exposure; at pH 5, branchial Na^+/K^+-ATPase activity was greater than at pH 7 and the inhibition of Na^+/K^+-ATPase activity caused by exposure to $CuSO_4$ at pH 7 was also not observed. There were some changes in haematology and depletion of plasma Na^+ at pH 7 and 5 due to Cu exposure, but there were few material-type or pH effects. Overall, the data show that the accumulation of Cu is greater from $CuSO_4$ than Cu-NPs; however, understanding of the effects of low pH on bioavailability of $CuSO_4$ may not be directly transferred to Cu-NPs without further consideration of the physico-chemical behaviour of Cu-NPs in acid water.

1. Introduction

Copper (Cu) is an essential metal that plays an important role in cellular metabolism (Linder and Hazegh-Azam, 1996) but it can also be acutely toxic to freshwater fish in the $\mu g\,l^{-1}$ range (see reviews by Handy, 2003; Grosell, 2012). Copper concentrations in freshwater environments typically range from about 0.2 to around 30 $\mu g\,l^{-1}$ (Bowen, 1985). For waterborne exposures, the target organs for dissolved Cu are primarily the gill, with the liver also being a central compartment in Cu metabolism (Grosell et al., 1996, 1997; Shaw et al., 2012). However, Cu toxicity to fish is influenced by water chemistry such as alkalinity, hardness, dissolved organic matter and pH (Erickson et al., 1996). There is general agreement that increasing water hardness or adding dissolved organic matter will reduce the toxicity of dissolved copper (Erickson et al., 1996; Laurén and McDonald, 1985a). The situation is less clear for water pH. In some circumstances, lowering water pH can protect against acute Cu toxicity; for example, LC_{50} values at pH 4.7, 5.7 and 7.0 were 66.0, 4.2, and 2.8 $\mu g\,l^{-1}$ in juvenile rainbow trout [*Oncorhynchus mykiss* (Cusimano et al., 1986)]. Such phenomena are explained by hydrogen ions competing for binding sites on the gill with Cu ions (the biotic ligand model; Playle and Dixon, 1993). Alternatively, lowering water pH might increase Cu toxicity (Erickson et al., 1996), and at pH 4 or less the toxic Cu^{2+} ion dominates chemical speciation in the water, promoting Cu uptake at the gill (Bury and Handy, 2010). However, between pH 5 and pH 7, a number of copper hydroxide

* Corresponding author at: School of Biological Sciences, Plymouth University, Drake Circus, Plymouth PL4 8AA, UK. Fax: +44 1752 584605.
E-mail address: r.handy@plymouth.ac.uk (R.D. Handy).

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complexes are formed which also alter the toxicity of Cu (Kamunde and Wood, 2004).

Acidic water alone is known to be toxic to freshwater fishes (Wood and Mcdonald, 1982). Low water pH is associated with excessive mucus secretion and respiratory distress (Plonka and Neff, 1969). Indeed, waterborne exposure to acid (Chevalier et al., 1985) or dissolved Cu (Figueiredo-Fernandes et al., 2007) can cause damage to the gills of fish. In both cases, loss of gill function can lead to disturbances to ionic regulation and gas exchange (Wood and McDonald, 1982; Laurén and McDonald, 1985b); combinations of acid and Cu have also been reported to produce haematological disturbances (Nussey et al., 2002). Copper is also known to cause toxicity to fishes via oxidative stress to the internal organs and/or via secondary hypoxia associated with gill injury (Hoyle et al., 2007; Eyckmans et al., 2011; Shaw et al., 2012).

Only a few recent studies have assessed the effects of copper nanoparticles (Cu-NPs) on fishes, and all of these studies are at neutral pH values (Griffitt et al., 2007; Shaw et al., 2012). The mechanisms of Cu-NP toxicity and whether or not there is involvement of the free metal ion remains unclear. A fraction of dissolved Cu may be released from Cu-NPs (*e.g.* Shaw et al., 2012), but for rainbow trout at least, $CuSO_4$ appears more acutely toxic than the equivalent concentration of Cu-NPs, with exposure to 100 $\mu g\,l^{-1}$ of $CuSO_4$ causing complete mortality, while the same concentration of Cu as Cu-NPs for 10 days caused only 19% mortality (Shaw et al., 2012). Although the magnitude of effects for $CuSO_4$ compared to Cu-NPs were greater, the types of sub-lethal effects were similar. Both showed Cu accumulation in the gills, transient changes in haematology and depletion of plasma Na^+, disturbances to tissue ion levels, and decreased branchial Na^+/K^+-ATPase activity (Shaw et al., 2012). Exposure to 0.25 $mg\,l^{-1}$ of dissolved Cu and either 0.25 or 1.5 $mg\,l^{-1}$ of Cu-NPs for 48 h in zebrafish showed similar effects that included inhibition of the branchial Na^+/K^+-ATPase activity and gill injury (Griffitt et al., 2007).

The effects of water pH on the sub-lethal toxicity of Cu-NPs have not been investigated in rainbow trout. It remains unclear whether or not the concept of pH-dependent toxicity will apply to nanomaterials in the same way as traditional dissolved metals (*e.g.* Playle and Dixon, 1993). The aim of the present study was to determine whether or not Cu accumulation and sub-lethal responses in rainbow trout upon exposure to Cu-NPs were affected by lowering water pH from 7 to 5. Experiments were also conducted to understand the effect of acidity on the rate of dissolution of dissolved Cu from Cu-NPs. In addition, given the dearth of detailed physiological data on the combined sub-lethal effects dissolved Cu and low pH, the $CuSO_4$ control in the present study also contributes to our understanding of metal ion/acid toxicity to trout.

2. Materials and methods

2.1. Experimental design

Juvenile rainbow trout were obtained from Torre Fisheries Ltd., Watchet, Somerset, UK, and held for 4 weeks prior to experimentation in a stock aquarium containing aerated, dechlorinated Plymouth tap water (see below) and under a photoperiod of 12 h light: 12 h dark. Trout were fed to satiation twice daily with a commercial feed (EWOS, Westfield, UK; 2–3 mm pellets) containing 8 $mg\,kg^{-1}$ Cu, as is standard for trout.

For logistical reasons, exposures were conducted first at pH 7 (7 day exposure), and then repeated one week later at pH 5. One day prior to the commencement of exposures, fish weighing 27.4 g ± 0.9 and with a total length of 13.8 ± 0.1 cm (means ± S.E.M., $n = 198$) were distributed randomly between nine aerated experimental glass aquaria containing 20 l of dechlorinated Plymouth tap water (11 fish/tank), in a triplicate design (3 tanks/treatment). Fish were exposed for up 7 days using a semi-static exposure regime (80% water change every 12 h with re-dosing after each change, *i.e.* twice/day) to: control (no added Cu); 20 $\mu g\,l^{-1}$ of Cu as $CuSO_4.5H_2O$; or 20 $\mu g\,l^{-1}$ copper nanoparticles (Cu-NPs). The stock water was adjusted manually to either pH 5 or pH 7 by adding 0.5 M H_2SO_4. Furthermore, the pH was checked every 4 h during experiments, and adjusted as necessary. The exposure concentration of 20 $\mu g\,l^{-1}$ of Cu as $CuSO_4$ or Cu-NPs was chosen as a sub-lethal dose that would produce physiological disturbances in trout based on our previous study (Shaw et al., 2012). The acidic pH value, pH 5, was chosen because Cu accumulation on the gill of trout (exposure) appears to be high at this pH (Playle and Dixon, 1993), whilst also avoiding the acute mortality associated with lower pH values. This pH also seemed to give reasonably stable dispersions of Cu-NPs (see Section 2.2).

Fish were not fed in the 24 h prior to, or during the experiment in order to minimise the risk of incidental ingestion of Cu-NPs or $CuSO_4$ with food, and to help maintain good water quality. Water samples were collected before and after each water change to verify the exposure concentrations (see Section 3.1) and monitor temperature, and dissolved oxygen (HACH HQ40d multi reader), and total ammonia (HI95715, Hanna Instruments). There were no treatment differences in water quality between tanks (One-Way ANOVA, $p > 0.05$). Data were (means ± S.E.M., $n = 576$ samples): temperature, 15.7 ± 0.2 °C; dissolved oxygen, 96 ± 0.5%; total ammonia, 0.97 ± 0.05 $mg\,l^{-1}$. The electrolyte composition of the dechlorinated Plymouth tap water was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian 725 ES, Melbourne, Australia), and was: 17.86 ± 0.01, 1.67 ± 0.18, 9.90 ± 0.25 $mg\,l^{-1}$ for Ca^{2+}, K^+ and Na^+ respectively (0.45, 0.04, and 0.43 $mmol\,l^{-1}$). Background total water Cu concentrations in the tanks of control fish were 2.94 ± 0.28 $\mu g\,l^{-1}$ (0.046 ± 0.004 $\mu mol\,l^{-1}$). Fish were randomly sampled on day 0 (initial fish from the stock), and days 4 and 7 for haematology and plasma ions, tissue electrolytes, and biochemistry.

2.2. Copper nanoparticles, stock suspensions, and dosing regimes

The Cu-NPs used here are from the same batch that was used by Shaw et al. (2012). Briefly, the powdered form of Cu-NPs was obtained from Sigma–Aldrich, UK, and had an average particle size <50 nm and 99.9% purity (manufacturer's information). One l stock suspensions of 1 g l^{-1} were prepared twice daily in low-density polyethylene (LDPE) bottles (in order to prevent sticking of Cu-NPs to glass) by dispersing the NPs in ultrapure (Milli-Q) water with stirring (magnetic stirrer IKA Werke RET basic C, at 300 rpm) for 12 h prior to dosing. This stock was then used to dose aquaria immediately following water changes. Due to the low concentrations of Cu-NPs used in the exposures with fish which exceeded the sensitivities of some analytical instruments, the effects of water pH on primary particle sizes [by transmission electron microscopy (TEM, JEOL 1200EXII)] and agglomerative state [by Nanoparticle Tracking Analysis (NTA, NanoSight LM10, Nanosight, Salisbury, UK)] were investigated in 1 g l^{-1} stocks prepared in ultrapure water and stirred for 12 h as described above, followed by dilution into pH-adjusted Plymouth tap water to a concentration of 0.1 g l^{-1} and stirring whilst gassing with air for a further 12 h *i.e.* to match the 12 h of stirring prior to dosing tanks and the additional 12 h between water changes. At 1 g l^{-1}, Cu-NPs had primary particle diameters of: 61 ± 4 nm for stock dispersions in Milli-Q water, and in dispersions at pH 7 and 5 in Plymouth tap water following dilution to 0.1 g l^{-1} the diameters were 60 ± 2 and 55 ± 1 nm, respectively (mean ± S.E.M, $n = 68–123$, Fig. 1). Measured mean agglomerate sizes of NP stock dispersions were: 138 ± 16 nm in Milli-Q water, 143 ± 3 nm at pH 7, and 114 ± 11 nm at pH 5.

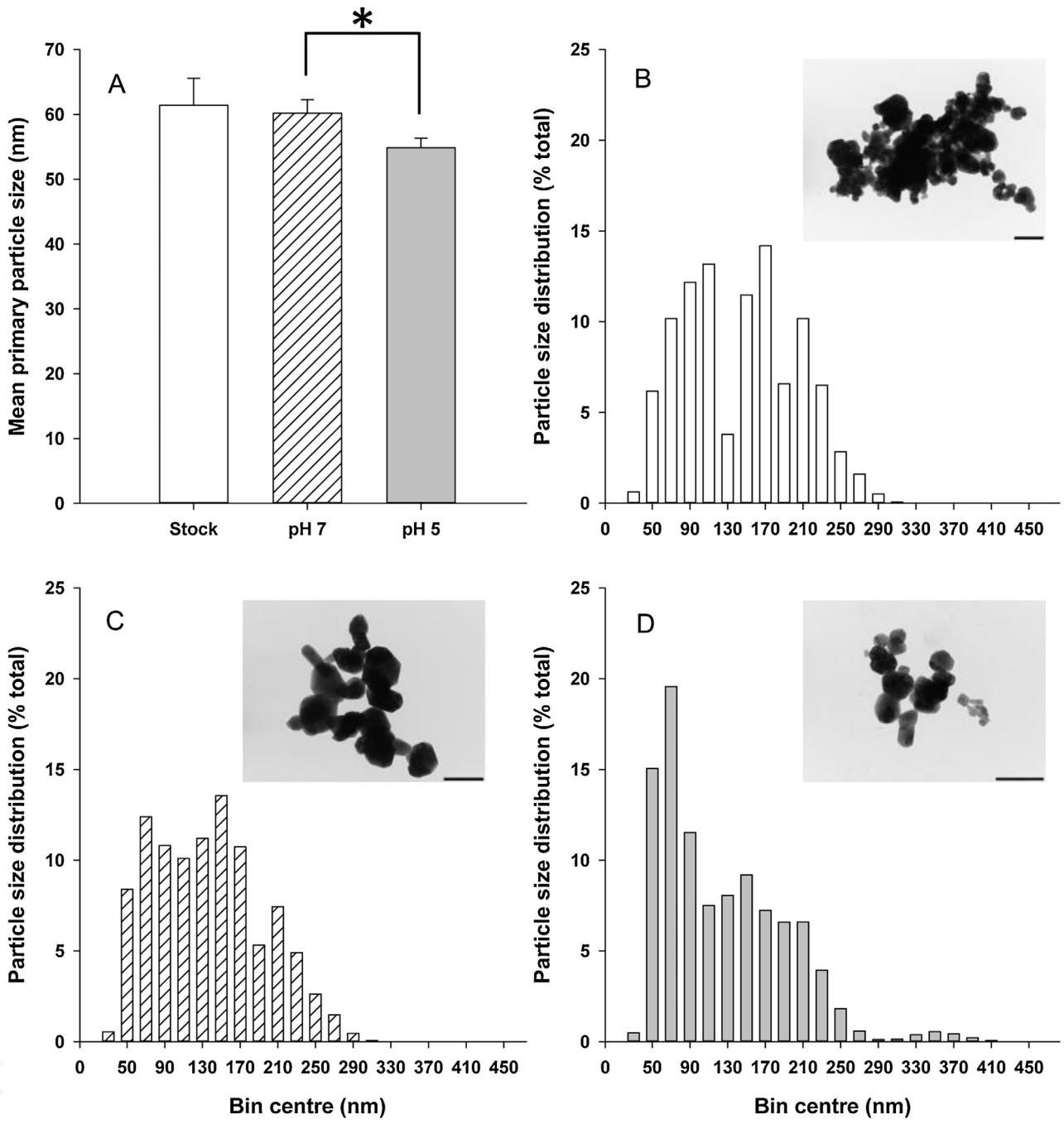

Fig. 1. Primary particle sizes and particle size distributions of 1 g l^{-1} Cu-NP stocks. After 24 h stirring, the primary particle sizes of Cu-NPs in aquarium water at pH 5 were significantly smaller than at pH 7 (* panel A, $p = 0.037$, Student's *t*-test). Data are means ± S.E.M, $n = 68$ and 123. Panels B, C and D show particle size distributions after 24 h (NanoSight LM10) in stocks (B) and at pH 7 (C) and pH 5 (D), respectively, and are presented as % of total particles measured. Insets are electron micrographs showing Cu-NPs. Scale bars are 200 nm.

A 1 g l^{-1} dissolved Cu stock solution was prepared by dissolving $CuSO_4 \cdot 5H_2O$ (Sigma–Aldrich) in 1 l of ultrapure (Milli-Q). The same $CuSO_4$ stock solution was used in both 7 day exposures at pH 7 and pH 5. Tanks were dosed with $CuSO_4 \cdot 5H_2O$ at 12 h intervals following water changes as described above for Cu-NPs.

2.3. Dissolution of Cu from Cu-NPs at different pH

Dialysis experiments were conducted to measure the dissolution rate of Cu from the Cu-NPs in dechlorinated Plymouth tap water at pH 7 and 5 (see Section 2.1) and as described by Handy et al. (1989) with modifications for NPs as reported by Besinis et al. (2012). All glassware and equipment were acid washed (5% nitric acid) and triple rinsed in deionised (Milli-Q) water before use. A stock of Cu-NPs was prepared and dispersed as described in Section 2.2 and then 8 ml of the stock diluted to 100 mg l^{-1} of Cu-NPs, or a no added Cu control (*i.e.* the test solution with no copper) were filled into dialysis bags [70 × 25 mm cellulose dialysis tubing with a 12 kDa molecular weight cut off (Sigma–Aldrich, St. Louis, USA)] and both ends of each dialysis bag secured with Medi-clips to prevent any leakage. The bags were then placed in a beaker containing 492 ml water at the appropriate pH (total volume 500 ml). The solutions in the beakers were gently agitated with a multi-point magnetic stirrer for 24 h at room temperature and samples of the

external solution taken from each beaker at 0, 0.5, 1, 2, 3, 4, 6, 8 and 24 h. The pH was monitored throughout. This is an extended dialysis period compared to the regimen used for the exposure (*i.e.* 80% water change at 12 h intervals) to better compare the behaviours of NPs at pH 7 and 5 and the fate of NPs in the residual 20% of water remaining in tanks between water changes. Samples were analysed by ICP-OES for total Cu concentrations.

2.4. Haematology and blood plasma analysis

Haematology and blood plasma analyses of trout were performed as described in Handy and Depledge (1999). Two fish were randomly collected from each tank ($n = 6$ fish/treatment and $n = 6$ initial fish) at days 4 and 7 from each pH experiment, and terminally euthanized with MS222 (0.2 g l^{-1}) pH buffered to match the exposure waters (pH 5 or pH 7). Fish were measured for length and weight and then whole blood was collected via caudal puncture into lithium heparinised syringes. Haematocrit (Hct) was calculated in duplicate by centrifuging whole blood in microhaematocrit tubes (13,000 rpm, 2 min, Heraeus pico 17 microcentrifuge) and calculating the ratio of packed red cells to supernatant by using a Hawksley reader (Hawksley, Sussex, UK). The haemoglobin (Hb) concentration was calculated by diluting 20 μl whole blood in 5 ml of Drabkin's reagent (Sigma–Aldrich, UK) and comparing to cyanmethemoglobin standards at 540 nm. A further 20 μl of whole blood was also fixed in 0.98 ml of Dacie's fluid (10 ml of 40% formaldehyde, 31.3 g trisodium citrate, 1.0 g brilliant crystal blue, diluted to 1 litre with Milli-Q water) for red and white blood cell counts. The remaining blood was centrifuged (13,000 rpm for 2 min, Micro Centaur MSE), and plasma collected and stored at −80 °C until subsequent analysis of plasma ions and osmometry. Plasma Na^+ and K^+ were analysed by flame photometry (Corning 420 Flame photometer), while plasma chloride was analysed by automated titration (Jenway PCLM 3Chloride Meter). Osmotic pressure was determined by the freezing-point depression method using 50 μl of plasma (Precision System micro osmometer, Natick, Massachusetts, USA).

2.5. Tissue ion analysis

Following blood sampling, fish were dissected for tissue metal analysis. Gill, liver, spleen and kidney were harvested and washed with Milli-Q water and processed for ion analysis according to Handy et al. (2000) with minor modification. Samples were placed on clean glass slides and dried to constant weight (Gallenkamp Oven BS Model OV-160) at 100 °C for 48 h, then the dried tissue was removed into polypropylene scintillation vials (VWR International Ltd., Poole, UK). Samples (typically 0.1–0.5 g dried tissue) were digested in 4 ml of concentrated nitric acid (analytical grade, Fisher Scientific, UK) at 70 °C for 3 h in a water bath, allowed to cool, then diluted to 16 ml with ultra pure water (Milli-Q). For very small tissue samples (less than 0.1 g dry weight) the volumes of reagents were reduced to 1 ml of nitric acid and then diluted to a final volume of 4 ml with Milli-Q water. Samples were then analysed for trace elements (*e.g.* Cu, Ca, Na, K, Mn and Zn) by ICP-OES And compared with matrix-matched standards prepared from Aristar® plasma emission grade solutions, with accuracy checked after every 10 samples during the analysis by running a blank or standard as a sample. Spike recovery tests were performed with both $CuSO_4$ and Cu-NPs using rainbow trout gill and liver digested as above and were (% mean ± S.E.M, $n = 6$): 94 ± 1 (Cu-NPs) and 102 ± 1 ($CuSO_4$) in the gill tissues and 92 ± 2 (Cu-NPs) and 102 ± 1 ($CuSO_4$) in the liver.

2.6. Biochemistry

Additional fish at days 0, 4 and 7 ($n = 6$ fish/treatment and initial fish) were euthanized as described in Section 2.4 and tissues (gill, liver, kidney, and spleen) were harvested and immediately snap frozen in liquid nitrogen and stored at −80 °C until required. Tissues (approximately 0.5 g) were weighed and homogenised (3 × 10 s with 2 min rest at 17,500 rpm, Cat X520D with a T6 shaft, medium speed, Bennett & Co., Weston-super-Mare) in five volumes of ice-cold isotonic buffer solution (in mmol l^{-1}; 300 sucrose, 0.1 ethylenediamine tetra acetic acid (EDTA), 20 HEPES [4-(2-hydroxyethyl) piperazine-1-ethane sulfonic acid], adjusted to pH 7.8 with a few drops of Tris (2-amino-2-hydroxyl-1,3-propanediol)). Crude homogenates were centrifuged for 2 min (13,000 rpm at 4 °C) after which the supernatant was stored in 0.5 ml aliquots at −80 °C until required. Tissue homogenates were analysed exactly as described in Smith et al. (2007) in triplicate wells per sample for Na^+/K^+-ATPase activity (15 μl of homogenate), thiobarbituric acid reactive substances (TBARS, 40 μl of homogenate), and total glutathione (GSH, 20 μl of homogenate).

2.7. Statistical analysis

The resulting data were analysed by SigmaPlot version 13.0 (Systat Software, Inc.) and StatGraphics Plus version 5.1 (StatPoint Technologies, Inc.). Throughout the experiment, no tank effects were seen, so data were pooled according to treatment for statistical analyses. Briefly, normally-distributed data (Shapiro–Wilk test, $\log_{10}$ transformed if required) that had equal variances (Brown–Forsythe test) were analysed according to independent variables treatment, pH, time, and their interactions by a 3-way ANOVA with Fisher's LSD test *post hoc*. Where the main effects could not be interpreted due to interactions between variables, and to detect simple effects, one-way ANOVA and a Fisher's LSD test *post hoc* was used. If data were not normally distributed and transformation failed, the Kruskal–Wallis test was used. Data are presented as means ± S.E.M. All statistical analyses used a probability level of 0.05 ($p < 0.05$) to reject the null hypothesis.

3. Results

3.1. Total Cu concentrations in water and tissues

Water samples taken within 10 min of dosing of experimental tanks confirmed exposures of fish to Cu. The total Cu concentrations in tanks were: 2.94 ± 0.05, 20.36 ± 0.27, 19.42 ± 0.12 μg l^{-1} at pH 7 and 3.23 ± 0.12, 20.53 ± 0.08, 19.62 ± 0.21 μg l^{-1} at pH 5 (for the control, 20 μg l^{-1} of either Cu as $CuSO_4$ or Cu-NPs treatments respectively, $n = 12$), representing recoveries of 97–103% of nominal concentrations of $CuSO_4$ and Cu-NPs. At the end of the 12 h exposure period between water changes measurable Cu concentrations in tanks had decreased in all treatments and were 85.7 and 77.7% at pH 7 and 88.6 and 83.8% at pH 5 for $CuSO_4$ and Cu-NPs treatments, respectively.

Dialysis experiments with Cu-NPs in Plymouth tap water were conducted at pH 7 and 5 (Fig. 2). The Cu-NPs at pH 7 rapidly reached equilibrium, with a maximum total Cu-NP dissolution of 7.5 ± 0.8%. However, for Cu-NPs at pH 5, no equilibrium was reached with a steady increase in dissolution of Cu across all time-points measured reaching a maximum of 20.9 ± 3.9% NP dissolution at 24 h. Concentrations of Cu in control beakers were unchanged over the 24 h period and were consistently below the limit of detection of this analysis run (<0.0083 mg l^{-1}).

The exposures were also confirmed by measuring total Cu concentrations in the tissues of fish (Fig. 3). A three-way ANOVA with

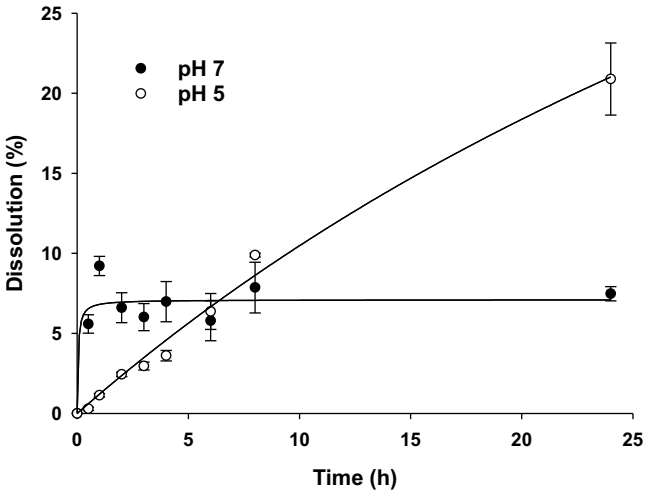

Fig. 2. Dissolution of Cu-NPs (% of measured total Cu) in dechlorinated Plymouth tap water adjusted to pH 7 (closed symbols) and 5 (open symbols) over 24 h. Data are means ± S.E.M, $n = 3$ replicates. Curves for dissolved Cu concentration were fitted by using a rectangular hyperbole function (one site, ligand binding to saturation) in SigmaPlot *v.* 13. The Cu concentrations in controls (no Cu-NPs, dialysis tubing only) were below the instrument detection limits and are not shown.

treatment, pH and day as between-subjects factors revealed main effects of treatment ($F_{2,60} = 69.8$, $p < 0.001$) and pH ($F_{1,60} = 8.79$, $p = 0.004$) on Cu accumulation in gills of fish. All other main effects and interactions were non-significant. Copper accumulation was observed in the gills of trout exposed to both $CuSO_4$ and Cu-NPs treatments, on both days 4 and 7 and also at both pH 7 and 5. *Post hoc* tests with the Fisher's LSD test indicated that Cu accumulation was greater in fish exposed to $CuSO_4$ than Cu-NPs *i.e.* a nano-effect ($p < 0.001$). A pH effect was observed for branchial Cu accumulation in fish exposed to $CuSO_4$ which showed significantly less accumulation of Cu at pH 5 compared to pH 7 (Fisher's LSD test, $p = 0.004$). Nano copper-treated fish did not show any pH effects. There was also a main effect of treatment ($F_{2,60} = 7.97$, $p < 0.001$) on Cu accumulation in livers of fish. *Post hoc* tests indicated higher Cu concentrations in livers of fish exposed to $CuSO_4$ ($p < 0.001$) and Cu-NPs ($p = 0.033$) compared to controls, but no evidence of a nano-effect ($p = 0.075$). Copper concentrations were lower in spleens of fish at pH 5 than pH 7 ($F_{1,60} = 19.1$, $p < 0.001$) but there were no effects of treatment (or day or interactions). Although, there were some effects of exposure time and pH on Cu accumulation in the kidney, there were no clear patterns of treatment related effects on Cu concentrations in the kidney.

3.2. Haematology and blood plasma analyses

Exposure of trout to Cu as $CuSO_4$ or Cu-NPs at neutral and acidic pH values caused changes in some blood parameters (Table 1). The main effects of treatment, day and pH on Hb concentrations, Hct values, as well as red and white blood cell counts could not be fully interpreted with 3-way ANOVA due to multiple significant interactions that were detected between factors; however, *post hoc* Fisher's LSD test and one-way ANOVA provided additional resolution. There was an effect of treatment on Hb concentrations (3-way ANOVA, $F_{2,60} = 3.35$, $p = 0.042$) that was qualified by an interaction between treatment and day ($F_{2,60} = 5.42$, $p = 0.007$) and treatment and pH ($F_{2,60} = 14.0$, $p < 0.001$). At pH 7 and at day 4, Hb concentrations were lower in fish exposed to $CuSO_4$ compared to both controls and Cu-NPs (one-way ANOVA, $p < 0.05$). These had partially recovered by day 7. An opposite effect of treatment on Hb concentrations was evident on day 7 at lower pH with elevations observed in fish exposed to both $CuSO_4$ and Cu-NPs compared to controls (one-way ANOVA, $p < 0.05$). Similar trends were also apparent in Hct and red blood cell counts. A 3-way ANOVA indicated greater Hct in fish at pH 5 compared to pH 7 ($F_{1,60} = 23.9$, $p < 0.001$) that was qualified by a three-way interaction between treatment, day and pH ($F_{2,60} = 6.58$, $p = 0.003$). Differences were detected with Fisher's LSD tests and indicated decreased Hct in fish exposed to $CuSO_4$ compared to both controls ($p = 0.003$) and fish exposed to Cu-NPs ($p = 0.001$) at day 4 and pH 7; however, Hct had increased (*i.e.* recov-

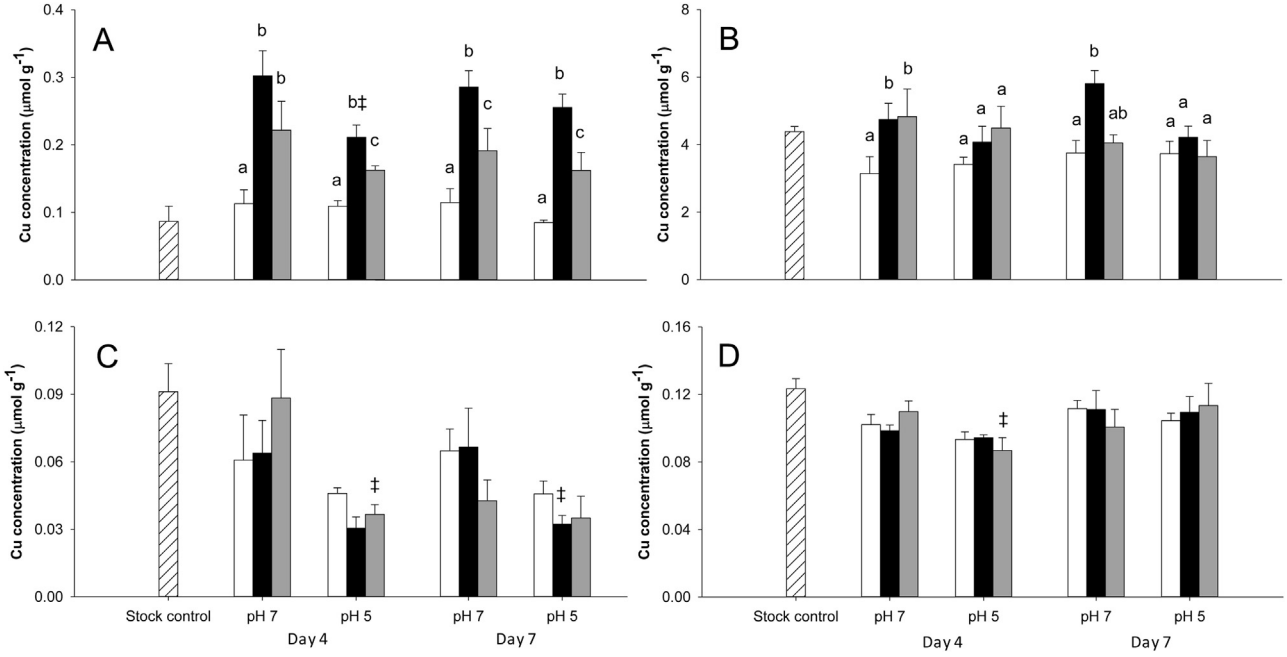

Fig. 3. Cu concentrations in (A) gills, (B) liver, (C) spleen and (D) kidney of trout after exposure to control (white bars), and 20 μg l^{-1} of either $CuSO_4$ (black bars) or Cu-NPs (grey bars) for 4 and 7 days at pH 7 and 5. Diagonal hatched bars are initial (day 0) fish. Data are means ± S.E.M., dry weight tissue, $n = 6$ fish/treatment. 3-way ANOVA and where appropriate one-way ANOVA with Fisher's LSD test was used to detect differences between treatments ($p < 0.05$). Different lowercase letters denote statistically significant differences between treatments within pH and time point. (‡) indicates a statistically significant difference between pH 7 and 5 within treatment and time point.

Table 1
Haematology and plasma ions in rainbow trout exposed to control (no added Cu), and 20 μg l^{-1} of either $CuSO_4$ or Cu-NPs at pH 7 and 5.

Parameter	Day 0 fish	Day	pH 7			pH 5		
			Control	$CuSO_4$	Cu-NPs	Control	$CuSO_4$	Cu-NPs
Haemoglobin (mg dl^{-1})	17.4 ± 0.5	4	19.8 ± 0.8^{a}	13.9 ± 0.5^{b}	18.8 ± 0.7^{a}	16.6 ± 1.0	17.7 ± 0.9	20.5 ± 1.5
		7	19.3 ± 1.0	16.9 ± 0.9^{+}	16.9 ± 1.2	14.9 ± 1.2^{a}	19.0 ± 0.9^{b}	18.5 ± 0.9^{b}
Haematocrit (%)	20.3 ± 0.8	4	23.3 ± 1.0^{a}	19.0 ± 0.4^{b}	23.7 ± 0.7^{a}	26.3 ± 1.0	27.7 ± 1.2	25.5 ± 0.3
		7	23.2 ± 1.3	25.7 ± 0.5^{+}	21.2 ± 0.9	24.7 ± 1.7	25.7 ± 1.5	25.2 ± 1.8
Red blood cells (× 10^{6})	0.57 ± 0.00	4	0.71 ± 0.04^{a}	0.54 ± 0.01^{b}	0.60 ± 0.01^{b}	0.73 ± 0.02	0.65 ± 0.03‡	0.68 ± 0.05
		7	0.72 ± 0.01^{a}	0.63 ± 0.01^{b+}	0.62 ± 0.14^{b}	0.57 ± 0.04‡	0.69 ± 0.05	0.67 ± 0.04
White blood cells (× 10^{3})	15.6 ± 0.8	4	18.3 ± 1.9	17.3 ± 0.7	15.8 ± 1.0	25.3 ± 3.4^{a‡}	17.2 ± 0.72^{b}	22.2 ± 2.5^{a‡}
		7	13.0 ± 0.2^{a‡}	17.2 ± 0.8^{b}	15.5 ± 0.5^{ab}	28.51 ± 2.1^{a‡}	35.0 ± 2.7$^{b+‡}$	34.7 ± 0.8^{b+}
Plasma Na^{+} (mmol l^{-1})	155.0 ± 0.5	4	148.8 ± 1.7^{a}	137.9 ± 1.6^{b}	135.4 ± 1.7^{b}	139.9 ± 2.9‡	133.6 ± 0.6	133.1 ± 1.9
		7	151.6 ± 2.6^{a}	130.0 ± 2.4^{b}	142.5 ± 4.5^{c}	126.7 ± 1.6$^{+‡}$	126.6 ± 1.1^{+}	126.9 ± 2.9$^{+‡}$
Plasma K^{+} (mmol l^{-1})	3.03 ± 0.01	4	2.89 ± 0.05^{a}	2.52 ± 0.08^{b}	2.49 ± 0.03^{b}	2.66 ± 0.18^{a}	2.86 ± 0.14^{a}	3.35 ± 0.17^{b‡}
		7	3.00 ± 0.01^{a}	2.82 ± 0.09^{ab+}	2.73 ± 0.09^{b+}	3.66 ± 0.18$^{+‡}$	3.69 ± 0.27$^{+‡}$	3.34 ± 0.06‡
Plasma Cl^{-} (mmol l^{-1})	160.3 ± 1.9	4	154.3 ± 3.2	151.4 ± 0.5	157.3 ± 2.0	146.8 ± 4.4	138.5 ± 5.4‡	145.8 ± 1.3‡
		7	158.1 ± 2.7^{ab}	157.4 ± 2.9^{a}	150.7 ± 2.6^{b}	142.9 ± 3.0‡	147.7 ± 2.6$^{+‡}$	145.9 ± 2.2

Data are means ± S.E.M. (n = 6 fish per treatment and time point). Different lowercase letters denote statistically significant differences between treatments within time point and pH. ‡ indicates a statistically significant difference between pH 7 and 5 within the type of Cu-treatment and time point (pH effects). $^{+}$ indicates a statistically significant difference between day 4 and 7 within the type of Cu-treatment and pH (time effects). Measurements of day 0 fish are shown for information only and were not included in statistical analyses. Data were analysed with 3-way ANOVA and where appropriate one-way ANOVA with Fisher's LSD test (p < 0.05).

ered) by day 7 (p < 0.001). Red blood cell counts were similarly diminished in fish exposed to both $CuSO_4$ and Cu-NPs compared to controls at pH 7 and on both days 4 and 7 (one-way ANOVAs, p < 0.05). Exposure to low pH had a pronounced effect on white blood cell counts (elevated, 3-way ANOVA, $F_{1,60}$ = 131.6, p < 0.001) which changed with the duration of exposure (increased by day 7, $F_{1,60}$ = 15.5, p < 0.001) but these were qualified by an interaction between these two factors ($F_{1,60}$ = 42.1, p < 0.001). Exposure to Cu also affected white blood cell counts. Specifically, counts were lower in fish exposed to $CuSO_4$ and Cu-NPs compared to controls at day 4, but the effect was reversed by day 7 (one way ANOVAs, p < 0.05).

Blood plasma electrolytes showed pH, treatment and time effects (Table 1). Plasma Na^{+} was affected by treatment ($F_{2,60}$ = 17.8, p < 0.001), day ($F_{1,60}$ = 10.6, p = 0.002) and pH ($F_{1,60}$ = 53.3, p < 0.001) although these effects were qualified by a 3-way interaction between factors ($F_{2,60}$ = 3.85, p = 0.027). At pH 7 both $CuSO_4$ and Cu-NPs exposed fish showed depleted Na^{+} compared to controls on day 4 (Fisher's LSD tests, p = 0.002 and p < 0.001, respectively). By day 7 a similar decrease in plasma Na^{+} of fish exposed to $CuSO_4$ and Cu-NPs was also evident and there was evidence of a nano-effect (worse with $CuSO_4$). In fish exposed at pH 5, plasma Na^{+} was observed to decrease from day 4 to day 7 across all treatments (Fisher's LSD test, p < 0.001, one-way ANOVA p < 0.05). The interpretation of effects of Cu exposures on plasma K^{+} concentrations were complicated by a 3-way interaction between treatment, day and pH (3-way ANOVA, $F_{2,60}$ = 5.23, p = 0.008). Although values remained largely in the normal range, there were a few small, but statistically significant changes. These included a decrease in the K^{+} of both the $CuSO_4$ and Cu-NP treatments at pH 7 compared to the control (Fisher's LSD tests p = 0.017 and p = 0.005, respectively). There was also a large elevation in plasma K^{+} in Cu-NPs exposed fish at pH 5 on day 4 compared to controls and also fish exposed to $CuSO_4$ (Fisher's LSD tests, p < 0.001 and p = 0.01, respectively). Overall, there was also a small pH-effect on plasma K^{+} concentration, with slightly elevated values at pH 5 compared to pH 7, in both the $CuSO_4$ and Cu-NPs treatments (ANOVA, p < 0.05). Plasma Cl^{-} concentrations also remained in the normal range, but with a few small, differences that were inconsistent over time. There was a pH-effect on plasma Cl^{-} concentration (3-way ANOVA, $F_{1,60}$ = 34.5, p < 0.001), with all treatments showing lower plasma Cl^{-} concentration at pH 5 compared to pH 7. There was no Cu-treatment or pH-effects on plasma osmolarity (data not shown).

3.3. Tissue electrolytes

There were some differences in the electrolyte concentrations in some of the tissues of trout during exposures to $CuSO_4$ or Cu-NPs, and at each pH value (Table 2). However, many of the changes were transient with no clear treatment or time-dependent trend. In the gill tissue, there were effects of treatment (3-way ANOVA, $F_{2,60}$ = 5.881, p = 0.005), day ($F_{1,60}$ = 12.3, p < 0.001) and pH ($F_{1,60}$ = 36.6, p < 0.001) although these were partially qualified by an interaction between treatment and day ($F_{2,60}$ = 3.37, p = 0.041). *Post hoc* Fisher's LSD test indicated elevated Na^{+} concentrations in $CuSO_4$ treated fish compared to controls (p < 0.001) and Cu-NPs treated fish (a nano effect, p = 0.003) at day 4 and simple effects were further investigated with one-way ANOVA (see annotations in Table 2). Exposure to acid caused some branchial Na^{+} depletion that was also evident in the pH 5/$CuSO_4$ and the pH 5/Cu-NP treatments (one-way ANOVA, p < 0.05), but with no material-type effect by the end of the experiment. There were also effects of treatment (3-way ANOVA, $F_{2,60}$ = 4.32, p = 0.018) and day ($F_{1,60}$ = 7.25, p = 0.009) on branchial K^{+} concentrations. At low pH, branchial K^{+} concentrations showed an increase in fish from the $CuSO_4$ and Cu-NP treatments compared to the control (one-way ANOVA, p < 0.05), but there was no pH-effect between materials. Branchial Zn concentrations were affected by pH of the water (3-way ANOVA, $F_{1,60}$ = 16.3, p < 0.001), but there were no main effects of treatment or day of exposure.

Hepatic Na^{+} showed a pH-effect which included a decrease in the Na^{+} concentration in livers of fish from both $CuSO_4$ and Cu-NP treatment at pH 5 when compared to pH 7 (3-way ANOVA, $F_{1,60}$ = 23.1, p < 0.001, one-way ANOVA p < 0.05, Table 2). Hepatic Na^{+} concentrations did not show Cu-treatment or time effects, except for a small material-type effect with a decrease in Na^{+} concentration in the Cu-NP treatment compared to the $CuSO_4$ treatment at pH 5 (3-way ANOVA, $F_{2,60}$ = 4.91, p < 0.001, one-way ANOVA, p < 0.05). There were some small, transient decreases in hepatic K^{+} concentrations in both the $CuSO_4$ and Cu-NP treatment at pH 7 on day 4 compared to controls (3-way ANOVA, significant interaction between treatment, time and pH, $F_{2,60}$ = 4.58, p = 0.014, *post hoc* Fisher's LSD tests between treatments, p < 0.001

Table 2
Electrolyte concentrations (μmol g^{-1} dry weight) in the gill and liver of rainbow exposed to control (no added Cu), and 20 μg l^{-1} of either $CuSO_4$ or Cu-NPs at pH 7 and 5.

Tissue	Tissue	Day 0 fish	Day	pH 7			pH 5		
				Control	$CuSO_4$	Cu-NPs	Control	$CuSO_4$	Cu-NPs
Gill	Na	231.6 ± 9.1	4	196.3 ± 7.5[a]	249.4 ± 5.9[b]	214.6 ± 7.8[a]	183.1 ± 10.8	200.3 ± 10.0[‡]	180.9 ± 14.3[‡]
			7	232.2 ± 10.2[+]	245.2 ± 3.1	241.4 ± 6.7[+]	196.2 ± 4.1[‡]	209.0 ± 8.2[‡]	211.5 ± 6.6[‡+]
	K	385.4 ± 4.8	4	372.4 ± 2.9	379.2 ± 8.9	388.6 ± 9.0	376.5 ± 12.2	390.8 ± 6.9	371.5 ± 8.4
			7	391.9 ± 5.5[a]	409.8 ± 9.6[ab+]	418.1 ± 7.5[b+]	365.8 ± 3.5[a]	409.3 ± 3.4[b]	408.6 ± 4.0[b+]
	Ca	138.0 ± 12.8	4	109.4 ± 21.0	127.3 ± 24.7	95.2 ± 19.6	43.1 ± 5.9[‡]	88.8 ± 22.1	54.5 ± 10.4
			7	107.7 ± 23.1	105.7 ± 25.7	123.3 ± 23.0	77.7 ± 16.4	52.6 ± 8.4	104.8 ± 22.2
	Zn	5.5 ± 0.9	4	6.7 ± 0.7	6.3 ± 1.2	6.1 ± 1.0	9.7 ± 2.0	8.6 ± 2.1	13.1 ± 2.2[‡]
			7	9.5 ± 1.6	2.9 ± 0.1	4.5 ± 1.2	9.1 ± 1.1	10.6 ± 1.5[‡]	10.3 ± 1.5[‡]
Liver	Na	76.0 ± 3.2	4	72.6 ± 3.4	75.1 ± 2.2	74.1 ± 1.4	67.4 ± 1.5[ab]	73.1 ± 1.8[a]	63.9 ± 1.9[b‡]
			7	69.5 ± 0.3	77.4 ± 0.8	74.2 ± 5.5	64.8 ± 3.0	67.9 ± 2.0[‡]	64.8 ± 2.5[‡]
	K	359.8 ± 14.5	4	414.7 ± 8.8[a]	393.5 ± 5.3[b]	384.2 ± 4.9[b]	363.6 ± 4.0[‡]	362.9 ± 6.7[‡]	362.1 ± 0.7
			7	406.4 ± 4.0	390.2 ± 5.6	409.8 ± 7.6[+]	369.5 ± 3.0[‡]	371.0 ± 4.3	359.9 ± 4.3[‡]
	Ca	4.8 ± 0.2	4	5.2 ± 0.1[a]	5.9 ± 0.1[a]	4.3 ± 0.1[b]	4.8 ± 0.1	4.1 ± 0.2[‡]	4.3 ± 0.3
			7	5.5 ± 0.3	5.3 ± 0.5	5.2 ± 0.2[‡]	4.1 ± 0.2[‡]	4.2 ± 0.2[‡]	4.0 ± 0.2[‡]
	Zn	1.3 ± 0.1	4	1.4 ± 0.1	1.5 ± 0.0	1.5 ± 0.1	1.3 ± 0.1[a]	2.1 ± 0.1[b‡]	2.0 ± 0.1[b‡]
			7	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	2.0 ± 0.1[+‡]	2.2 ± 0.1[‡]	2.0 ± 0.1[‡]

Data are means ± S.E.M. ($n = 6$ fish per treatment and time point). Different lowercase letters denote statistically significant differences between treatments within time point and pH. [‡] indicates a statistically significant difference between pH 7 and 5 within the type of Cu-treatment and time point (pH effects). [+] indicates a statistically significant difference between day 4 and 7 within the type of Cu-treatment and pH (time effects). Measurements of day 0 fish are shown for information only and were not included in statistical analyses. Data were analysed with 3-way ANOVA and where appropriate one-way ANOVA with Fisher's LSD test ($p < 0.05$).

and $p = 0.011$, respectively), but no clear material-type effect. Hepatic Ca^{2+} concentrations were affected by Cu exposures and pH (3-way ANOVA, $F_{2,60} = 5.45$, $p = 0.007$, $F_{1,60} = 45.7$, $p < 0.001$, respectively). Noteworthy was a small decrease at pH 7 in the Cu-NP treatment only, compared to controls and $CuSO_4$ exposed fish (one-way ANOVA, $p < 0.05$). Effects of Cu exposures, day of exposure and pH on hepatic Zn concentrations were qualified by a 3-way interaction between variables ($F_{2,60} = 7.57$, $p < 0.001$). Nevertheless, *post hoc* testing with Fisher's LSD tests indicated increases in hepatic Zn at day 4 and pH 5 of both Cu treatments and controls (both $p < 0.001$) but no evidence of a nano effect.

The effects of $CuSO_4$ and Cu-NPs on electrolytes in kidney and spleen were minimal. An exception was a pronounced decrease in K^+ observed in kidney of fish exposed to $CuSO_4$ at pH 7 that was not observed in Cu-NPs exposed fish. Measured concentrations of K^+ in kidney were: 418.3 ± 7.8, 245.3 ± 11.6 and 420.8 ± 13.0 μmol g^{-1} in control, $CuSO_4$ and Cu-NPs exposed fish, respectively. This effect was not observed in fish exposed to $CuSO_4$ at pH 5 (data not shown).

3.4. Na^+/K^+-ATPase activity

Exposure to $CuSO_4$ or Cu-NPs at pH 7 and 5 caused perturbations in Na^+/K^+-ATPase activity in gill of trout (Fig. 4). A 3-way ANOVA revealed main effects of treatment ($F_{2,60} = 3.73$, $p = 0.030$) and pH ($F_{1,60} = 25.0$, $p < 0.001$). This was qualified by interactions between treatment, day and pH ($F_{2,60} = 3.99$, $p = 0.024$). At day 4, the gill of trout exposed to $CuSO_4$ at pH 7 showed an inhibition of Na^+/K^+-ATPase activity compared to controls (Fisher's LSD test, $p < 0.001$), with a material-type effect (worse with $CuSO_4$ than Cu-NP treatment, Fisher's LSD test, $p = 0.002$), although activity had returned to control levels, by day 7 (Fisher's LSD test, $p < 0.001$). At pH 5, exposure to $CuSO_4$ did not cause depletion in the activity of the Na pump although there was a clear trend toward inhibition at day 7. In kidney, the main effects of treatment, day and pH on Na^+/K^+-ATPase activity could not be interpreted with 3-way ANOVA due to interactions between all factors ($F_{2,60} = 3.24$, $p = 0.046$). One-way ANOVA indicated elevated Na^+/K^+-ATPase activity in fish exposed to $CuSO_4$ and Cu-NPs compared to controls at pH 5 on day 4 ($p < 0.05$), and in fish exposed to $CuSO_4$ compared to controls and fish exposed to Cu-NPs *i.e.* a nano effect at pH 7 on day 7 (Kruskal–Wallis test, $p < 0.05$).

3.5. TBARS and total glutathione content

Trout did not show any Cu-treatment-dependent, or pH-dependent, changes in TBARS in the gill, kidney or spleen (gill, Fig. 5; data not shown for kidney and spleen). In the liver, some small, transient, effects on TBARS were observed. A 3-way ANOVA revealed main effects of treatment ($F_{2,60} = 5.56$, $p = 0.006$), day ($F_{1,60} = 15.5$, $p < 0.001$) and pH ($F_{1,60} = 7.23$, $p = 0.009$). These main effects were not qualified by interactions between factors. There was lower TBARS in fish exposed to Cu-NPs compared to $CuSO_4$ (Fisher's LSD test, $p = 0.002$) but not compared to controls (Fisher's LSD test, $p = 0.053$). Specifically, exposure to Cu-NPs at pH 7 for 4 days showed a material-type effect (more depletion with Cu-NPs than $CuSO_4$; ANOVA, $p < 0.05$). At pH 5, there was also a material-type effect on the TBARS value of the Cu-NP treatment at day 7 compared to the equivalent $CuSO_4$ treatment (a larger decrease with the Cu-NP treatment). There was generally no clear Cu-treatment or pH-effects on the total glutathione (GSH) concentration in the kidney, spleen, or gill over time. In the liver, hepatic GSH concentrations did not show a Cu-treatment effect, but there was a pH-effect which included more depletion at pH 5 with both Cu treatments compared to that at pH 7 (3-way ANOVA, $F_{1,60} = 35.1$, $p < 0.001$, Fig. 5).

4. Discussion

This study is one of the first reports detailing the effects of acidic water on the sub-lethal toxicity and accumulation of Cu from Cu-NP exposure in rainbow trout. In itself, exposure to acid water was observed to cause changes in the ionoregulatory physiology of control fish. Additional effects in trout were also attributable to exposures to $CuSO_4$ and Cu-NPs. Gill burdens of Cu increased upon exposure to both $CuSO_4$ and Cu-NPs, but with greater accumulation from $CuSO_4$ (a material-type effect). Both forms of Cu also caused perturbations in ion homeostasis, including Na^+ balance and decreased branchial activity of Na^+/K^+-ATPase from exposure to

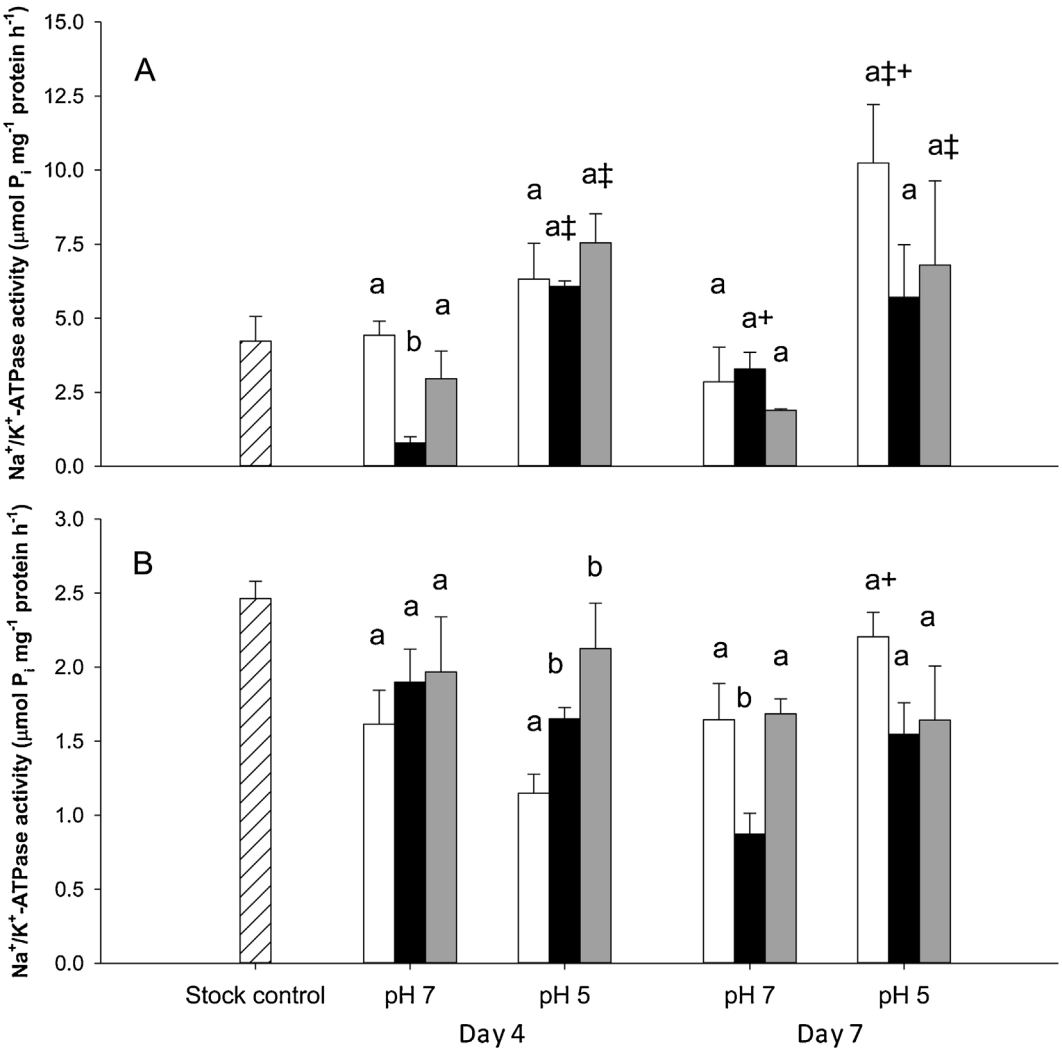

Fig. 4. Na^+/K^+-ATPase activity in crude homogenates of (A) gills and (B) kidney of trout after exposure to control (white bars), and 20 $\mu g\,l^{-1}$ of either $CuSO_4$ (black bars) or Cu-NPs (grey bars) for 4 and 7 days at pH 7 and 5. Diagonal hatched bars are initial (day 0) fish. Data are means ± S.E.M. ($n = 6$ fish per treatment). 3-way ANOVA and where appropriate one-way ANOVA with Fisher's LSD test was used to detect differences between treatments ($p < 0.05$). Different lowercase letters denote statistically significant differences between treatments within pH and time point. (‡) indicates a statistically significant difference between pH 7 and 5 within the type of Cu-treatment and time point. (+) indicates a statistically significant difference between day 4 and 7 within the type of Cu-treatment and pH.

$CuSO_4$. In $CuSO_4$-exposed fish, these effects were more pronounced at pH 7 than pH 5, an effect explained by the decreased bioavailability of dissolved Cu at the gill under acidic conditions (at day 4). However, gill Cu accumulation was not significantly lower at pH 5 compared to pH 7 in fish exposed to Cu-NPs. In acidic water, Cu-NPs underwent greater dissolution which may have offset the reduced bioavailability of dissolved Cu. Together, these data indicate that $CuSO_4$ was more toxic to trout than Cu-NPs and the toxicity of the Cu-NPs was mediated by the release of the Cu ion; however, the physico-chemical behaviour of Cu-NPs under variable environmental conditions is an important consideration in risk assessment in freshwaters.

4.1. Confirming copper exposure and pH effects on Cu-NPs

Exposure to $CuSO_4$ and Cu-NPs was confirmed by ICP-OES analysis of water samples for total Cu concentrations, and the measured concentrations were within 3% of the nominal concentrations. Similar to our previous findings with $CuSO_4$ and Cu-NPs (Shaw et al., 2012), the measured total Cu concentrations in the water also suggested that a 12 h water change was sufficient to maintain the exposure, and would meet likely regulatory test requirements for NPs (*e.g.* approximately 80% or greater of the nominal exposure concentration, Handy et al., 2012). Waterborne exposure to both forms of Cu at pH 7 and 5 was also confirmed by increases observed in the total Cu concentrations in/on the gills by ICP-OES (Fig. 3). Irrespective of pH, there was also a material-type effect with higher total Cu concentrations in the gills of fish exposed to $CuSO_4$ rather than an equal concentration of Cu-NPs. This finding is consistent with our previous experiment on trout where higher total Cu concentrations were observed in the gills of fish exposed to $CuSO_4$ than Cu-NPs after 10 days (Shaw et al., 2012). At neutral pH at least, Shaw et al. (2012) argued in favour of a greater bioavailability of dissolved Cu from $CuSO_4$, and likely limited dissolution of soluble Cu species from Cu-NPs at the gill surface. However, in the present study, exposures were also conducted at pH 5. For the $CuSO_4$ treatment, the gills showed less Cu accumulation at pH 5, compared to the exposure at pH 7. This observation is consistent with the biotic ligand model, where H^+ ions with their high ionic mobility in solution compared to all other cations (Handy and Eddy, 1991; Playle and Dixon, 1993), and greater concentration in the present study (10 $\mu mol\,l^{-1}$ H^+ compared to about 0.3 $\mu mol\,l^{-1}$ of total Cu in the water), should out-compete Cu for binding sites on the gill.

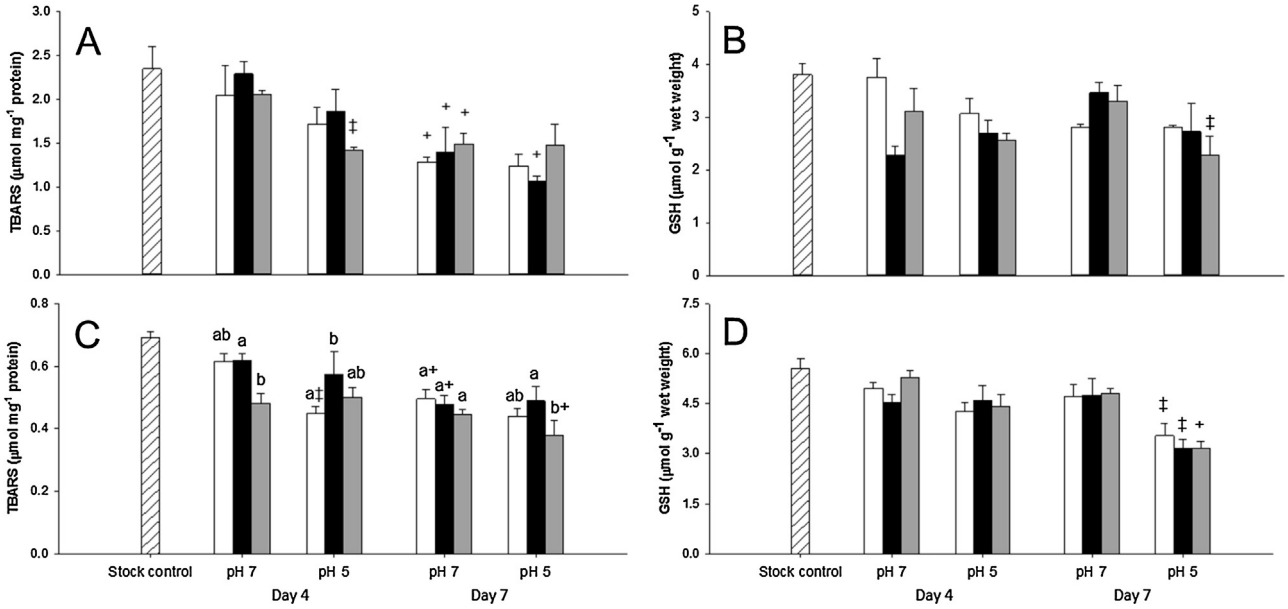

Fig. 5. Thiobarbituric acid reactive substances (TBARS) (left panel) and total glutathione content (right panel) in crude homogenates from gill (A and B) and liver (C and D) after exposure to control (white bars), 20 μg l^{-1} of Cu as either $CuSO_4$ (black bars) or Cu-NPs (grey bars) for 4 and 7 days at pH 7 and 5. Diagonal hatched bars are initial (day 0) fish. Data are means ± S.E.M. (n = 6 fish per treatment). 3-way ANOVA and where appropriate one-way ANOVA with Fisher's LSD test was used to detect differences between treatments ($p < 0.05$). Different lowercase letters denote a statistically significant difference between treatment within time point and pH. (‡) indicates a statistically significant difference between pH 7 and 5 within the type of Cu-treatment and time point. (+) indicates a statistically significant difference between day 4 and 7 within the type of Cu-treatment and pH.

In contrast to $CuSO_4$, there was no pH-effect on the accumulation of Cu in the gill from Cu-NP exposures, despite the fact that the physico-chemistry and dialysis experiments indicated a pH-effect on the particles themselves. In the stock dispersions, the primary particle diameter decreased from about 60 nm at pH 7 to 54 nm at pH 5, suggesting that the Cu-NPs had partially dissolved in the acid. This was also confirmed by dialysis experiments which showed a greater increase in the total Cu concentration in the external part of the beaker (Fig. 2), which could be interpreted as increased dissolution of Cu from Cu-NPs at low pH. The mean hydrodynamic diameter of aggregates of particles was also smaller, and with a reduced particle number concentration at pH 5 compared to pH 7 in the stock dispersions (Fig. 1), also supporting the notion of dissolution. However, any apparent dissolution should increase the dissolved Cu concentration in the water to promote Cu accumulation in the gill at low pH, but this was not observed with Cu-NP exposure (Fig. 3). This phenomenon requires further investigation, but it possible that the high H^+ concentration at low pH simply out competes any Cu ions released from the particles (*i.e.* the BLM for the dissolved metal fraction is more important than particle chemistry for metal bioavailability). Unfortunately, it was not possible to measure particle size distributions directly from the aquarium water because the exposure concentrations were far below the useful detection limit of NTA, or any other method for measuring particle size distributions (see Handy et al., 2012). It is also possible that the low pH in the aquarium water increased the polyanionic charge of macromolecules such as humic acids (Plymouth water typically has around 1 mg l^{-1} of total dissolved organic carbon). This would provide a soft colloid sink (Town et al., 2012) for chelating any Cu ions released from the Cu-NPs, resulting in the observed no-effect of pH on metal accumulation at the gill.

4.2. Copper accumulation by the internal organs at pH 7 and pH 5

The body distribution and internal target organs for traditional dissolved forms of Cu are reasonably understood for studies with radio-labelled ^{64}Cu. For teleost fishes in freshwater, Cu is absorbed across the gill and transferred to the liver as the central compartment for Cu homeostasis (Grosell et al., 1997, 1998a,b). The liver regulates the biliary excretion of Cu and therefore only limited amounts of Cu are passed on to other internal organs (*e.g.* Kamunde and Wood, 2004). In our previous study, Shaw et al. (2012) showed that exposure to 20 μg l^{-1} of Cu as $CuSO_4$ for 10 days caused measurable increases in the total Cu concentration in the liver of rainbow trout, but with no detectable increases in the muscle, brain or spleen. Similar observations are made in the present study at the same Cu concentration at pH 7 with the liver being the only internal organ to show an elevated tissue Cu concentration by the end of the experiment (Fig. 3). There was a pH-effect, in that the fish exposed at pH 5 did not show an elevated hepatic Cu concentration (consistent with less accumulation by the gill).

For the Cu-NP treatment, no measurable increases in total Cu in the internal organs were observed, apart from a transient increase in the total Cu concentration in the liver at day 4 at pH 7, which was also found in the equivalent $CuSO_4$ treatment (Fig. 3). The absence of measurable increases of total Cu concentrations in the internal organs following Cu-NP exposure is also consistent with our previous reports at neutral water pH (Shaw et al., 2012). This may also indicate that the measurable increases in Cu gill burdens may, at least partially, be from the superficial binding of Cu-NPs to the branchial epithelium (*i.e.* not intracellular Cu) and/or mucus; exposure to low pH water (~pH 5) causes mucus discharges from the gills of salmonids (Ledy et al., 2003). Since there was no observable Cu accumulation from the Cu-NP exposure in the internal organs at pH 5, and only transient changes in the liver at pH 7, a pH-effect cannot be reliably discerned.

4.3. Haematology and ionoregulatory disturbances during waterborne exposure

The effects of acid water on the osmoregulation and haematology of trout is well-known. The acute effects include inhibition of active Na^+ and Cl^- influx at the gill, and a general passive leak of electrolytes across the branchial epithelium caused by the disrup-

tion of paracellular tight junctions (McDonald, 1983; Wood, 1989; Wood et al., 1998). This may result in some haemodynamic changes including the swelling of red blood cells (Weaver et al., 1999). In the present study, fish at pH 5 showed lower plasma Na^+ and Cl^- concentrations and some dilution of red blood cells (Table 1), consistent with sub-lethal effects of acid on fishes.

Copper is well known to interfere with sodium balance. Exposure to $CuSO_4$ at neutral pH causes osmoregulatory dysfunction at the gills, with inhibition of the branchial Na^+/K^+-ATPase leading to plasma sodium depletion, and subsequent changes in haematology in trout (Laurén and McDonald, 1985b; Shaw et al., 2012). In the present study, and as expected, inhibition of Na^+/K^+-ATPase and depletion of plasma Na^+ were observed at pH 7 with $CuSO_4$ (Fig. 4; Table 1). Perturbations in tissue concentrations of K^+ were also observed. Elevation of gill tissue Na^+ is also consistent with observed plasma Na^+ depletion and inhibition of branchial Na^+/K^+-ATPase. A similar observation was reported in trout exposed to $CuSO_4$ (Shaw et al., 2012). Nonetheless, there are relatively few reports of the combined effects of acid/dissolved Cu on the blood of rainbow trout (but see Laurén and McDonald, 1985a; Hughes and Nemcsok, 1988). Of particular note, inhibition of Na^+/K^+-ATPase activity was not observed at pH 5 on day 4 and there was only a non-significant trend toward lower activity at day 7 and this is consistent with the lower bioavailability of Cu at pH 5 and slower rate of gill accumulation over the course of the exposure. In the current study, the effect of lowering the pH on both $CuSO_4$ and Cu-NPs exposed-fish was greater decreases in gill tissue Na^+ concentrations compared to the $CuSO_4$ and Cu-NPs treatment at pH 7; an effect not seen in control fish, at least at day 4. This means that the osmoregulatory disturbance is altered at lower pH values.

In our previous study on Cu-NPs at neutral pH, rainbow trout showed similar disturbances to the blood as fish exposed to $CuSO_4$, but with some additional transient changes in plasma Na^+ and haematocrits (Shaw et al., 2012). These effects were mostly within the normal physiological range of trout, and were interpreted as not being clinically significant. The present study is consistent with these findings, with some relative changes in plasma ion concentrations and haematocrits occurring later during the exposure in fish exposed to Cu-NPs compared to $CuSO_4$ at pH 7. Increased haematocrits have also been previously reported in both European perch (*Perca fluviatilis*; Rask and Virtanen, 1986) and banded sunfish (*Enneacanthus obesus*; Gonzalez and Dunson, 1987) at low pH and have been suggested to be in response to the decreased oxygen binding-affinity of haemoglobin at acid pH (the Bohr effect).

Some observations were also made on white blood cell counts. Copper is known to alter immune parameters in the blood of fish (Dethloff and Bailey, 1998) and modulate the immune system (Handy, 2003). Both exposure to acid or $CuSO_4$ alone increased the total white cell count (Table 1), consistent with an immune-stimulation or inflammation effect. At low pH, the effect of $CuSO_4$ on the white blood cell count was much greater, doubling the number of cells (Table 1). This implies the effects of acid and $CuSO_4$ are additive. A similar effect was noted with combination of acid/Cu-NPs, even though lower Cu accumulation was noted in the Cu-NP treatment. This phenomenon requires further investigation, but may be related to inflammation associated with gill injury from $CuSO_4$ and Cu-NPs (Al-Bairuty et al., 2013).

4.4. Oxidative stress during waterborne exposure

Oxidative stress is a well-known result of copper exposure (Hoyle et al., 2007; Eyckmans et al., 2011), and has also been reported for acid stress in fish toxicity (*e.g.* Mai et al., 2010). In the current study, there were mostly no changes in the gill and other internal organs, but some effects on the liver, likely relating to the function of this organ (Fig. 5). Exposure of fish to $CuSO_4$ at pH 5 caused an increase in TBARS levels in the liver, although the glutathione content of the liver was conserved (Fig. 5) and values were similar to controls under other conditions in the experiment (*i.e.* at pH 7). At acidic pH, the liver showed no measurable Cu accumulation from $CuSO_4$ treatment (Fig. 3), but a systemic hypoxia arising from gill injury due to Cu (Mustafa et al., 2012) or acid exposure could generate secondary ROS leading to oxidative stress in the liver. Similar disparate effects were also apparent in fish exposed to Cu-NPs.

5. Conclusions

The current study provides one of the first detailed effects of water pH on the sub-lethal toxicity of Cu-NPs in rainbow trout. Overall, our findings demonstrate that the accumulation of Cu and associated sub-lethal responses in trout were affected by lowering the water pH from 7 to 5. It has also indicated that the effects of Cu-NPs are less or similar to those known for Cu metal salts at both pH values. For example, some physiological results (Cu accumulation in the gill, haematology and plasma electrolytes) showed that the effect of $CuSO_4$ was greater than Cu-NP treatments at both pH values. This arose from the greater bioavailability of Cu from $CuSO_4$ compared to Cu-NP at both pH values. Nevertheless, the demonstration that acidic water caused increased metal ion dissolution from Cu-NPs has important implications for our understanding of the fate of Cu-NPs under variable environmental conditions. Salmonids inhabit a range of freshwater environments including acidic and low ionic strength waters that are often typical of upland streams where spawning occur. Understanding how water chemistry affects NP toxicity is therefore necessary for a robust risk assessment approach to nanomaterials in the environment.

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