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Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

It is unclear whether copper nanoparticles are more toxic than traditional forms of dissolved copper. This study aimed to describe the pathologies in gill, gut, liver, kidney, brain and muscle of juvenile rainbow trout, *Oncorhynchus mykiss*, exposed in triplicate to either a control (no added Cu), 20 or 100 $\mu g\,l^{-1}$ of either dissolved Cu (as $CuSO_4$) or Cu-NPs (mean primary particle size of 87 ± 27 nm) in a semi-static waterborne exposure regime. Fish were sampled at days 0, 4, and 10 for histology. All treatments caused organ injuries, and the kinds of pathologies observed with Cu-NPs were broadly of the same type as $CuSO_4$ including: hyperplasia, aneurisms, and necrosis in the secondary lamellae of the gills; swelling of goblet cells, necrosis in the mucosa layer and vacuole formation in the gut; hepatitis-like injury and cells with pyknotic nuclei in the liver; damage to the epithelium of some renal tubules and increased Bowman's space in the kidney. In the brain, some mild changes were observed in the nerve cell bodies in the telencephalon, alteration in the thickness of the mesencephalon layers, and enlargement of blood vessel on the ventral surface of the cerebellum. Changes in the proportional area of muscle fibres were observed in skeletal muscle. Overall the data showed that pathology from $CuSO_4$ and Cu-NPs were of similar types, but there were some material-type effects in the severity or incidence of injuries with Cu-NPs causing more injury in the intestine, liver and brain than the equivalent concentration of $CuSO_4$ by the end of the experiment, but in the gill and muscle $CuSO_4$ caused more pathology.

1. Introduction

The pathological effects of waterborne copper, and target organs for Cu toxicity are reasonably well known in fish (Wilson and Taylor, 1993; Handy, 2003; Grosell et al., 2007; Mustafa et al., 2012). The gill is considered the main route for waterborne Cu uptake, and injuries include oedema, epithelial lifting and fusion of the lamellae during acute toxicity (Sola et al., 1995), and changes in the proportions of epithelial cells and mucocytes are noted in chronic exposure (Dang et al., 1999). For dietary Cu exposure, the gill does not show acute pathology, but instead, changes can occur in the intestinal mucosa including intestinal cell proliferation and apoptosis (Berntssen et al., 1999). The liver is the central compartment for handling Cu in teleost fish (Grosell et al., 1996), and Cu is excreted in the bile. However, waterborne (Baker, 1969; Figueiredo-Fernandes et al., 2007) or dietary (Handy et al., 1999) exposure to Cu can produce liver pathology in fishes. There are concerns that Cu may be immunotoxic, although studies on the haematopoietic system of trout with Cu are limited, melanomacrophage aggregates are noted in trout kidney during Cu exposure (Handy, 2003).

Copper is also known to effect excitable tissue such as nerve and muscle. Additions of Cu alter the electrical properties of epithelia (e.g., Na channels in frog skin, Flonta et al., 1998), and while excess Cu in the CNS results in brain pathology in mammals (e.g., Wilsons disease, Menkes, 1999), and vacuole formation in the brain of trout has been observed from dietary Cu exposure (Handy, 2003). Fish do show altered behaviour and locomotor activity during Cu exposure (Handy et al., 1999; Campbell et al., 2002), but the relative contributions of CNS and skeletal muscle pathology to such events is less clear.

Relatively recently, a new form of Cu metal has been engineered comprising of Cu nanoparticles (Cu-NPs), which are one type of metal-containing nanomaterials (review on "nano metals", Shaw and Handy, 2011). However, despite an emerging body of literature on the ecotoxicity of nanomaterials (Moore, 2006; Handy et al., 2008; Klaine et al., 2008; Kahru and Savolainen, 2010; Handy et al., 2011), less attention has been given to organ pathologies in aquatic species. Some evidence is emerging on Cu-NPs in fish and waterborne exposure to high concentrations (1.5 $mg\,l^{-1}$ Cu-NPs for 48 h) caused oedema in the gills of zebrafish (Griffitt et al., 2007). Other nanomaterials also cause gill pathology (TiO_2 NPs, Federici et al.,

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2007; Single-walled carbon nanotube, Smith et al., 2007; Nano-Fe, Li et al., 2009). Data on internal organ pathologies from Cu-NPs in fish are generally lacking, but other metal NPs had been reported to cause pathologies. For example, liver tissue shows fatty change with lipidosis, and abnormal nuclei in the hepatocytes from trout following waterborne exposure to TiO_2 (Federici et al., 2007), and apoptosis in liver tissue is reported in zebra fish with Ag-NPs (Choi et al., 2010). The brains of rainbow trout exposed to 0.1 mg l^{-1} TiO_2 NPs for 14 days also exhibited some necrotic cells in the cerebrum (Federici et al., 2007).

Overall, the relative hazard of pathology from nano forms of Cu compared to traditional metal salts is unknown. Therefore, the main goal of the current study was to determine the effects of dissolved Cu and Cu-NPs on the organ integrity and histology of the gill, gut, liver, kidney, brain and muscle of rainbow trout following waterborne exposure to these materials. The second objective was to compare and contrast the effects of Cu metal with Cu-NPs, to identify any nano-specific pathologies.

2. Materials and methods

2.1. Experimental design

The experimental design and water quality are based on the method used by Federici et al. (2007) for TiO_2 NPs. Briefly, juvenile rainbow trout (mean ± S.E.M., n = 210; 29.4 ± 1.0 g) were exposed, in triplicate tanks, to 20 μg l^{-1} and 100 μg l^{-1} of Cu metal either as $CuSO_4 \cdot 5H_2O$ or nano copper (Cu-NPs) for 0, 4, 10 days using a semi-static waterborne exposure regime (80% water change every 12 h with re-dosing after each change). Water samples were taken before and after each water change for pH, temperature, saturated oxygen (HACH HQ40d multi reader), total ammonia (HACH LANGE GMBH LCK kit 304 read on a HACH LANGE GmbH DR 2800 spectrophotometer) and water hardness (Ca and Mg measured by inductively coupled plasma optical emission spectroscopy (ICP-OES)). As there were no significant differences between any tanks in water quality, data were pooled and were (mean ± S.E.M., n = 240–528 samples); pH, 6.98 ± 0.004; temperature, 16.0 ± 0.01 °C; oxygen saturation, 90.9 ± 0.2%; total ammonia, 0.85 ± 0.05 mg l^{-1} (equivalent to 0.049 ± 0.002 mmol l^{-1} of total ammonia, <0.1 μmol l^{-1} as NH_3), total hardness (mg l^{-1} $CaCO_3$), 52.02 ± 1.2. Photoperiod was 12 h light:12 h dark. The electrolyte composition of the dechlorinated Plymouth tap water used was 9.89, 1.56, and 18.05 mg l^{-1} Na^+, K^+ and Ca^{2+} respectively (0.43, 0.04, and 0.45 mmol l^{-1} respectively). Background Cu levels in the water were 6.04 ± 0.27 μg l^{-1} (0.095 ± 0.004 μmol l^{-1}). Fish were not fed 24 h prior to, or during the experiment, to avoid confounding the exposure with potential food particles in the water as well as to minimise the risk of the Cu-NPs absorbing to faecal material and to help maintain water quality. The entire experiment had ethical approval under the Animals (Scientific Procedures) Act 1986 in the UK and fish were also subject to independent health checks. Fish were randomly sampled on day zero (initial stock fish), then days 4 and 10 for haematology, plasma analysis, tissue electrolytes, tissue biochemistry (presented in Shaw et al., 2012) and histology (presented here).

2.2. Stock solutions and particle characterisation

Stock solutions of $CuSO_4$ and dispersions of nano Cu were prepared as well as characterised exactly as described in detail by Shaw et al. (2012) using the same stocks. Briefly, powder form of nano Cu was obtained from Sigma–Aldrich (manufacturer's information: 99.9% purity, mean particle size of 50 nm). A fresh 50 ml stock solution of 1.0 g l^{-1} Cu-NPs was made at 6 pm daily by dispersing the NPs in ultrapure water (Millipore, 18.2 MΩ cm resistance, ion free and unbuffered) without solvents and stirring (magnetic stirrer IKA Werke RET basic C, at 300 rpm) for 4 h in a low-density polyethylene (LDPE) plastic container. This stock was then used to dose the fish at 10 pm following the evening water change and again at 10 a.m. the following morning (the stock was stirred for a further hour prior to the morning dosing event). A 10 ml subsample was then taken from the stock for analysis of total Cu by ICP-OES, and NTA, the remaining stock discarded. The measured primary particle size in the stock solution was 87 ± 27 nm (mean ± S.D., n = 50, JEOL 1200EX II transmission electron microscope), and mean aggregate size by nanoparticle tracking analysis (NTA, Nanosight LM10) was 216 ± 122 nm (mean ± S.D., n = 6) with a mode of 48 nm. Particle size distribution measurements by NTA in the tank water during the experiments were not possible due to the low concentrations and interference from other natural colloids in the water (see Handy et al., 2012 for discussion). Inductively coupled plasma optical emission spectroscopy (Varian 725 ES) analysis revealed no metal impurities (data not shown).

A 1 g l^{-1} $CuSO_4$ stock solution was prepared by dissolving 3.929 g $CuSO_4 \cdot 5H_2O$ (Sigma–Aldrich) in 1 l of ultrapure (Milli-Q) water with stirring for 30 min (magnetic stirrer IKA Werke RET basic C, at 300 rpm). Nominal dosing concentrations in the experimental tanks were achieved by adding either 0.4 or 2.0 ml of the $CuSO_4$ stock for the 20 and 100 μg l^{-1} Cu (as $CuSO_4$) treatments respectively (with subsequent redosing following each 12 h water change).

2.3. Histopathology

Histological examinations were performed as described in Smith et al. (2007) with minor modifications. Fish were examined at the start (initial fish at day 0, n = 5) and at the end of the exposure (day 10, n = 6 fish/treatment, 2 fish taken randomly from each of the triplicate tanks from each treatment). However, for ethical and veterinary reasons during the experiment, some interim observations were also made at day 4 (n = 3 fish/treatment, one from each triplicate tank). Fish were terminally anaesthetised with an overdose of buffered MS222 and tissue were collected and fixed into buffered formal saline (250 ml 40% formaldehyde, 10 g $NaH_2PO_4 \cdot 1H_2O$, 16.5 g NaH_2PO_4 (anhydrous), diluted to 2.5 l with distilled water and buffered to pH 7.2) in the following order: the second and third gill arch, the hind part of intestine, liver, a transverse section of the body just anterior to anal fin (including kidney, bone, and muscle section) and the whole brain. Tissue were processed and stained for routine wax histology, with the gills decalcified prior to wax processing (Rapid Decalcifier, CellPath Plc, UK). Routine examinations were made on sections (7 μm) with gills stained with Mallory's trichrome and gut, liver, kidney, muscle and sagittal sections of brain stained with haematoxylin and eosin (H&E). Photographs were taken using an Olympus Vanox - T microscope with Olympus digital camera (C-2020 Z). Sub-sets of slides were scored independently to check for observer bias (negligible). Slides were also processed in batches containing controls and treatments to eliminate staining artefacts.

Quantitative histological measurements were made in several tissues of organs. For the gills, the percentage of injured filaments was counted from two randomly selected primary filaments from the middle of the second gill arch, and at least 80–100 secondary lamellae were counted in each specimen. The total number of secondary lamellae with lesions, and the percentage incidence of each type of lesion were counted. For the gut epithelium, morphometrics included manual measurements of the diameter of villi, columnar cell width and columnar cell nuclei width. Similarly, dimensions of hepatocytes in the liver, nerve cells in the brain, and epithelial cells in the kidney tubules were also measured. At least ten cells on each

Table 1
Copper concentrations in the tissue of rainbow trout exposed to control (no added Cu), 20 μg l^{-1} Cu as $CuSO_4$, or 20 or 100 μg l^{-1} Cu as Cu-NPs at day 10 of the exposure.

Tissue	Treatment			
	Control	20 μg l^{-1} Cu (as $CuSO_4$)	20 μg l^{-1} Cu-NPs	100 μg l^{-1} Cu-NPs
Gills	0.03 ± 0.01^a	0.17 ± 0.02^b	0.091 ± 0.03^c	0.08 ± 0.01^c
Liver	1.79 ± 0.42^a	3.15 ± 0.63^b	1.52 ± 0.32^a	1.95 ± 0.31^a
Intestine	0.13 ± 0.04^a	0.05 ± 0.01^a	0.10 ± 0.03^a	0.69 ± 0.21^b
Spleen	0.07 ± 0.01^a	0.05 ± 0.01^a	0.13 ± 0.07^a	0.06 ± 0.01^a
Brain	0.13 ± 0.02^a	0.09 ± 0.01^a	0.10 ± 0.01^a	0.10 ± 0.01^a
Muscle	0.03 ± 0.01^a	0.14 ± 0.12^a	0.02 ± 0.01^a	0.03 ± 0.01^a

Data are mean ± S.E.M., μmol Cu g^{-1} dry weight tissue, $n = 6$ fish/treatment. Different letters denote a statistically significant difference between treatments at day 10, with identical letters indicating no significant difference (ANOVA or Kruskal–Wallis, $P < 0.05$). Note there were no 100 μg l^{-1} Cu (as $CuSO_4$) exposed fish present at day 10.

image per specimen were measured. In some organs the dimension of the tissues were also measured manually, in triplicate from three random images from each fish, including the total length and thickness of each layer in mesencephalon and cerebellum regions of the brain and the diameter of renal corpuscles (glomerulus and Bowman's capsule). Fractional areas of tissues in organs were counted manually from randomly selected area on a section from each fish (image) using the point counting method of Weibel et al. (1966). Where the fractional volume (area) $V_i = P_i/P_T$; and P_i is the number of points counted, P_T is the total number of points on the counting grid. This method was used to calculate the proportions of hepatocytes area and sinusoid space in the liver; the proportion of renal corpuscles, renal tubule and haematopoeitic tissue in the kidney; the proportion of nerve cell in the telencephalon brain area and the proportion of muscle fibres and space among the muscle fibres.

2.4. Statistical analysis

Data were analysed by using StatGraphic Plus version 5.1, one way analysis of variance (ANOVA) was used to identify treatment-effects at the end of the experiment (day 10), and where possible at day 4. The least squares difference (LSD) post hoc test was used to identify differences between treatment, or time-effects where appropriate. Bartlett's test was used to check the validity of each ANOVA. In addition, 2-way ANOVA was used to check for treatment × time effects in the data. The Student's *t*-test was sometimes used to investigate the differences between pairs of data, where appropriate. For non-parametric data, the Kruskal–Wallis test was used for data that could not be transformed. Results are presented as mean ± S.E.M. All statistical analysis used the default 5% rejection level.

3. Results

Waterborne copper exposure was confirmed by ICP-OES analysis of water samples following the dosing of the experimental tanks. Copper concentrations were (mean ± S.E.M., $n = 12$ water samples per treatment) 3.01 ± 0.02, 22.3 ± 1.7, 102.3 ± 5.7, 19.7 ± 2.8, and 100.8 ± 6.9 μg l^{-1} (for the control, 20 and 100 μg l^{-1} Cu as $CuSO_4$ and Cu-NP treatments respectively), representing recoveries of 111.5, 102.3, 98.5, and 100.8% of the nominal concentrations respectively. Waterborne exposure to both dissolved Cu and Cu-NPs caused some mortality in the experiment with the former being far more toxic. Full details are reported elsewhere (Shaw et al., 2012). Briefly, exposure to 100 μg l^{-1} dissolved Cu caused 80% mortality in 96 h and for ethical reasons this treatment was stopped at day 4. The other treatments continued for 10 days with cumulative total mortalities of 4.8, 16.7, 7.1, and 19.0% for control, 20 μg l^{-1} Cu as $CuSO_4$ and 20 and 100 μg l^{-1} Cu as Cu-NPs respectively. Significant accumulation of Cu was observed on/in the gills of all the Cu treatments, with both Cu-NP groups accumulating less Cu than the $CuSO_4$-exposed fish by the end of the experiment (Table 1). There was no statistical difference in branchial Cu accumulation between the high and low Cu-NP concentrations (Table 1). Elevated levels of Cu were observed in the intestine of fish exposed to the high concentration of Cu-NPs only. At the end of exposure to 20 μg l^{-1} $CuSO_4$, the concentration of Cu increased in the liver, but not in any of the other internal organs compared to controls. Similarly, there were no statistically significant increases of Cu in any of the internal organs from either Cu-NP treatment compared to controls at the end of the experiment (Table 1).

3.1. Histological observation on the gill

Gill morphology of trout was normal in all the unexposed control animals (Fig. 1). Exposure to waterborne copper sulphate caused gill injury. At day 4, trout from the 100 μg l^{-1} $CuSO_4$-treatment showed signs of acute Cu toxicity (laboured respiration, disequilibrium), and, upon histological examination, the gills showed areas of hyperplasia at the base of the secondary lamellae, oedema of the gill epithelium, lamellar fusion, clubbed tips, the occasional aneurism in the secondary lamellae, and swollen mucocytes (Fig. 1). Exposure to the 100 μg l^{-1} Cu-NP treatment produced similar gill pathologies to those observed with $CuSO_4$, although the extent of the injuries appeared less severe at day 4 in the fish exposed to Cu-NPs (Fig. 1). No gills were examined from the 100 μg l^{-1} Cu as $CuSO_4$ treatment at day 10 because no fish survived. After 10 days of exposure to 20 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs and 100 μg l^{-1} of Cu-NPs, the lesions were of similar types, but generally worse than the same treatment at day 4 (Fig. 1). Typically, each lesion was observed in at least 4/6 of the fish examined per treatment, with all Cu-exposed fish having more than one type of lesion.

A quantitative analysis of the gill injuries confirmed some material-type effects in the incidence of some lesions. For example, at day 10, the proportion of gill lamellae showing fusion increased with 20 μg l^{-1} of either Cu as $CuSO_4$ or Cu as Cu-NPs compared to controls and were all statistically significant from each other (ANOVA, $P < 0.05$). Values were (mean percentage ± S.E.M., $n = 6$ fish); not observed (control), 7.1 ± 0.5 (Cu as $CuSO_4$), and 2.7 ± 0.4% (Cu as Cu-NPs). There was also a material-type effect for the incidence of swollen mucocytes after 10 days for the 20 μg l^{-1} of $CuSO_4$ and Cu-NP treatments (Student's *t*-test, $P < 0.05$). Values were (mean percentage ± S.E.M., $n = 6$ fish) 10.2 ± 1.3 ($CuSO_4$) and 14.2 ± 1.9% (Cu-NPs) for swollen mucocytes.

3.2. Histological observation on the gut

The gut of fish from the fresh water control showed normal histology (Fig. 2). Exposure to $CuSO_4$ caused mostly minor injuries to the gut mucosa. At day 4, trout from the 100 μg l^{-1} Cu as $CuSO_4$ or Cu-NPs showed the occasional areas of oedema, some swelling of goblet cells, the occasional villus tip with some

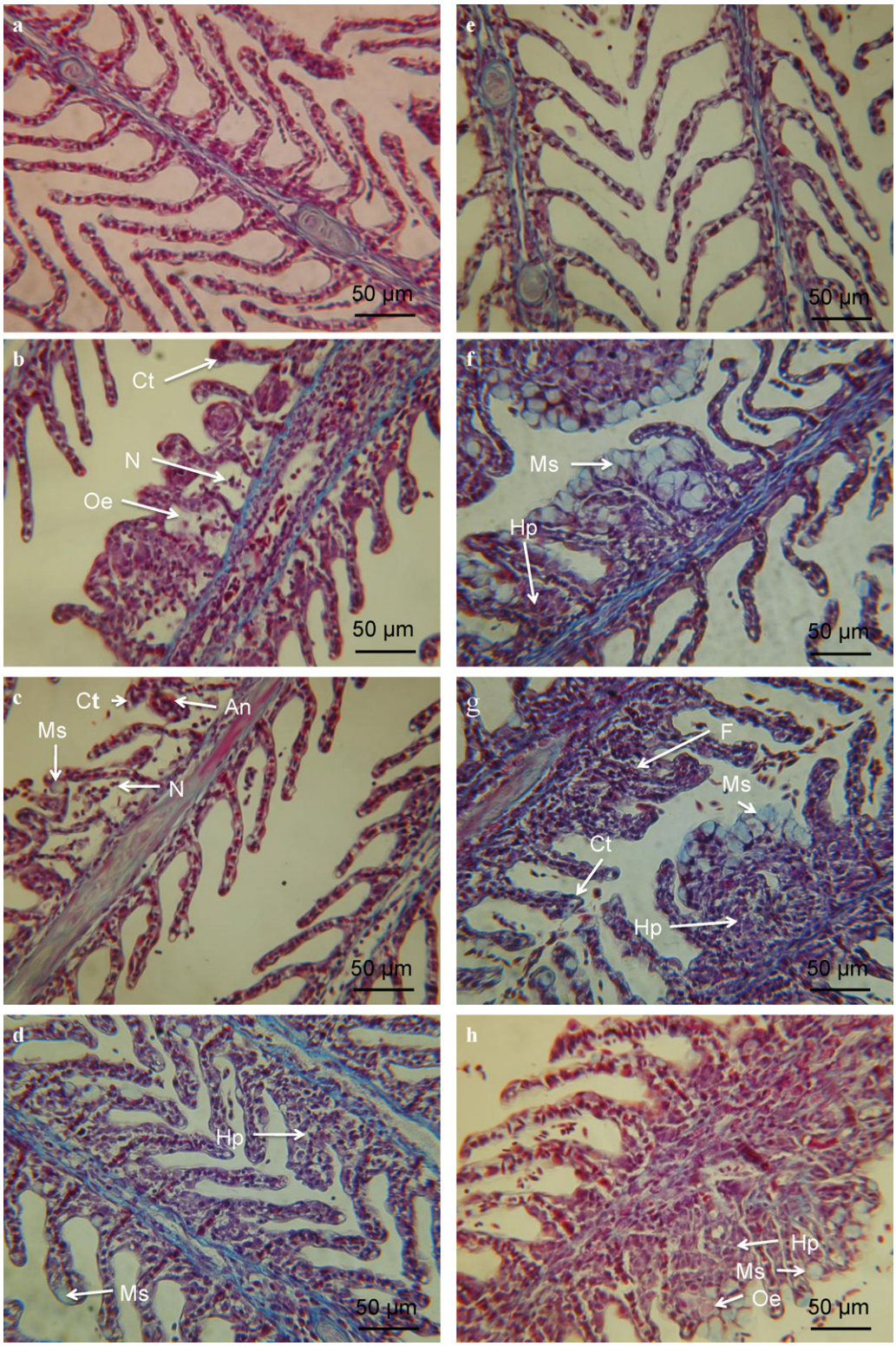

Fig. 1. Gill morphology in rainbow trout following waterborne exposure to $CuSO_4$ or Cu-NPs for 4 days (left column) and 10 days (right column). For day 4 the panels include (a) control, (b) 100 $\mu g\,l^{-1}$ Cu as $CuSO_4$, (c and d) 100 $\mu g\,l^{-1}$ Cu as Cu-NPs. For day 10 the panels include (e) control, (f) 20 $\mu g\,l^{-1}$ Cu as $CuSO_4$ (g) 20 $\mu g\,l^{-1}$ Cu as Cu-NPs and (h) 100 $\mu g\,l^{-1}$ Cu as Cu-NPs. The gills of control fish showed normal histology, whilst all treatments showed injuries that include oedema (Oe), necrosis (N), clubbed tips (Ct), aneurism (An), mucocytes swollen (Ms), hyperplasia (Hp) and fusion (F). These injuries were greater with $CuSO_4$ than Cu-NPs at day 4 but the situation was reversed by day 10 with some types of lesion (see text for details). Scale bar indicates magnification; sections were 7 µm thick and stained with Mallory's trichrome. Note the termination of the 100 $\mu g\,l^{-1}$ $CuSO_4$ treatment after 4 days for ethical reasons.

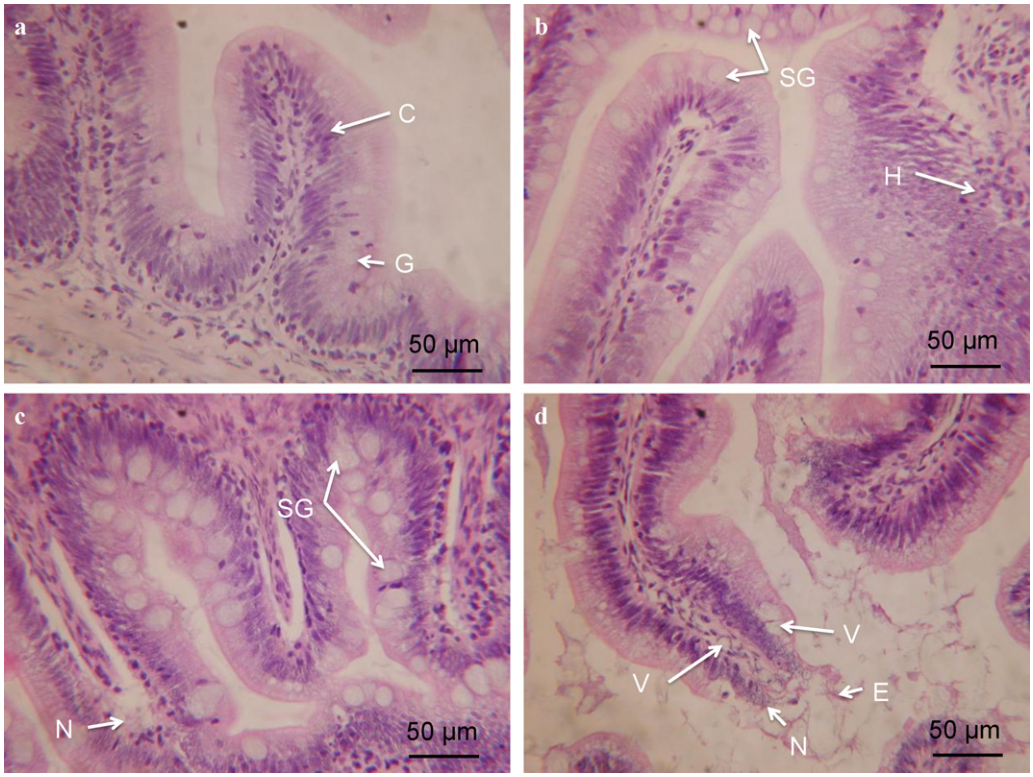

Fig. 2. Gut morphology in rainbow trout following waterborne exposure to (a) control, (b) 20 μg l^{-1} Cu as $CuSO_4$, (c) 20 μg l^{-1} Cu as Cu-NPs, and (d) 100 μg l^{-1} Cu as Cu-NPs for 10 days. The gut of control fish showed normal histology with columnar cells (C) and goblet cells (G). At day 10, all Cu treatments showed similar type of injuries, but those from Cu-NPs were worse than $CuSO_4$. These injuries include the appearance of vacuoles in the lamina propria (V), hyperplasia (H), necrosis in the mucosal layer (N), swelling of goblet cells (SG), and erosion of villi (E). Scale bar indicates magnification; sections were 7 μm thick and stained with haematoxylin and eosin (H&E). Note the termination of the 100 μg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

erosion of the surface, and the tips of a few villi showing vacuole formation and/or evidence of a few necrotic cells (in all three fish examined/treatment, data not shown). At day 10, fish exposed to 20 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs exhibited similar types of intestinal changes to those observed above in 4/6 fish examined from each treatment, with the injuries appearing slightly worse in the equivalent Cu-NP treatment (Fig. 2). There was also a concentration-effect within the Cu-NP treatment at 10 days with the 100 μg l^{-1} of Cu as Cu-NPs causing more frequent erosion of the villus tips, numerous swollen goblet cells, more vacuole formation in the epithelium, and more necrotic cells (observed in all 6 fish examined) compared to the lower NP concentration.

A quantitative analysis of gut mucosa dimensions confirmed a small material-type effect for only the highest Cu concentrations at the end of the experiment. After 10 days of exposure, the width of intestinal villi was statistically significant increased for all Cu-treatments compared to controls but not between the 20 μg l^{-1} Cu as $CuSO_4$ or Cu-NP treatments (ANOVAs, $P < 0.05$). Values were (μm, mean ± S.E.M., $n = 6$); control, 118.7 ± 0.9; 20 μg l^{-1} Cu as $CuSO_4$, 149.4 ± 1.9; 20 μg l^{-1} Cu as Cu-NPs, 150.0 ± 0.9; 100 μg l^{-1} Cu as Cu-NPs, 153.9 ± 2.0. The thickness of the intestinal mucosa was also measured. Only exposure to 100 μg l^{-1} Cu as Cu-NPs for 10 days showed an increase in the thickness of mucosa layer (μm, mean ± S.E.M., $n = 6$; control, 66.7 ± 1.5; treated, 74.7 ± 0.9) compared to the unexposed control (ANOVA, $P < 0.05$).

3.3. Histological observation on the liver

The livers of control fish showed normal histology (Fig. 3). At day 4, fish exposed to 100 μg l^{-1} Cu as $CuSO_4$ or Cu-NPs showed similar types of lesion in all three fish examined per treatment, including the occasional cell with pyknotic nuclei or cytoplasmic vacuoles indicative of the early stages of necrosis in a few cells, small foci of hepatitis-like cell injury were observed as well as increase the number of melanomacrophage deposits in all three fish examined/treatment compared to controls (data not shown). After 10 days, the types of lesions in the Cu-exposed fish were similar to those observed in their respective treatments at day 4; although in addition the occasional separation of the endothelium from the walls of blood vessels was observed and changes in the sinusoid space was now also evident (see measurements below). One or more of these lesions were observed in 5/6 fish examined in each of the $CuSO_4$ or Cu-NPs treatment (Fig. 3).

A quantitative analysis confirmed changes in liver sinusoid space (data not shown for day 4). At the end of the experiment (day 10), exposure to 20 μg l^{-1} Cu as $CuSO_4$ caused a small but statistically significant increase in the proportion of sinusoid space (% mean ± S.E.M., $n = 6$; control, 13.6 ± 0.8; Cu treated, 15.8 ± 0.5, ANOVA, $P < 0.05$), compared to the unexposed control. Exposure to 20 or 100 μg l^{-1} Cu as Cu-NPs had the opposite effect to $CuSO_4$ at day 10 with a small but statistically significant decrease in the proportion of sinusoid space (% mean ± S.E.M., $n = 6$; 20 μg l^{-1} of Cu as Cu-NPs, 9.3 ± 0.3; 100 μg l^{-1} of Cu as Cu-NPs, 9.5 ± 0.6), compared to the unexposed controls (ANOVA, $P < 0.05$). The material-type effect was statistically significant for the changes in sinusoid space. In the nano treatments the increase in hepatic area (decrease in sinusoid space) was accompanied by a fall in the ratio of the nucleus:hepatocyte diameter (mean ± S.E.M., $n = 6$; control, 0.36 ± 0.01; 20 μg l^{-1} of Cu as Cu-NPs, 0.34 ± 0.01; 100 μg l^{-1} of Cu as Cu-NPs, 0.32 ± 0.01), indicating cellular hypertrophy as the cause of the increased hepatic area in the livers with the concomitant decrease of sinusoid space.

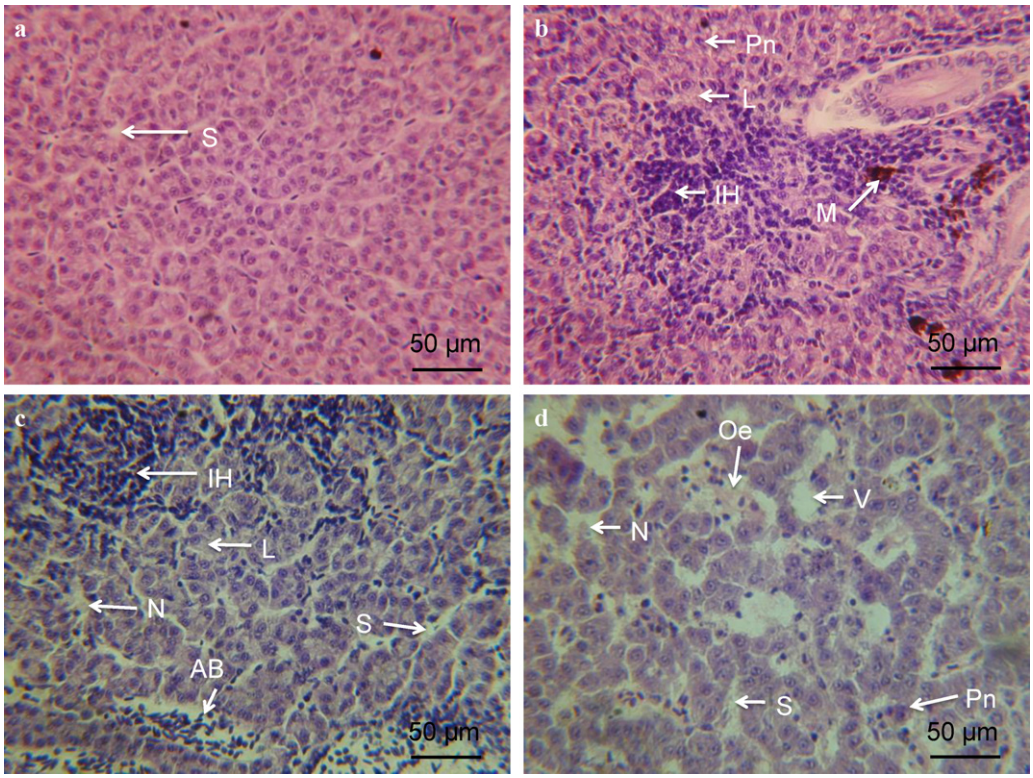

Fig. 3. Liver morphology in rainbow trout following waterborne exposure to (a) control, (b) 20 $\mu g\,l^{-1}$ Cu as $CuSO_4$, (c) 20 $\mu g\,l^{-1}$ Cu as Cu-NPs, and (d) 100 $\mu g\,l^{-1}$ Cu as Cu-NPs for 10 days. The livers of control fish showed normal histology with sinusoid space (S). Both materials caused similar types of injuries, although these were worse in the equivalent Cu-NP treatment by day 10. These injuries include cells with pyknotic nuclei (Pn), foci of hepatitis-like injury (IH), foci of melanomacrophages (M), lipidosis (L), vacuole formation (V), necrosis (N), oedema in the tissue (Oe), and aggregation of blood cell (AB). Scale bar indicates magnification; sections were 7 μm thick and stained with haematoxylin and eosin. Note the termination of the 100 $\mu g\,l^{-1}$ $CuSO_4$ treatment after 4 days for ethical reasons.

3.4. Histological observation on the kidney

The kidneys of control fish showed normal histology (Fig. 4). The incidental histology for veterinary purposes in the 100 $\mu g\,l^{-1}$ Cu as $CuSO_4$ or Cu-NPs treatment at day 4 showed occasional degeneration of renal tubules, a few necrotic cells in the haematopoietic tissue, minor elevation in the number of melanomacrophage deposits throughout the kidney, as well as some enlargement in Bowman's space (data not shown). After 10 days of exposure to 20 $\mu g\,l^{-1}$ of Cu as $CuSO_4$ or Cu-NPs and 100 $\mu g\,l^{-1}$ of Cu-NPs, fish showed the same pathologies as above in five out of six fish examined from each treatment (Fig. 4).

A quantitative analysis of the alteration observed in the kidney confirmed these observations (data not shown for day 4). For example, after 10 days there was a statistically significant increase (ANOVA, $P<0.05$) in the diameter of Bowman's corpuscle in all treatments compared to the unexposed control (μm, mean ± S.E.M., $n=6$; control, 73.8 ± 1.4; 20 $\mu g\,l^{-1}$ Cu as $CuSO_4$, 81.3 ± 1.6; 20 $\mu g\,l^{-1}$ Cu as Cu-NPs, 79.9 ± 1.7; 100 $\mu g\,l^{-1}$ Cu as Cu-NPs, 86.1 ± 0.9), with a concentration-effect within the nano treatments, but no material type effect compared to $CuSO_4$. Copper exposure, regardless of material type or concentration doubled the size of the Bowman's space (μm, mean ± S.E.M., $n=6$): control, 5.3 ± 0.7; 20 $\mu g\,l^{-1}$ Cu as $CuSO_4$, 13.7 ± 1.3; 20 $\mu g\,l^{-1}$ Cu as Cu-NPs, 13.3 ± 2.2; 100 $\mu g\,l^{-1}$ Cu as Cu-NPs, 14.6 ± 0.9, ANOVA, $P<0.05$). The number of melanomacrophage deposits in the kidney after 10 days showed a statistically significant increase in all treatments compared to controls, and also with a material type effect (ANOVA, $P<0.05$; mean ± S.E.M., $n=6$; counts/image at ×200 magnification, total area of Image 212,400 μm^2): control, 277 ± 4; 20 $\mu g\,l^{-1}$ of Cu as $CuSO_4$, 341 ± 3; 20 $\mu g\,l^{-1}$ of Cu as Cu-NPs, 366 ± 4; 100 $\mu g\,l^{-1}$ of Cu as Cu-NPs, 352 ± 2.

3.5. Histological observation on the brain

The brains of fish were removed whole, and the histology is reported here for the forebrain (telencelon), mid-brain (mesencephalon) and hind brain (metencelon or cerebellum) regions. The telencephalon of control fishes showed normal structure, but exposure to $CuSO_4$ or Cu-NPs caused similar types of pathology, and this was evident in the 100 $\mu g\,l^{-1}$ Cu as $CuSO_4$ or Cu-NPs treatment as early as day 4 with occasional necrotic nerve cell bodies, occasional cells with pyknotic nuclei and/or vacuole formation in the cell body, as well as apparently enlarged nerve cells (hydropic change) in all three fish examined (data not shown). However, by day 10, the 20 $\mu g\,l^{-1}$ Cu as $CuSO_4$ or Cu-NPs and 100 $\mu g\,l^{-1}$ Cu-NPs treatment also showed the same pathologies as above in four to six fish out of six fish examined/treatment (data not shown).

A quantitative analysis of the telencephalon at day 10 confirmed a statistically significant increase in the proportion of enlarged (hydropic change) nerve cells (% mean ± S.E.M., $n=6$): control, 3.0 ± 0.5; 20 $\mu g\,l^{-1}$ of Cu as $CuSO_4$, 9.7 ± 1.3; 20 $\mu g\,l^{-1}$ of Cu as Cu-NPs, 10.2 ± 1.4; 100 $\mu g\,l^{-1}$ of Cu as Cu-NPs, 10.6 ± 0.7), and increased incidence of necrosis (% mean ± S.E.M., $n=6$; control, 0.4 ± 0.3; 20 $\mu g\,l^{-1}$ of Cu as $CuSO_4$, 7.0 ± 1.4; 20 $\mu g\,l^{-1}$ of Cu as Cu-NPs, 10.5 ± 1.1; 100 $\mu g\,l^{-1}$ of Cu as Cu-NPs, 9.9 ± 1.3) compared to the control (ANOVA or Kruskal–Wallis, $P<0.05$), and with a small but statistically significant material type effect for the incidence of necrosis (more in the nano treatments than $CuSO_4$).

The mesencephalon region of the trout brain was normal in the control animals (Fig. 5), but after 4 days of exposure to the highest $CuSO_4$ or Cu-NPs concentration showed similar injuries including apparent enlargement of blood vessels, minor alterations in the thickness of mesencephalon layers and occasional rupture of the periventriculare layer in all three fish examined (data not

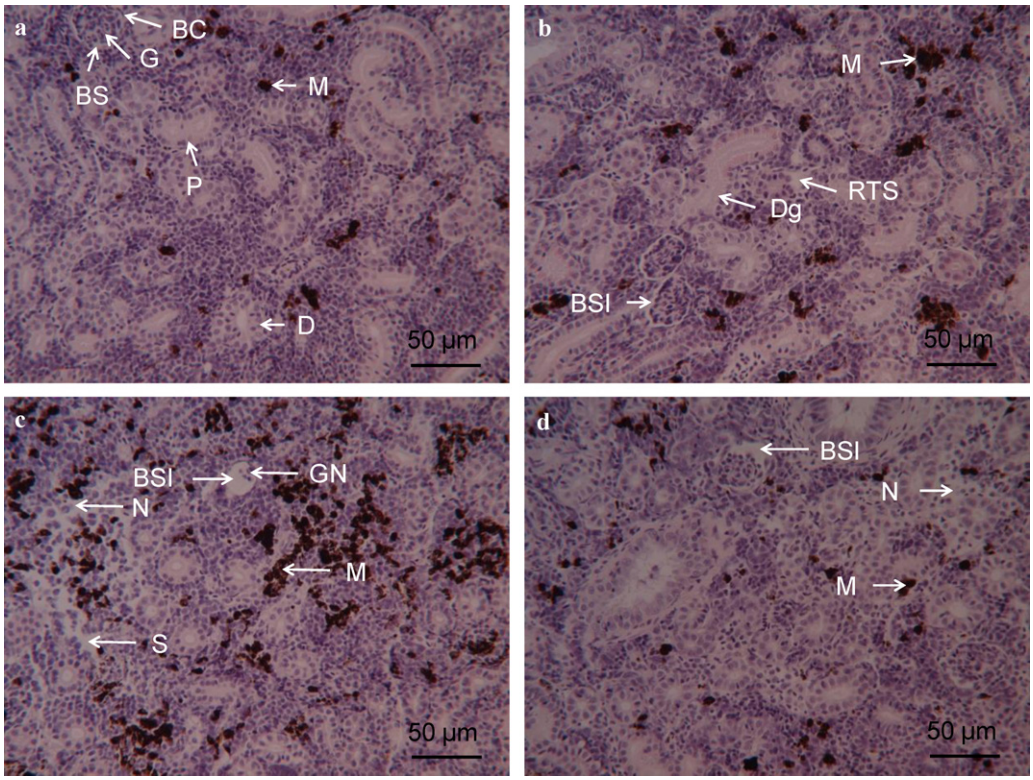

Fig. 4. Kidney morphology in rainbow trout following waterborne exposure to (a) control (b) 20 $\mu g\,l^{-1}$ of Cu as $CuSO_4$, (c) 20 $\mu g\,l^{-1}$ of Cu as Cu-NPs, and (d) 100 $\mu g\,l^{-1}$ of Cu as Cu-NPs for 10 days. Kidney of control fish showed normal histology with parietal epithelium of Bowman's capsule (BC), glomerulus (G), Bowman's space (BS), proximal tubules (P), distal tubules (D), and melanomacrophages (M). The types of pathologies were similar for $CuSO_4$ and Cu-NPs, but with more melanomacrophage centres in the latter. These injuries include degeneration of renal tubule (Dg), increased Bowman's space (BSI), melanomacrophage aggregate (MA), sinusoids were enlarged (S), renal tubular separation (RTS), necrosis of haematopoietic tissue (N), and glomerular necrosis (GN). Scale bar indicates magnification, section were 7 μm thick and stained with haematoxylin and eosin. Note the termination of the 100 $\mu g\,l^{-1}$ $CuSO_4$ treatment after 4 days for ethical reasons.

shown). After 10 days, these alterations were also evident in 5/6 fish from the 20 $\mu g\,l^{-1}$ of Cu as $CuSO_4$ or Cu-NPs and 100 $\mu g\,l^{-1}$ of Cu-NPs treatment. A quantitative analysis of the mesencephalon layers confirmed these observations (Fig. 5). Exposure to 100 $\mu g\,l^{-1}$ Cu-NPs for 10 days caused a statistically significant increase in the thickness of stratum periventriculare and a decrease in the stratum fibrosum et griseum and stratum griseum centrale compared to the unexposed control (ANOVA or Kruskal–Wallis, $P < 0.05$) (Fig. 5). There was a concentration-effect within the nano treatments with the 100 $\mu g\,l^{-1}$ Cu-NPs being worse than 20 $\mu g\,l^{-1}$ Cu-NPs (Fig. 5).

The metencephalon (cerebellum) regions of trout brain showed normal structure in the controls (Fig. 6). Exposure to 100 $\mu g\,l^{-1}$ of Cu as $CuSO_4$ or Cu-NPs for 4 days showed similar alteration that included: increases in blood vessel diameter on the ventral surface of the cerebellum and occasional necrotic cells between the molecular and granular layer of cerebellum cortex in all three

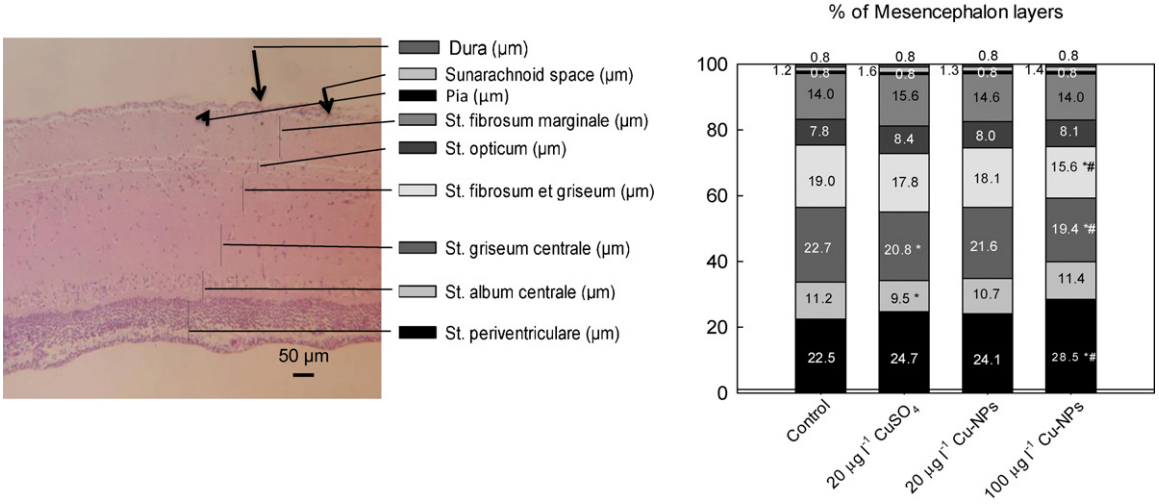

Fig. 5. The proportion of alteration in the thickness of mesencephalon tissue layers of the brain in rainbow trout following waterborne exposure to control, 20 $\mu g\,l^{-1}$ of Cu as $CuSO_4$, 20 or 100 $\mu g\,l^{-1}$ of Cu as Cu-NPs for 10 days. Data are proportion means as a % ± S.E.M., $n = 6$ fish/treatment. (*) Significant difference from control within treatment (ANOVA, $P < 0.05$). (#) Significant difference between low and high concentration of Cu-NPs (t-test, $P < 0.05$). Note the termination of the 100 $\mu g\,l^{-1}$ $CuSO_4$ treatment after 4 days for ethical reasons.

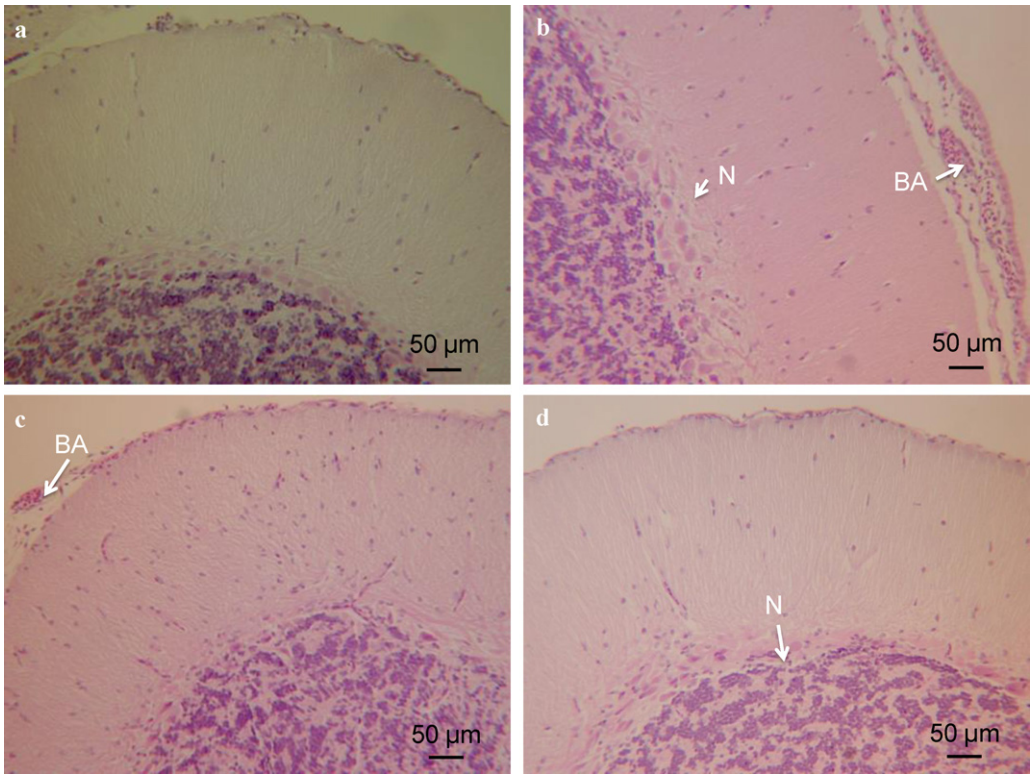

Fig. 6. Cerebellum morphology in the brain of rainbow trout following waterborne exposure to (a) control, (b) 20 μg l^{-1} of Cu as $CuSO_4$, (c) 20 μg l^{-1} of Cu as Cu-NPs, or (d) 100 μg l^{-1} of Cu as Cu-NPs for 10 days. The cerebellum of control fish showed normal histology with normal surface architecture and layers of the cerebellum. All Cu-treatments showed similar types of injuries, but the severity was worse for the equivalent Cu-NP exposure by day 10. These injuries include blood vessel abnormality on the ventral surface (BA), necrosis between the molecular and granular layers (N). Scale bar indicates magnification, section were 7 μm thick and stained with haematoxylin and eosin. Note the termination of the 100 μg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

fish treatments (data not shown). The same alterations were also evident after 10 days of exposure to 20 μg l^{-1} of Cu as $CuSO_4$ (5/6 animals), 20 μg l^{-1} of Cu as Cu-NPs (4/6 fish), 100 μg l^{-1} of Cu as Cu-NPs (4/6 fish) (Fig. 6).

3.6. Histological observation on the muscle

Histology of the trout skeletal muscle shows normal anatomy in the controls (Fig. 7). Exposure to 100 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs for 4 days showed an increase in the extracellular space and a proportional decrease in the area of the sections muscle fibre in all three fish examined (data not shown). These changes were also evident by day 10 in the 20 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs and 100 μg l^{-1} of Cu-NPs treatment (between four to five out of six animals/treatment (Fig. 7). A quantitative analysis of the skeletal muscle confirmed these observations with the proportion of muscle bundle area decreasing in all the Cu-treatments compared to the unexposed control (ANOVA, $P < 0.05$), and with a small but statistically significant material-type effect at the 20 μg l^{-1} concentrations. Values for the proportion of muscle bundle area in skeletal muscle were (%, mean ± S.E.M., $n = 6$): control, 68.6 ± 0.3; 20 μg l^{-1} Cu as $CuSO_4$, 64.8 ± 0.3; 20 μg l^{-1} Cu as Cu-NPs, 66.4 ± 0.3; 100 μg l^{-1} Cu as Cu-NPs, 65.4 ± 0.3, with the remainders being extracellular space and connective tissue.

4. Discussion

This study details the effects of dissolved Cu compared to Cu-NPs on the organ integrity of rainbow trout. Overall, the results showed that dissolved Cu and Cu-NPs cause similar types of injuries in gill, gut, liver, kidney, brain and muscle. At day 4, these injuries were greater with $CuSO_4$ than Cu-NPs, but by day 10 the pathology with Cu-NPs was comparable or worse than equivalent $CuSO_4$ treatment, suggesting a delay in the appearance of Cu-NP pathology compared to $CuSO_4$.

4.1. Pathological observation in the gill

Copper is a well-known respiratory and ionoregulatory toxicant in fish (reviews, Handy, 2003; Grosell et al., 2007) and there is some evidence that waterborne exposure to metal NPs may result in particle accumulation in or on the epithelial cells (e.g., TiO_2, Moger et al., 2008). The current study showed that $CuSO_4$ and Cu-NPs caused similar types of gill injuries (e.g., hyperplasia, oedema, lamellar fusion, clubbed tips, etc., Fig. 1), but these pathologies appeared earlier (by day 4) in the highest $CuSO_4$ concentration and with greater injuries than the equivalent concentration of Cu-NPs. The gills of $CuSO_4$-treated fish also had more Cu accumulation than the equivalent nano treatment by the end of the experiment (Table 1). Together, these data suggest that the Cu salt is more bioavailable and/or bioreactive than the nano form.

Mucus secretion is often the first line of defence to metal exposure in the gills and can temporarily protect the underlying epithelium from injury (Handy and Maunder, 2009). Mucus secretion and swollen mucocytes were observed in this study (Fig. 1), and has been noted in rainbow trout with other nanomaterials (SWCNT, Smith et al., 2007; TiO_2 NPs, Federici et al., 2007). However, in the present study mucus secretion was not sufficient to protect the gills from the pathology arising from either $CuSO_4$ or Cu-NP exposure (Fig. 1). The types of gill pathology reported here for Cu-NPs have also been reported for waterborne exposures with other nanomaterials in trout (SWCNT, Smith et al., 2007; TiO_2 NPs, Federici et al., 2007), and for TiO_2 NPs in carp (Hao et al., 2009). Oedema appears to be a common feature of the gill pathology for nano metals, and

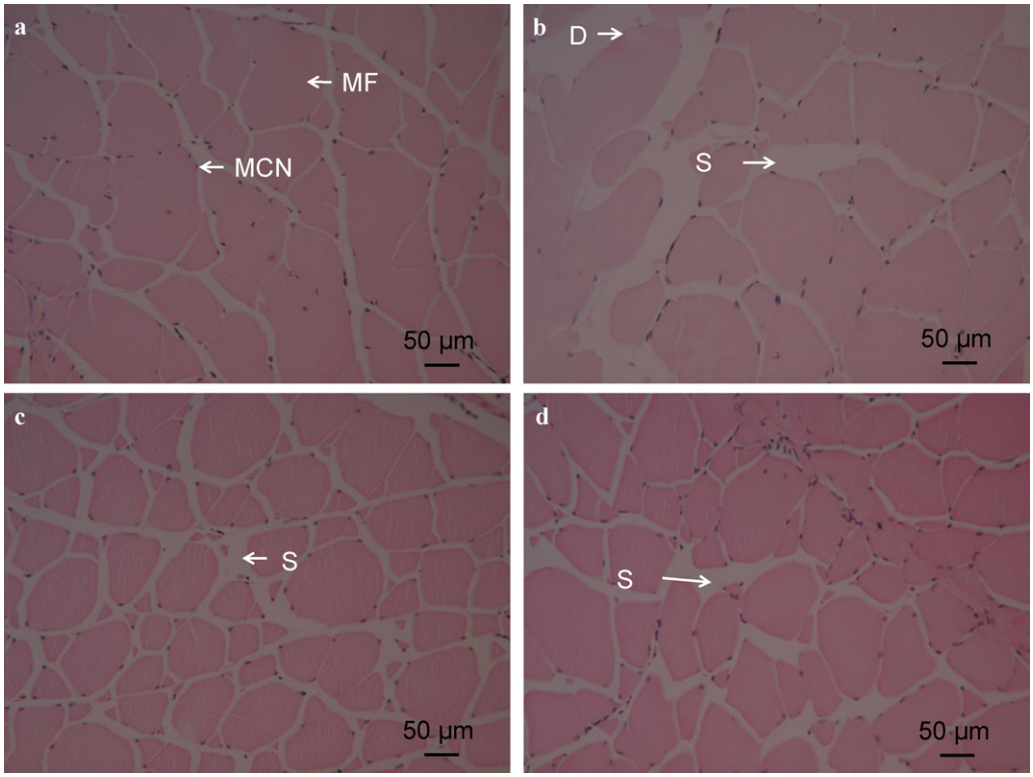

Fig. 7. Muscle morphology in rainbow trout following exposure to (a) control (b) 20 $\mu g\,l^{-1}$ of Cu as $CuSO_4$, (c) 20 $\mu g\,l^{-1}$ of Cu as Cu-NPs, and (d) 100 $\mu g\,l^{-1}$ of Cu as Cu-NPs for 10 days. The muscle of control fish showed normal architecture with the muscle cell nucleus (MCN), and muscle fibres (MF). All Cu treatments showed similar types of injuries, but with slightly more pathology in the equivalent $CuSO_4$ treatment by day 10. These alterations include increased extracellular space between muscle bundles (IS), and the occasional area of degeneration within individual muscle bundles (D). Scale bar indicates magnification, section were 7 μm thick and stained with haematoxylin and eosin. Note the termination of the 100 $\mu g\,l^{-1}$ $CuSO_4$ treatment after 4 days for ethical reasons.

for dissolved metals this is usually explained by inhibition of the branchial Na^+, K^+-ATPase which leads to solute accumulation in the epithelial cell and the osmotic influx of water. This explanation might also apply to Cu-NPs since they also inhibit branchial Na^+, K^+-ATPase (Shaw et al., 2012), although measurements of intracellular free Cu ion activity from Cu-NPs in gill cells would be needed to confirm this hypothesis.

The gill also functions in respiratory gas exchange. Waterborne exposure to $CuSO_4$ is known to cause swelling of the secondary lamellae of the trout gill, resulting in an increase in the diffusion distance across the epithelium for gas exchange and a systemic hypoxia (e.g., arterial oxygen partial pressures of <5 kPa within 24 h, Wilson and Taylor, 1993). The hyperplasia, fusion and lifting of the gill epithelium suggests that Cu-NPs, like $CuSO_4$, could also compromise oxygen uptake and contribute to a systemic hypoxia. Recently Bilberg et al. (2010) also showed that exposure to silver NPs can reduce blood pO_2 in fish. The increased incidence of aneurism in the branchial vasculature with Cu-NPs reported here would also suggest some interruptions of gill perfusion that might contribute to a systemic hypoxia. Further research on the exercise performance and swimming behaviour of fish exposed to Cu-NPs is required to determine the functional significance of the gill pathology. Nonetheless, animals with acute branchial oedema and epithelial lifting would be unlikely to survive in the wild.

4.2. Pathological observation in the gut epithelium

The gut is an important route of Cu uptake in teleost fishes (e.g., Handy et al., 1999) and the anatomy of fish gut lends itself to particle uptake as a relatively leaky epithelium (compared to the gills) which also forms absorptive vacuoles for taking up nutrients (see Clearwater et al., 2005 for an anatomical review relating to metals). In the present study, the fish were unfed and the normal gut morphology in time-matched controls at the end of the experiment suggests this short period without food was not a cause of pathology. Likely stress-induced drinking with gut pathology has been noted in trout with other nanomaterials (SWCNT, Smith et al., 2007; TiO_2 NPs, Federici et al., 2007). Notably while the types of gut pathologies for $CuSO_4$ and Cu-NPs were similar (Fig. 2), the injuries were more severe in the Cu-NP treatment by the end of the experiment; and the quantitative analysis confirmed this difference in severity. The mechanism and biological importance of the difference in magnitude of the material-type effect is less clear. The copper concentrations in the gut tissue at the end of the exposure for the 20 $\mu g\,l^{-1}$ of Cu treatments were similar for both $CuSO_4$ and Cu-NPs (Table 1), and there was no material-type effect on intestinal Na^+, K^+-ATPase or thiobarbituric acid reactive substances (TBARS) at the end of the experiment (Shaw et al., 2012); although the reduction in sodium pump activity and elevated TBARS implicated osmoregulatory disturbances and oxidative stress as mechanisms for both $CuSO_4$ and Cu-NPs (Shaw et al., 2012).

4.3. Pathological observation in the liver

The liver is a central compartment for Cu metabolism in fish and a target organ for Cu accumulation (Grosell et al., 1996; Handy et al., 1999). The types of injuries reported here for $CuSO_4$ (some patches of cellular necrosis, changes in sinusoid space, etc., see Fig. 3) are well-known (Baker, 1969; Figueiredo-Fernandes et al., 2007; Handy et al., 1999) and often explained by Cu-induced oxidative stress in the liver tissue (Hoyle et al., 2007). Exposure to Cu-NPs

produced the same types of pathologies, but the severity was different. For example in the 20 $\mu g\,l^{-1}$ treatments, Cu-NPs cause greater alteration in the proportion of hepatic area and sinusoid space than $CuSO_4$ after 10 days, but this occurred even though the livers from the Cu-NP treatment showed no statistical increase of new Cu accumulation in the tissue (Table 1). This suggests, more likely, that a secondary systemic hypoxia is contributing to changes in the liver with the Cu-NP treatments. This notion is supported by the decrease in sinusoid space in the Cu-NP treatments implying some redirection of blood flow to other internal organs. Functionally, the aspects of energy metabolism affected in the liver are likely to be similar for both materials given that the *types* of pathology are similar. For example, the decrease in hepatocyte diameter relative to nuclei diameter suggests (with both substances) that liver cells are more metabolically active, and the increased presence of melanomacrophage deposits supports this notion as they are known to be involved with recycling of endogenous materials from damaged cells (Haaparanta et al., 1996). In the absence of methods to identify Cu-NPs in the liver cells (trout livers can synthesis endogenous Cu-containing particles, Lanno et al., 1987), it remains uncertain if these melanomacophage deposits are also involved in the removal of Cu-NPs from the tissue.

4.4. Pathological observation in the kidney

There are relatively few reports of renal pathology from waterborne Cu exposure in rainbow trout (Handy, 2003), but the pathologies reported here including some damage to the epithelial cells of the renal tubules, changes in the Bowman's space, and an increase in the foci of melanomacrophage deposits (see Fig. 4). These pathologies are broadly similar to previous reports on fishes. For example in the Nile tilapia (*Oreochromis niloticus*) following 7 days of exposure to 46 $mg\,l^{-1}$ Cu, pathologies included tubular swelling, atrophy of the glomerulus, and necrosis of the renal epithelium (Kosai et al., 2009). Nano copper showed similar types of renal pathology as $CuSO_4$ (Fig. 4). At least one study in mice has also shown damage to renal proximal tubules, swollen glomeruli accompanied by loss of extracellular space in the Bowman's capsule after 72 h of oral gavage of 108–1080 $mg\,kg^{-1}$ Cu-NPs (Chen et al., 2006); and while some details may be different, these pathologies are broadly consistent with the findings here in trout. Unfortunately, partly due to the small size of the fish in the present study, it was not possible to determine total Cu in the urine or kidney tissue. It is therefore uncertain if the pathologies from the $CuSO_4$ or Cu-NPs are caused by direct target organ toxicity from internalised metal in the renal tissue, or an indirect effect.

In freshwater-adapted teleost fishes, the osmoregulatory strategy is to produce a large volume of dilute urine to compensate for the osmotic influx of water across the body surfaces. Renal filtration rate is therefore critical to survival. The almost doubling in the size of the Bowman's space with either $CuSO_4$ or Cu-NPs implies that water is being drawn osmotically into the space, presumably from glomerular filtration, but is not likely to be moving along the obstructed tubule lumens (damaged epithelial cells) and it seems likely that urine flow would be compromised. This is consistent with a dilution of plasma electrolytes in the same fish (Shaw et al., 2012). However, at least one study has shown the maintenance of creatinine clearance in trout exposed to mg levels of TiO_2 NPs (Scown et al., 2009), implying that glomerular filtration rate (GFR) can be maintained in some exposures with less toxic NPs. The only notable material-type effect of Cu-NPs on renal pathology was an increase in the number of melanomacrophage deposits compared to the $CuSO_4$ treatment and unexposed controls. This is largely a haematopoietic response and is well known for metals in fish kidney and for $CuSO_4$ (Handy, 2003), but the observed material-type effect implies there may be some additional inflammation stress on the haematopoeitic system with the nano form. However with so little known about the effects of metal NPs on the renal physiology of fishes (review on fish physiology, Handy et al., 2011), much more research is needed.

4.5. Pathological observation in the brain

There are relatively few detailed reports of regional brain pathologies in fishes from toxic metals, and most of these have been for dietary exposures (Hg, Berntssen et al., 2003; Cu, Handy, 2003). In this study, histological examination of the main regions of the brain following exposure to $CuSO_4$ showed injuries that include occasional necrosis of some nerve cells and the occasional vacuole formation in the telencephalon; minor alteration in the thickness of mesencephalon with parts of the preventriculare layer being damaged; as well as enlargement of blood vessels on the ventral surface of the cerebellum (Figs. 5 and 6). Vascular injury on the surface of the brain, necrotic cell bodies and small foci of vacuoles in the optic lobe and cerebrum of trout have also been observed with SWCNT (Smith et al., 2007). The functional consequences of changes in the brain for animal behaviour and locomotion remain unclear in this experiment. However, previous studies on dietary Cu where the appearance of vacuoles and necrotic cells in the brain also occurred (without measurable increases in brain Cu content) resulted in changes in circulating serotonin/melatonin levels (Handy, 2003) and loss of circadian locomotor activity patterns (Campbell et al., 2002). The latter has potentially severe ecological consequences for a predator that feeds at dawn and dusk, as well as a big impact on their social status and competitiveness within a school of fishes (Campbell et al., 2005). It is therefore a substantial ecological concern that the same brain pathologies are evident for Cu-NPs (Figs. 5 and 6). Moreover, the quantitative analysis confirmed that the effects of Cu-NPs were greater at day 10 than the equivalent concentration of $CuSO_4$.

The accumulation of new Cu in the brain of fishes was not observed in the present study, and like previous report of Cu in fishes, the effects on the brain are best explained by indirect toxicity on neuro-endocrine functions and/or by hypoxia (Handy, 2003). Some recent work on rodents has shown that the administration of Ag-, Cu-, or Al/Al_2O_3-NPs can cause disruption of the blood brain barrier (BBB) and brain oedema (Sharma et al., 2010). While the possibility of direct Cu or Cu-NP entry into the brain cannot be completely excluded here, systemic hypoxia resulting from damaged gills seems a more likely explanation. The enlarged blood vessels on the surface of the brain are consistent with an attempt to increase blood flow to offset the effects of hypoxia. Cu-NPs and $CuSO_4$ caused some changes in the relative thickness of the layers in the midbrain. This cannot be due to differential tissue growth over 10 days and is almost certainly related to oedema. The rupture of preventricular layer points to an enlargement of the adjacent ventricle (with fluid) or inflammation within the cerebral–spinal fluid compartment. The aetiology of Cu and Cu-NP pathology in the brain needs further investigation, but it is clear from these observations that several physiological dysfunctions (osmotic, metal homeostasis, oxidative stress, vascular) may be contributing to the pathology.

4.6. Pathological observation in the muscle

Copper is known to cause a decrease in the size of skeletal muscle fibres with a concomitant increase in extracellular space in the muscles of gold fish (Vogel, 1959), and these observations are also consistent with the findings here for $CuSO_4$ in trout (Fig. 7). The functional consequences for trout exposed to $CuSO_4$ are well known and can include spending more time at lower swimming speeds (Campbell et al., 2002; Handy et al., 1999), as well as depletion of muscle glycogen stores (Campbell et al., 2005). These effects

usually occur without appreciable increases of new Cu accumulation in the muscle (Handy et al., 1999), implying indirect toxic effects of $CuSO_4$ on muscle function. This was also the case in the present study with no measurable increase in the total Cu concentrations in the muscle from either $CuSO_4$ or Cu-NP exposures (Table 1). However, the effects of Cu-NPs on muscle have not been previously reported. Exposure to Cu-NPs caused similar types of injuries to $CuSO_4$ including with a decrease in the relative proportion of muscle fibre area compared to controls (Fig. 7). The quantitative histological analysis of trout muscle fibre dimensions confirmed a small but statistically significant material-type effect, with the $CuSO_4$ causing slightly more changes in the muscle structure than the Cu-NPs. This implies the functional consequences for muscle function during Cu-NP exposure may be similar to those known for $CuSO_4$, but probably less severe. However, swimming speed and its relationship with muscle biochemistry needs further investigation with Cu-NPs.

In conclusion, this study has demonstrated from the viewpoint of pathology that the target organs for Cu-NPs are similar to those for $CuSO_4$. It has also demonstrated that the *types* of pathologies caused by Cu-NPs are similar to those known for Cu metal salts, but there may be a short time delay in the appearance of the Cu-NP pathologies. There are also some organ-specific material-type effects with Cu-NPs causing more injury in the intestine, liver and brain than the equivalent concentration of $CuSO_4$ by the end of the experiment. However the reverse was true in the gill and muscle where Cu-NPs are less effective at producing pathology, and little difference between the materials in the kidney. For $CuSO_4$, it is well known that organ pathology is not necessarily associated with direct Cu accumulation in all of the internal organs, because of secondary oxidative stress and hypoxia during the metal exposure. This aspect needs further investigation for Cu-NPs, but like $CuSO_4$, it would be prudent to consider the possibility that some injuries could occur without the presence of exogenous Cu particles or Cu metal in the internal organs.

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