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Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects

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Abstract

Mammalian studies have raised concerns about the toxicity of carbon nanotubes (CNTs), but there is very limited data on ecotoxicity to aquatic life. We describe the first detailed report on the toxicity of single walled carbon nanotubes (SWCNT) to rainbow trout, using a body systems approach. Stock solutions of dispersed SWCNT were prepared using a combination of solvent (sodium dodecyl sulphate, SDS) and sonication. A semi-static test system was used to expose rainbow trout to either a freshwater control, solvent control, 0.1, 0.25 or 0.5 mg l^{-1} SWCNT for up to 10 days. SWCNT exposure caused a dose-dependent rise in ventilation rate, gill pathologies (oedema, altered mucocytes, hyperplasia), and mucus secretion with SWCNT precipitation on the gill mucus. No major haematological or blood disturbances were observed in terms of red and white blood cell counts, haematocrits, whole blood haemoglobin, and plasma Na^+ or K^+. Tissue metal levels (Na^+, K^+, Ca^{2+}, Cu, Zn and Co) were generally unaffected. However some dose-dependent changes in brain and gill Zn or Cu were observed (but not tissue Ca^{2+}), that were also partly attributed to the solvent. SWCNT exposure caused statistically significant increases in Na^+K^+-ATPase activity in the gills and intestine, but not in the brain. Thiobarbituric acid reactive substances (TBARS) showed dose-dependent and statistically significant decreases especially in the gill, brain and liver during SWCNT exposure compared to controls. SWCNT exposure caused statistically significant increases in the total glutathione levels in the gills (28%) and livers (18%), compared to the solvent control. Total glutathione in the brain and intestine remained stable in all treatments. Pathologies in the brain included possible aneurisms or swellings on the ventral surface of the cerebellum. Liver cells exposed to SWCNT showed condensed nuclear bodies (apoptotic bodies) and cells in abnormal nuclear division. Overt fatty change or wide spread lipidosis was absent in the liver. Fish ingested water containing SWCNT during exposure (presumably stress-induced drinking) which resulted in precipitated SWCNT in the gut lumen and intestinal pathology. Aggressive behaviour and fin nipping caused some mortalities at the end of the experiment, which may be associated with the gill irritation and brain injury, although the solvent may also partly contributed to aggression. Overall we conclude that SWCNTs are a respiratory toxicant in trout, the fish are able to manage oxidative stress and osmoregulatory disturbances, but other cellular pathologies raise concerns about cell cycle defects, neurotoxicity, and as yet unidentified blood borne factors that possibly mediate systemic pathologies.

Keywords: Single walled carbon nanotubes; SWCNT; Gill; Respiration; Brain pathology; Liver pathology; Haematology; Na^+K^+-ATPase; TBARS; Glutathione; Rainbow trout; Copper; Zinc

1. Introduction

Nanotechnology has been defined as using materials and structures with nanoscale dimensions, usually in the range 1–100 nm (Masciangioli and Zhang, 2003; Roco, 2003). However from the view point of toxicology, this definition is not absolute and studies have included aggregates of nanomaterials, as well as individual particles (Handy and Shaw, 2007). Manufactured nanomaterials have numerous industrial applications including electronics, optics, and textiles, as well as applications in medical devices, drug delivery systems, chemical sensors, biosensors, and in environmental remediation (Kong et al., 2000; Masciangioli and Zhang, 2003; Freitas, 2005; Aitken et al., 2006). The materials are often custom made for the particular application, and it is therefore no surprise that there are a wide variety of nanomaterials and nanoparticles. Toxicological research on nanomaterials has currently focused on two major groups of materials. These include the effects of carbon-

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based nanomaterials such as carbon nanotubes (CNTs) or carbon nanospheres ("fullerenes") (Lam et al., 2004; Oberdörster, 2004; Cui et al., 2005), and the effects of metal or metal oxide nanoparticles (Bermudez et al., 2004; Chen et al., 2006; Sayes et al., 2006). There are many variations in structure within each of these major types of engineered nanomaterials, and CNTs are commercially available as single walled (SWCNT) or multi-walled carbon nanotubes (MWCNTs) of different sizes (Roco, 2003).

Most of the emerging literature on the toxicity of nanoparticles has focused on respiratory exposure in mammalian models and the implications for human health; and these studies confirm that nanoparticles can have toxic effects (review, Handy and Shaw, 2007). Some toxic effects in mammals have been attributed to CNTs. For example, mice exposed to a single intratracheal instillation (i.t.) dose of 0.1 or 0.5 mg SWCNT, experienced over 55% mortalities within 7 days of exposure, with a dose-dependent incidence of lung pathologies (epithelioid granulomas, inflammation injuries, necrosis) presenting during the 90-day post-exposure follow up (Lam et al., 2004). Warheit et al. (2004) made similar post-exposure observations with SWCNT, and it is now recognised that CNTs are not easily cleared from the lung (e.g. MWCNT in rat lung 60 days after exposure, Muller et al., 2005). There have also been a range of *in vitro* studies using human or mammalian cell lines to investigate toxic mechanisms (see Handy and Shaw, 2007), which suggest that oxidative stress, inflammation reactions, and immunotoxicity may be key features of nanoparticle toxicity. For example, Barlow et al. (2005) exposed bovine serum to ultrafine carbon black particles and demonstrated that chemical anti-oxidants delayed macrophage aggregation responses. Shvedova et al. (2003) showed that SWCNT caused antioxidant depletion, free radical formation, and the accumulation of peroxide products with a loss of cell viability in human epidermal keratinocytes (HEK) cells.

These mammalian studies, and the apparent persistence of CNTs in tissues, raises concerns that nanomaterials may also be toxic to wildlife (Owen and Depledge, 2005). This has at least been partially confirmed in a few ecotoxicity studies on fish and invertebrates using carbon-based nanomaterials. Oberdörster (2004) showed that juvenile largemouth bass (*Micropterus salmoides*) exposed to 0.5 or 1 mg l^{-1} C_{60} fullerenes for up to 48 h (dissolved in tetrahydrofuran, THF) showed elevated lipid peroxidation products in the brain (but not the gill or liver) and a small reduction in the total glutathione pool of the gills. In a subsequent study on C_{60} fullerenes (without THF dispersion), Oberdörster et al. (2006) showed that 2.5–5 mg l^{-1} of C_{60} delayed moulting in *Daphnia*. Lovern and Klaper (2006) estimate the lethal concentrations of C_{60} fullerenes (48 h LC_{50}) to *Daphnia* was between 460 μg l^{-1} and 7.9 mg l^{-1} depending on the method of preparation of the nanomaterial. Although these examples raise concerns about ecotoxicity, there are few ecotoxicological studies on CNTs, and there have been no systematic investigations of the toxic effects of CNTs to rainbow trout.

In this study we used SWCNTs and similar dosimetry to that used in mammalian studies, and adopt a body systems approach to give the first detailed overview of the toxic effects of CNT in trout. Our aim was to measure functional responses in key areas of physiology (e.g. ventilation, osmoregulation, haematology) as well as documenting organ pathologies and biochemical responses during aqueous exposure. We therefore measured a range of end points including behaviours, gill ventilation rates, haematology and plasma ions, trace element profiles in the major organs, a suite of histopathology, as well as biochemical measurements relating to physiological fuction (e.g. Na^+K^+-ATPase activity) or oxidative stress (TBARS, glutathione content).

2. Materials and methods

2.1. Experimental design

Juvenile rainbow trout ($n = 180$) were obtained from Hatchlands Trout Farm, Rattery, Devon, and held for 4 weeks in stock aquaria with flowing, aerated, dechlorinated Plymouth tap water (see below). Stock animals were fed to satiation on a commercial trout food. Fish weighing 30.0 g ± 5.0 (mean ± S.E.M., $n = 180$) were then graded into fifteen experimental glass aquaria (12 fish/tank), in a triplicate design (3 tanks/treatment), and allowed to rest for 24 h prior to the commencement of the experiment. Fish were exposed in triplicate to one of the following treatments for 10 days using a semi-static exposure regime (80% water change every 12 h with re-dosing after each change): control (freshwater only, no CNT or solvent), solvent control (0.15 mg l^{-1} sodium dodecyl sulphate, SDS), 0.1, 0.25 or 0.5 mg l^{-1} SWCNT (see below for stock solutions). These SWCNT concentrations were selected after considering the doses used to produce epithelial injury in rat lung (Lam et al., 2004), and the sub-lethal effects of low mg amounts of fullerenes in largemouth bass and fathead minnows (Oberdörster, 2004; Oberdörster et al., 2006). In this experiment, the 0.15 mg l^{-1} concentration of SDS (also performed in triplicate tanks) represented the highest amount of solvent added to the highest SWCNT concentration. However, the 0.1 and 0.25 mg l^{-1} SWCNT contained less solvent (see stock solutions below) and we therefore also performed an additional solvent control experiment with the range of SDS concentrations used in triplicate (see stock solutions below) to verify that there was no dose-effect that could be attributed to the solvent (none was observed, data not shown).

Fish were not fed 24 h prior to, or during the experiment in order to minimise the risk of the CNT absorbing to food or faecal material, and to help maintain water quality. Water samples were collected immediately before and after each water change for pH (YSI 63 pH meter), total ammonia (HI 95715, Hanna Instruments), dissolved oxygen (YSI 85 D.O. meter). There were no treatment differences in water quality between tanks (ANOVA, $P > 0.05$). Values were (means ± S.E.M., $n = 253$-261 samples); total ammonia, 0.83 ± 0.18 mg l^{-1}; pH, 7.16 ± 0.01; oxygen saturation, 83 ± 0.13 %; temperature, 15.5 ± 0.3 °C. Photoperiod was 12 h light: 12 h dark. The electrolyte composition of the dechlorinated Plymouth tap water used was 0.3, 0.1, and 0.4 mmol l^{-1} for Na^+, K^+ and Ca^{2+} respectively. Fish were randomly sampled on day 0 (initial fish from the stock), day 4, and day 10 for haematology, plasma ions, tissue electrolytes,

histopathology, and biochemistry (see below). Behaviours were noted throughout the experiment, and ventilation rates (30 s visual count of opercular movements, in triplicate on each fish) were measured prior to sampling days to ensure fish had not been disturbed. The entire experiment was subject to ethical approval, and was independently monitored by a fish health expert.

2.2. SWCNT stock solution and dosing

Powder form of single-walled carbon nanotubes were obtained from Cheap Tubes Inc. (Vermont, USA), and had (manufacturer's information) 1.1 nm mean outside diameter, 5–30 μm length, and the powder was a minimum of 96.3% carbon, (maximum impurities were: Al 0.08, Cl 0.41, Co 2.91, and S 0.29%). However, analysis of stock solutions revealed these impurities were below detection (data not shown), and the batch purity was high. Stock solutions of dispersed CNTs were prepared using a combination of solvent (SDS) and sonication after considering the recommendations of the manufacturer, the solubility of CNTs in various solvents (Ham et al., 2005), and the potential toxicity of these solvents to fish. SDS was selected because the chemistry indicated moderately good dispersion of carbon nanotubes with SDS (Ham et al., 2005) while the maximum concentration of SDS we used (0.15 mg l^{-1} in the fish tank for solvent controls and the highest CNT concentration) was at least an order of magnitude below the lethal threshold for trout (Abel, 1976). After a series of trials to optimise sonication times and detergent concentrations (data not shown), a 0.5 g l^{-1} SWCNT stock solution was prepared by dispersing the SWCNT in 3 g l^{-1} SDS with sonication for 2 h in a bath-type sonicator (35 kHz frequency, Fisherbrand FB 11010, Germany), and manual inversion of the container every 20 min.

Dispersion was confirmed by transmission electron microscopy (TEM, JEOL 1200EXII). The dispersion was very good at the final working concentrations (0.5, 0.25 and 0.1 mg l^{-1} SWCNT) and the particle size was approaching the detection limit of the TEM in the presence of SDS (at least <5 nm). However, without added SDS the SWCNTs were not dispersed and clearly visible as polymerised strands about 50–100 nm thick (Fig. 1). The addition of SDS was therefore essential. Dispersion was also confirmed by optical methods. Spectral scans of the sonicated CNT solutions containing SDS (190–800 nm, Perkin Elmer UV/VIS Spectrometer, Lambda Bio 20), gave the typical profile expected with a distinct peak at about 260 nm (Fig. 1), and was similar to previous reports for carbon-based nanoparticles (e.g. Oberdörster, 2004).

In order to achieve working concentrations of 0.1, 0.25, and 0.5 mg l^{-1} SWCNT in the fish tanks, each tank was dosed with 4, 10 or 20 ml of the 0.5 g l^{-1} SWCNT stock solution respectively. This also gave nominal SDS concentrations of 0.03, 0.075, 0.15 mg l^{-1}, and in compliance with ethical approval we included one solvent control concentration of 0.15 mg l^{-1} SDS (in triplicate, for the worst case scenario for solvent effect) in the SWCNT experiment. Ham et al. (2005) raised concerns that adding excess solvent beyond that just needed to disperse nanoparticles could cause particle swelling or tube deformity. We therefore decided not to add extra SDS to make the same nominal solvent concentration in all tanks (an approach typically used in non-replicated regulatory toxicity tests). Instead, we performed an addition, triplicated, solvent control experiment using the nominal SDS concentrations above compared to a water only control, and dosed exactly as above but without the SWCNTs. The aeration and water flow in the tank dispersed each dose around the tank in seconds in all experiments. Volumes of SWCNT stock were adjusted on re-dosing to reflect the 80% water change every 12 h. Although the contents and composition of the stock solutions were confirmed (Fig. 1), and renewing the test media every day would maintain the exposure, we were unable to routinely quantify the sub mg l^{-1} levels of SWCNT in the fish tank water because the technology is not yet available. Even advanced optical techniques such as dynamic light scattering methods (Kammer et al., 2005) cannot quantify concentration below 1–2 mg l^{-1} levels of nanoparticles, especially against the background of the many other natural colloids/ultra fine particles that are normally present in water (Lead and Wilkinson, 2006). Attempts were made to exploit the 260 nm spectral peak for routine measurement of CNT levels in freshwater. However, the absorbance of SWCNTs in freshwater at 260 nm was only linear down to about 2.5 mg l^{-1} (with or without UV excitation, data not shown), and therefore could not be used.

2.3. Haematology and blood plasma analysis

Two fish were randomly collected from each tank (6 fish/treatment, and $n = 7$ for initial fish) at days 0, 4 and 10 and carefully anaesthetised with buffered MS222. Haematological measurements were carried out as described by Handy and Depledge (1999). Briefly, whole blood was collected via the caudal vein into heparinised syringes, and the fish weighed and total length was recorded. Haematocrit value (HCT) and haemoglobin concentration (Hb, using 20 μl of blood in 5 ml Drabkin reagent) were determined immediately. Whole blood (20 μl) was fixed in Dacie's fluid for red and white blood cell counts. A blood smear was also made and air dried for later examination, along with spleen prints made during dissection (data not shown). The remaining blood was centrifuged (13000 *rpm* for 2 min, Micro Centaur MSE), and serum collected and stored at −20 °C until subsequent analysis of plasma ions and osmometry. Plasma Na^+ and K^+ were analysed by flame photometry (Corning 480 Flame Photometer) as described by Burke et al. (2003). Osmotic pressure was determined by the freezing-point depression method using 50 μl of plasma (Precision Systems micro osmometer, Natick, Massachusetts, USA).

2.4. Tissue ion analysis

Following blood sampling, fish were terminally anaesthetised with MS222 and dissected for tissue ion analysis. Gill, liver, skinned muscle from the flank, and whole brain were harvested and processed for ion analysis according to Handy et al. (1999), with slight modifications. Samples were oven dried to a constant weight to determine moisture content. Samples were digested in 4 ml of concentrated nitric acid for 8 h at 50 °C, then diluted to

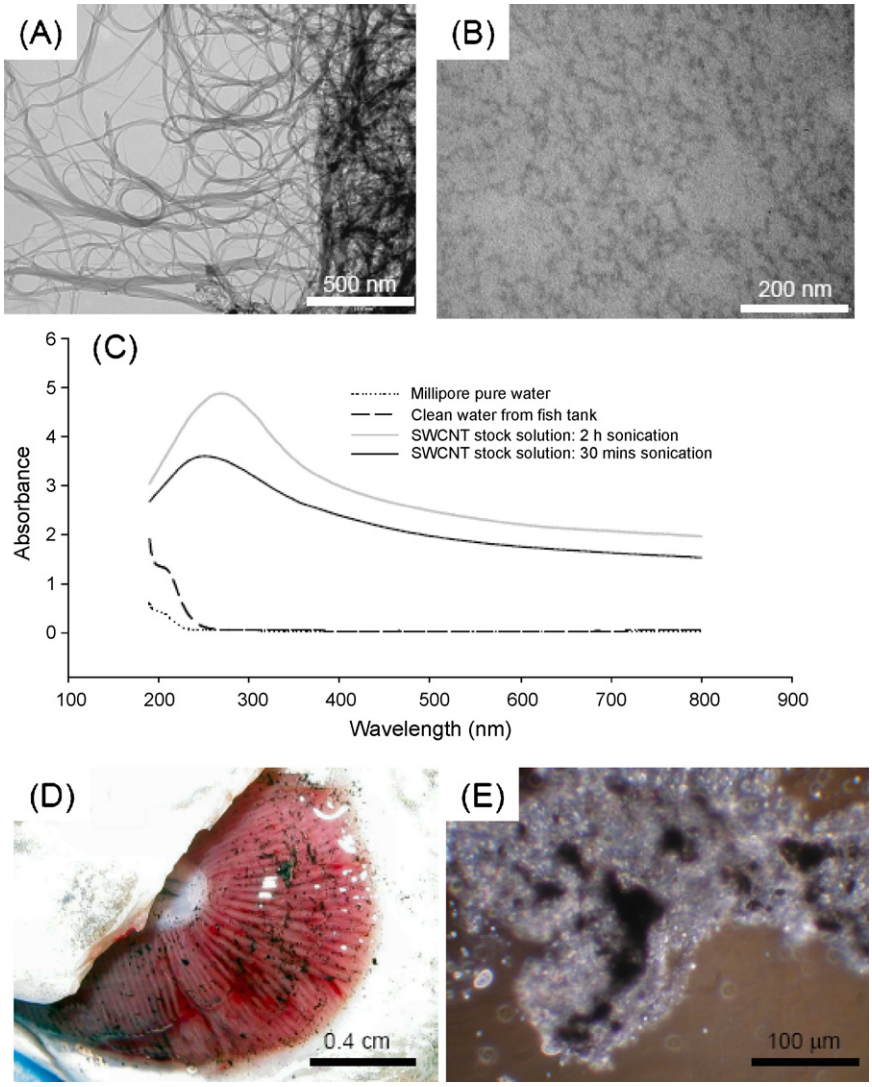

Fig. 1. Dispersion and aggregation of SWCNT. (A) Electron micrograph (60,000 × magnification) showing polymerisation of a 0.5 g l^{-1} SWCNT stock solution into discrete carbon strands in the absence of solvents; (B) dispersion of the same stock solution with 3 g l^{-1} sodium dodecyl sulphate, SDS, with nanoparticle dispersion so fine that particle size is approaching the limit of the electron microscope (150,000 × magnification); (C) absorbance spectra of SDS-dispersed SWCNT stock solution after sonicating for 30 min and 2 h compared to clean water from a fish tank, and millipore water; (D) secreted fish mucus rapidly aggregates previously dispersed SWCNT on the surface of the gills (example fish from 0.5 mg l^{-1} SWCNT treatment); (E) phase contrast photograph of a mucus smear (magnification ×40) showing aggregates of nanoparticles associated with the mucoproteins.

16 ml with ultra pure (ion free) water. Samples were analysed for Zn, Cu, Mn, and Co by inductively coupled plasma mass spectrometry (ICP-MS, Fisons Instruments, VG Plasma Quad PQ2 ICP-MS), and for Ca (Varian SpectrAA_{50}), Na and K (F-AA/AE Spectrometer, Double Beam GBC902) by flame atomic absorption spectroscopy. Analytical grade standards and reference materials were used, and mass spectrometry samples also included internal standards (1% yttrium and indium).

2.5. Biochemistry

An additional two fish were randomly collected from each tank (6 fish/treatment, and $n = 7$ for fish at the onset of the experiments) at days 0, 4 and 10 for biochemistry. Gill, liver, intestine, and whole brain, were removed and immediately snap frozen in liquid nitrogen and stored at −80 °C until required. Tissues (about 0.5 g, or whole brain) were homogenised (Cat X520D with a T6 shaft, medium speed, Bennett & Co, Weston-super-Mare) in 5 volumes (2.5 ml) of ice-cold isotonic buffer (in mmol l^{-1}; 300 sucrose, 0.1 ethylenediamine tetra acetic acid (EDTA), 20 4-(2-hydroxyethyl)piperazine-1-ethane sulfonic acid (HEPES), adjusted to pH 7.8 with a few drops of Tris (2-amino-2-hydroxylmethyl-1,3-propanediol)). Crude homogenates were stored in 0.5 ml aliquots at −80 °C until required. Tissues were analysed at the end of the experiment (day 10) for Na^+K^+-ATPase activity to determine possible effects on osmoregulation, and thiobarbituric acid reactive substances (TBARS) and total glutathione to assess oxidative stress.

The Na^+K^+-ATPase and TBARS assays were performed according to Bouskill et al. (2006). Briefly for Na^+K^+-ATPase with minor modifications, each sample (15 μl, in triplicate) was dispensed into 400 μl of both a K^+-containing buffer and a K^+

-free buffer (plus 1.0 mmol l^{-1} ouabain), then incubated at 37 °C for 10 min. The reaction was stopped by adding 1 ml of ice cold trichloroacetic acid and 1 ml of colour reagent was added to each tube (9.6% w/v $FeSO_4 \cdot 6H_2O$, 1.15% w/v ammonium heptamolybdate dissolved in 0.66 M H_2SO_4), and colour allowed to develop for 20–30 min at room temperature. Absorbances were measured at 630 nm (Dynex MRX microplate reader) against 0–0.5 mmol l^{-1} phosphate standards. The TBARS assay was performed using 40 μl of homogenate (in triplicate), exactly as described by Bouskill et al. (2006). Absorbances were read at 530 and 630 nm (Dynex MRX microplate reader), corrected for turbidity, and read against standards (0.5–25 nmol ml^{-1} 1,1,3,3-tetraethoxypropane).

Total glutathione (GSH) was also determined (according to Owens and Belcher, 1965). Briefly, 20 μl of gill or intestine tissue homogenate, blank or standard (0–20 μmol l^{-1} reduced glutathione), was added in triplicate to a microplate well containing 20 μl of 10 mmol l^{-1} DTNB (5,5′-dithiobis-(2-nitrobenzoicacid)), 260 μl of assay buffer (100 mmol l^{-1} K_2HPO_4, 5 mmol l^{-1} EDTA, pH 7.5), and 20 μl of 2 U ml^{-1} glutathione reductase (Sigma Chemicals, Poole, UK). The reaction was commenced by the addition of 20 μl of 3.63 mmol l^{-1} NADPH, with changes in absorbance at 412 nm (Dynex MRX microplate reader) recorded over 6 min, and total GSH (μmol g^{-1} wet weight tissue) determined using the standard calibration curve.

2.6. Histopathology

At days 0 and 10, fish (7 initial fish at day 0, and 6 fish/treatment at day 10) were dissected for histology. Fish were carefully terminally anaesthetised with an overdose of buffered MS222 and tissues were collected into buffered formal saline in the following order: gill filaments from the second gill arch, whole intestine, liver, spleen, a transverse section of the body just anterior to anal fin (including kidney and bone section), and the whole brain. Tissue were fixed, processed and stained for wax histology (8 μm sections) as previously described (Handy et al., 2002). Gills were decalcified prior to processing (Rapid Decalcifier, CellPath Plc, UK). Routine observations were made on sections stained with Mallory's trichrome, and brain sections were stained with toluidine blue. Photographs were produced using an Olympus Vanox-T microscope and an Olympus digital camera (C-2020 Z). Gill injuries were quantified by manually counting the number of injuries observed in each slide. Two primary filaments were selected from the middle of the second gill arch from each fish, and at least 100 secondary lamellae were counted in each specimen.

2.7. Statistical analysis

All data were analysed using StatGraphics Plus version 5.1. There were no dose-effects of the SDS in the solvent control experiment and therefore the 0.15 mg l^{-1} SDS concentration used in the main experiment was representative of the solvent effect for the main statistical analysis. No tank effects were observed within treatments in any experiments, so data were pooled by treatment for statistical analysis. After checking for kurtosis, skewedness and unequal variance (Bartlett's test), data were tested for treatment or time effects by ANOVA followed by Fisher's 95% least-squares difference, at 95% confidence limits. This analysis included comparison of both control and solvent control effects compared to each SWCNT concentration. For non-parametric data the Kruskal–Wallis test was used and differences located by notched box and whisker plots.

3. Results

3.1. Respiratory distress, behaviour and mortality

Fish showed signs of gill irritation and mucus secretion during exposure to SWCNT, which was not observed in the solvent or water only control. By day 4 fish were showing clear signs of elevated mucus secretion with strands of sloughed mucus, which were coated with SWCNT, appearing in the tanks. Visual inspection of the gills and mucus smears on day 4 showed a thin layer of secreted mucus on the surface of gills from fish treated with SWCNT, but not the controls. *In situ* examination of the gills of exposed fish showed black granular deposits of about 0. 5 mm diameter in the mucus layer, that were clearly precipitated SWCNT (Fig. 1). Subsequent light microscopy examinations of branchial mucus smears showed that the SWCNT precipitates were associated with the mucoproteins and not just in solution in the mucus (Fig. 1). The mucus secretion continued until the end of the experiment. The fish exposed to SWCNT also showed elevated ventilation rates compared to the controls (Fig. 2), but no mortalities had occurred at day 4. Fish in the 0.5 mg l^{-1} SWCNT treatment showed a continued rise in ventilation rate with exposure time, and fish in the 0.1 and 0.25 mg l^{-1} treatments showed sustained increases in ventilation above that of the controls until the end of experiment (Fig. 2).

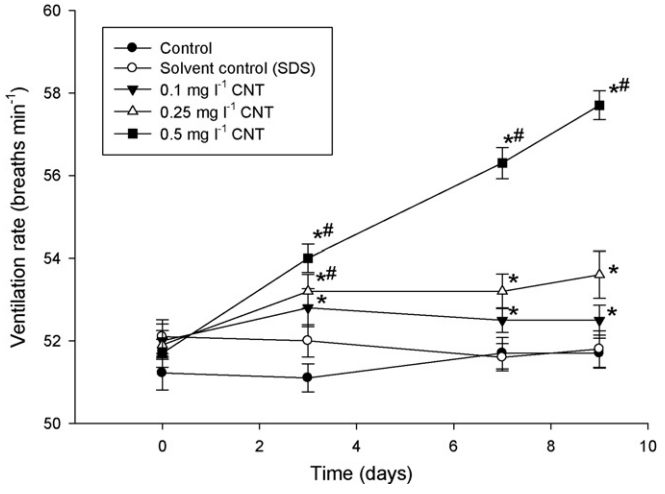

Fig. 2. The effects of exposure to SWCNT on ventilation rates in rainbow trout. Data are means ± S.E.M. ($n = 18$ fish/treatment). (*), significantly different from the control and solvent control within time point (ANOVA, $P < 0.05$). (#), significantly different from previous time point within treatment (ANOVA, $P < 0.05$). Note: there were no statistical differences between the control and solvent controls.

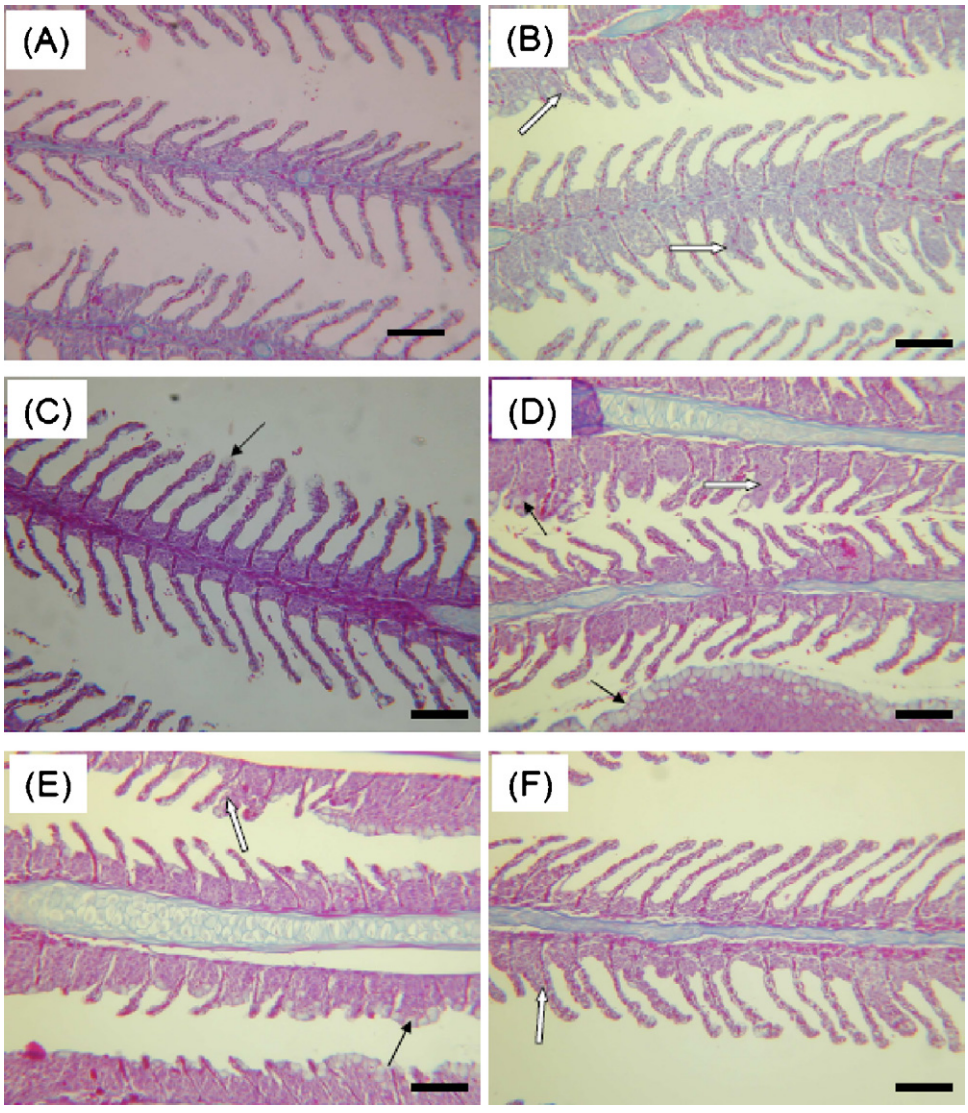

Fig. 3. Gill morphology in trout after 10 days of exposure to (A) control; (B) solvent control; (C) and (D) 0.1 mg l^{-1}; (E) 0.25 mg l^{-1}; and (F) 0.5 mg l^{-1} SWCNT, using a semi-static exposure method. The gills of control fish show normal histology, while some oedema (osmotic swelling) of some secondary lamellae are present in other treatments including the solvent control (white arrows). Some fish in 0.1 mg l^{-1} SWCNT did not show oedema (panel C), but swollen mucocytes (black arrow) about to discharge their contents. Fish exposed to 0.25 mg l^{-1} SWCNT showed a more persistent thickening of the epithelial tissue along the primary filament, which did not always extend to swelling along the length of the secondary lamellae (panel E). This was not observed at the highest SWCNT concentration, where the morphology was improved (panel F) compared to the lower doses (panels E & D). Scale bar 80 μm, sections were 8 μm thick and stained with Mallory's trichrome.

Histological examination of the gills at the end of the experiment (Fig. 3) showed normal anatomy in the freshwater controls, with a normal background incidence of injuries on <4% of the secondary lamellae (<4% of filaments with swollen tips, <4% of the secondary lamellae showing any signs of oedema at the base, <1% aneurisms, hyperplasia completely absent). Exposure to SWCNT resulted in some increases in the incidence of oedema in the secondary lamellae, changes in mucocyte morphology, and hyperplasia in the primary lamellae (Fig. 3). The proportions of secondary lamellae with oedema were (mean percentage ± S.E.M., $n = 3$–6 fish): 3.9 ± 2.8, 20.1 ± 1.7, 5.2 ± 1.6, 14.5 ± 4.3 and 16.6 ± 2.9% of secondary lamellae for control, solvent control, 0.1, 0.25 and 0.5 mg l^{-1} SWCNT respectively. The solvent control, 0.25 and 0.5 mg l^{-1} SWCNT had significantly higher incidence of oedema than the freshwater control (*t*-tests, $P < 0.05$). One third of the fish in the lowest SWCNT concentration, and half the fish in 0.25 mg l^{-1} SWCNT, showed evidence of enlarged mucocytes on every primary filament. This was not observed at the highest SWCNT concentration (mucocytes mainly discharged), or in the solvent control and freshwater control. The primary filaments of fish exposed to 0.1 and 0.25 mg l^{-1} SWCNT also showed a uniform thickening relative to secondary lamellae length which appeared to be caused by increased epithelial cell numbers (hyperplasia) at the base of the lamellae, and this effect was absent from all other treatments. Neither exposure to the solvent or SWCNT increased the incidence of aneurisms on the secondary lamellae (<1% throughout).

Fish showed some marked increases in aggressive behaviours, and in two fish tanks in particular (one solvent con-

trol, one 0.1 mg l^{-1} SWCNT tank) this led to a breakdown of the social group with fighting and mortalities towards the end of the experiment. For ethical reasons we stopped the experiment on day 10, after considering the gill irritation, increases in ventilation rate and aggression. In the first 3 days of exposure, all fish showed normal position holding and schooling behaviour, with only a background level of occasional fin nipping. After day 7 the fish were more aggressive in the SWCNT treatments and the solvent control tanks, with fin nipping, and changes in position holding in the water flow so that the more aggressive fish were at the front of the school and near the bottom of the tank. By day 7, 3 fish had died in the highest SWCNT concentration, and 1–2 fish in the other SWCNT treatments or solvent control. By the end of day 9 aggression was particularly evident in two tanks (a solvent control and 0.1 mg l^{-1} SWCNT tank), and this aggression caused mortality. A total of 1, 6, 9, 5 and 5 fish mortalities were recorded in the control, solvent control, 0.1, 0.25 and 0.5 mg l^{-1} SWCNT respectively. We therefore stopped the experiment the next day (day 10). All the dead fish showed fin nipping, especially to the dorsal fin. Mucus smears from the flank and gills of the moribund fish did not show fungal or bacterial infections. Mortality did not correlate with water quality variables, which all remained within the normal range for trout (no tank or treatment effect on water quality, ANOVA $P > 0.05$).

3.2. Haematology and plasma ions

SWCNT did not cause any major disturbance to haematology, and there were no clear treatment or time-dependent trends that emerged during the experiment, although a few individual data points were statistically different from the freshwater control or solvent control (Table 1). Notably, exposure to the highest SWCNT concentration caused a statistically significant decrease in haematocrit and whole blood haemoglobin content by day 10 compared to the freshwater control (ANOVA, $P < 0.05$). Mean erythrocyte haemoglobin content (MEH) was not effected (data not shown), except for a statistically significant decrease in MEH at the highest SWCNT concentration compared to the solvent control at day 10 (mean ± S.E.M., $n = 5$–6; control, 9.23 ± 0.77; solvent control, 11.38 ± 0.57; 0.5 mg l^{-1} SWCNT, 7.79 ± 0.78 μg $cell^{-1}$). White blood cell counts tended to decrease with time, but there was no treatment effect compared to controls (Table 1). There were no effects of the solvent on haematology, apart from a small transient decrease in haematocrit and red cell counts on day 4 (Table 1). Plasma osmolarity was not effected by either solvent or SWCNT exposure (data not shown), but there were some statistically significant effects on individual data points for plasma Na^+ and K^+, although no clear dose or time-dependent trend over the whole experiment was evident (Table 1).

3.3. Tissue electrolytes and trace metals

Fish tissues (gill, muscle, liver, whole brain) were analysed for major tissue electrolytes (Na^+, K^+, Ca^{2+}) and trace elements (Cu, Zn and Co). There was no time or treatment effects on tissue Na^+ levels (data not shown, Kruskal–Wallis test, $P > 0.05$) and no clear treatment-dependent trends in tissue K^+ levels (data

Table 1
Haematology and plasma ions in rainbow trout exposed to control (no SWCNT), solvent control, 0.1, 0.25, or 0.5 mg l^{-1} SWCNT for up to 10 days

Parameter	Time (days)	Treatment				
		Control	Solvent Control (SDS)	0.1 mg l^{-1} SWCNT	0.25 mg l^{-1} SWCNT	0.5 mg l^{-1} SWCNT
Haemoglobin (g dl^{-1})	0	5.60 ± 0.21 (7)				
	4	6.09 ± 0.23 (6)	5.31 ± 0.38 (6)	5.29 ± 0.24 (6)	4.07 ± 0.21 (6)$^{*S+}$	4.75 ± 0.55 (6)*
	10	4.85 ± 0.53 (6)	4.32 ± 0.26 (6)$^{+}$	4.43 ± 0.63 (6)$^{+}$	4.59 ± 0.40 (6)	3.42 ± 0.30 (6)$^{*\#+}$
Haematocrit (%)	0	28.6 ± 1.2 (7)				
	4	29.1 ± 2.1 (6)	23.3 ± 1.4 (6)*	27.8 ± 1.1 (6)	21.8 ± 0.7 (6)$^{*+}$	27.4 ± 2.2 (6)^{D}
	10	26.7 ± 2.1 (5)	25.8 ± 1.4 (6)	22.7 ± 3.0 (6)$^{\#+}$	25.2 ± 1.8 (6)	21.0 ± 1.6 (6)$^{*\#+}$
Red blood cell count (cells × 10^6 mm^3)	0	0.64 ± 0.04 (7)				
	4	0.67 ± 0.08 (6)	0.48 ± 0.04 (5)$^{*+}$	0.61 ± 0.02 (5)*	0.52 ± 0.04 (6)*	0.51 ± 0.05 (6)*
	10	0.54 ± 0.04 (5)	0.39 ± 0.02 (6)$^{+}$	0.40 ± 0.09 (6)$^{\#+}$	0.57 ± 0.06 (6)^{SD}	0.45 ± 0.04 (6)$^{+}$
White blood cell count (cells × 10^3 mm^3)	0	9.87 ± 1.28 (7)				
	4	10.62 ± 1.98 (6)	8.93 ± 1.82 (6)	10.80 ± 1.84 (5)	8.02 ± 1.35 (6)	6.92 ± 1.70 (6)
	10	6.02 ± 0.78 (6)	5.10 ± 0.59 (6)	5.04 ± 0.70 (6)$^{\#+}$	4.05 ± 0.43 (6)$^{\#+}$	6.56 ± 0.76 (6)^{D}
Plasma Na^+ (mmol l^{-1})	0	125.0 ± 2.0 (7)				
	4	114.0 ± 11.5 (5)	137.0 ± 4.4 (5)$^{*+}$	124.2 ± 7.7 (5)	138.0 ± 4.8 (5)$^{*+}$	129.4 ± 2.7 (5)
	10	113.8 ± 9.5 (5)	117.7 ± 16.9 (4)	120.7 ± 3.6 (5)	130.3 ± 4.0 (5)	133.6 ± 3.3 (5)$^{*+}$
Plasma K^+ (mmol l^{-1})	0	3.7 ± 0.2 (5)				
	4	3.3 ± 0.5 (5)$^{+}$	3.5 ± 0.2 (5)$^{+}$	2.8 ± 0.3 (5)$^{+}$	2.9 ± 0.1 (4)$^{*S+}$	3.2 ± 0.2 (5)$^{D+}$
	10	4.2 ± 0.7 (5)	5.1 ± 0.1 (4)$^{\#}$	3.8 ± 0.4 (5)$^{\#}$	3.8 ± 0.3 (5)$^{\#}$	3.5 ± 0.2 (5)$^{S\#}$

Data are means ± S.E.M. (n fish/treatment). (*), significant difference from control within rows (ANOVA or Kruskal Wallis, $P < 0.05$). (S), significant difference from solvent control within rows (ANOVA or Kruskal Wallis, $P < 0.05$). (#), significant difference between day 4 and day 10 within treatment (time-effect, ANOVA or Kruskal Wallis, $P < 0.05$). (D), significantly different from the previous SWCNT concentration within row (dose-effect within time point, ANOVA or Kruskal Wallis, $P < 0.05$). (+), significantly different from initial fish (stock fish at time zero, ANOVA or Kruskal Wallis, $P < 0.05$).

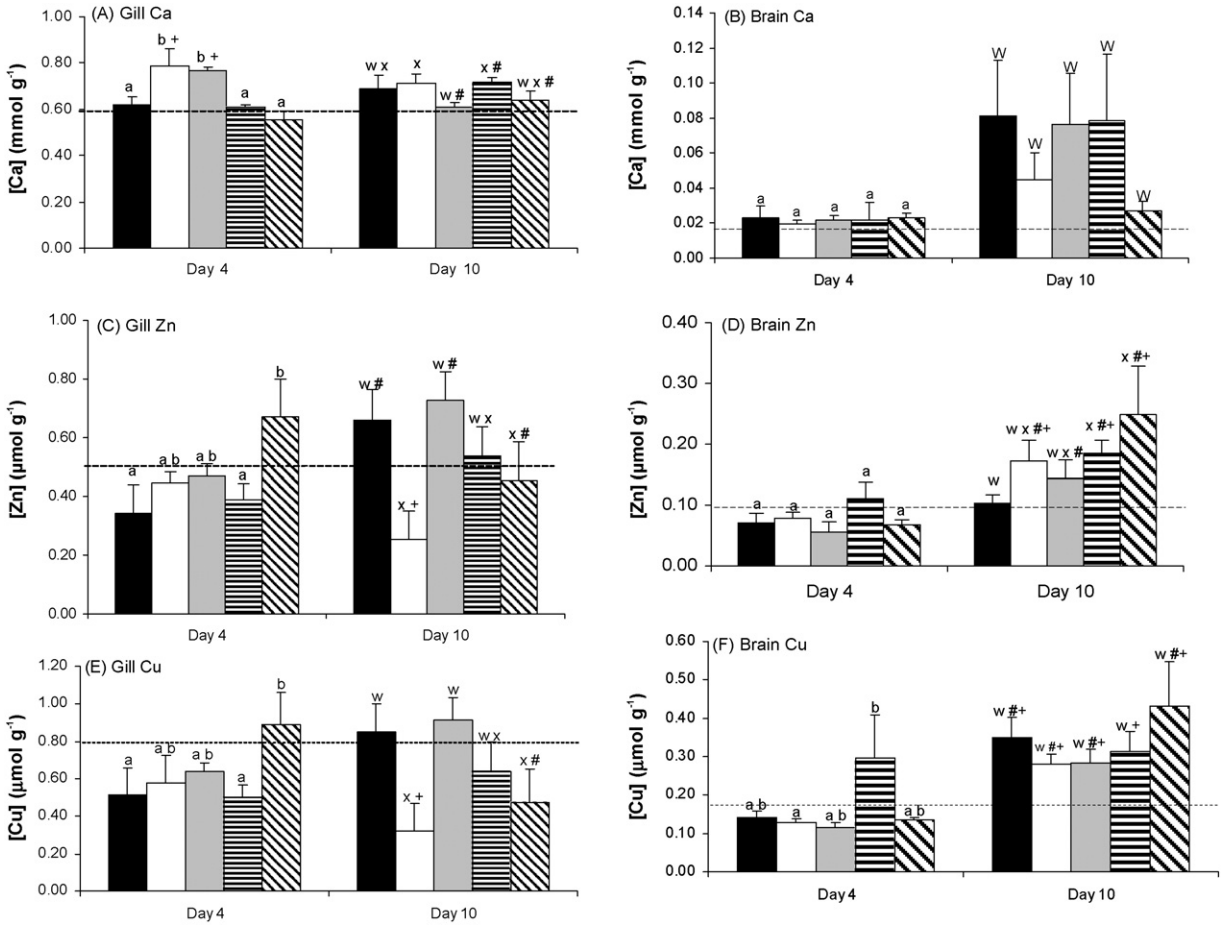

Fig. 4. Trace metal levels in the gill (left hand panels) and brain (right hand panels) of trout after 4 and 10 days exposure to control (black bar), solvent control (white bar), 0.1 (grey bar), 0.25 (horizontal bars) or 0.5 mg l^{-1} SWCNT (diagonal bars). Data are means ± S.E.M., $n = 5$–7 fish, for Ca^{2+} (panels A and B), Zn (panels C and D), and Cu (panels E and F). Note, the units for Ca^{2+} are mmol g^{-1} dry weight of tissue, while Zn and Cu are expressed as µmol g^{-1} dry weight of tissue. Different letters within time point indicate significant differences between treatments within each tissue (ANOVA or Kruskal–Wallis test, $P < 0.05$). (#) significant time effect within treatment compared to day 4 (ANOVA or Kruskal–Wallis test, $P < 0.05$). Dotted line indicates the mean value for initial stock fish, (+) significantly different from initial fish by ANOVA or Kruskal–Wallis test, $P < 0.05$).

not shown) except a trend of increasing K^+ in the gills at day 10 (significant increase over the control but not solvent control, Kruskal–Wallis test, $P = 0.018$), but even these gill K^+ data were in the expected range for trout with values ranging between 280–390 µmol g^{-1} dry weight of tissue. Similar observations were made for Ca^{2+}, Cu and Zn with no treatment-dependent trends (data not shown), except in the gills and the brain (Fig. 4). The gills showed a progressive dose-dependent decrease in Cu and Zn, but not Ca^{2+} after 10 days exposure to SWCNT. Brain Ca^{2+}, Cu and Zn levels showed more variability at day 10, and there was a dose-dependent increase in brain Zn levels at the end of the experiment (Kruskal–Wallis test, $P < 0.05$). Tissue cobalt levels remained low, about 0.1 µmol g^{-1} dry weight or much less in all tissues examined, with no SWCNT-dependent effects (data not shown).

3.4. Na^+K^+-ATPase

SWCNT exposure caused some increases in Na^+K^+-ATPase activity (Fig. 5). Aqueous exposure to SWCNT caused elevation of Na^+K^+-ATPase activity in the gills by the end of the experiment, with the largest increases in the 0.1 and 0.25 mg l^{-1} SWCNT treatments (Kruskal–Wallis test, $P = 0.001$). Elevation of Na^+K^+-ATPase activity was also noted in the intestine with exposure to 0.1 mg l^{-1} SWCNT compared to the controls (ANOVA, $P = 0.002$). There were no treatment effects on Na^+K^+-ATPase activity in the whole brain (ANOVA $P > 0.5$). There were no effects of the solvent on Na^+K^+-ATPase activity.

3.5. TBARS and total glutathione

Fish exposed to SWCNT generally showed a decrease in TBARS compared to controls at the end of the experiment (Fig. 6). In the gills, exposure to 0.25 mg l^{-1} SWCNT caused the greatest decrease in TBARS (55% decrease, statistically significant compared to either control, ANOVA $P < 0.05$). The intestine also showed a decrease in TBARS (ANOVA, $P < 0.05$), with the biggest reduction (30% fall) at the highest SWCNT concentration. The brain tissue showed a dose-dependent decrease in TBARS, with a maximum decrease of 55% compared to the freshwater controls (ANOVA, $P = 0.003$). The liver showed the largest effect, with a dose-dependent decrease in TBARS with

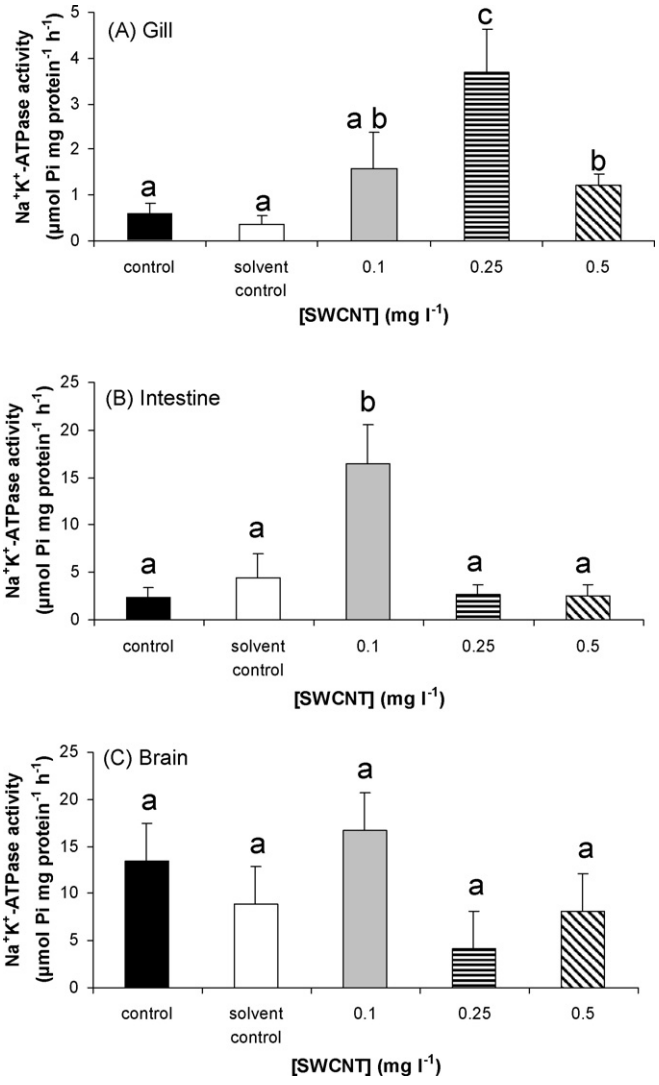

Fig. 5. Na^+K^+-ATPase activity in crude homogenates from (A) gill; (B) intestine; and (C) whole brain of rainbow trout after 10 days exposure to control (black bar), solvent control (white bar), 0.1 (grey bar), 0.25 (horizontal bars) or 0.5 mg l^{-1} SWCNT (diagonal bars). Data are means ± S.E.M., $n = 5$–7 fish/treatment. Different letters indicate significant differences between treatments within each tissue (ANOVA or Kruskal–Wallis test, $P < 0.05$). Note the smaller *y*-axis scale in panel (A).

the biggest response at the highest SWCNT concentration (72% fall) by the end of the experiment (Kruskal–Wallis test, $P = 0.04$). There were no effects of the solvent on TBARS, except in the intestine where the solvent also caused a reduction in TBARS compared to the freshwater control.

Total glutathione levels were measured in the gill, intestine, whole brain and liver homogenates. None of the tissues examined showed glutathione depletion. Levels of total glutathione in the intestine and brain were stable with no treatment-dependent effects (ANOVAs $P > 0.05$). Values ranged between 0.29–0.49 and 0.61–0.82 µmol g^{-1} wet weight of tissue for intestine and brain respectively. The gill and liver of fish exposed to 0.1 or 0.25 mg l^{-1} SWCNT showed statistically significant rises in total glutathione compared to the freshwater control (ANOVA or Kruskal–Wallis test, $P < 0.05$), with the 0.25 mg l^{-1} SWCNT dose also being higher (statistically significant) than the solvent control. Glutathione levels in the gills were (mean ± S.E.M., $n = 6$): 1.51 ± 0.61, 1.97 ± 0.77, 2.46 ± 0.52, 2.53 ± 0.162, 1.83 ± 0.88 for the freshwater control, solvent control, 0.1, 0.25 and 0.5 mg l^{-1} SWCNT respectively. Glutathione levels in the livers were (mean ± S.E.M., $n = 6$): 1.84 ± 0.21, 1.95 ± 0.55, 2.30 ± 0.54, 2.22 ± 0.15, 1.67 ± 0.16 for the freshwater control, solvent control, 0.1, 0.25 and 0.5 mg l^{-1} SWCNT respectively. No differences between total glutathione levels in the freshwater controls and solvent controls were observed in any tissue.

3.6. Histology of the liver, brain and intestine

The livers of fish from the freshwater control and solvent controls showed normal histology, and apart from some foci of lipidosis during SWCNT exposure there were no signs of fatty change that could be associated with oxidative stress (Fig. 7). However, all fish from the 0.25 mg l^{-1} SWCNT treatment showed changes to nuclear morphology with condensed nuclear bodies that have the appearance of apoptotic bodies, and cells also show nuclear division with condensed nuclear material. This was also evident to a lesser extent at the highest SWCNT concentration (observed in 4 out of the 6 fish). All the SWCNT treatments also showed some cells where the nucleus was very diffuse (early stages of cellular necrosis).

The brains of fish were removed whole, and the gross proportions of the fore, mid and hind brain appeared normal. External examination showed no evidence of gross inflammation (e.g. no blistering of the dura or swelling of the bulk tissue), although blood vessels were prominent in some fish exposed to 0.5 mg l^{-1} SWCNT. Histological examination confirmed that gross anatomy was generally normal in all the brains examined from all treatments. However, there were some localised and minor cellular pathologies (individual necrotic cell bodies, small foci of vacuolation) mainly in the optic lobe and cerebrum in fish exposed to SWCNT of 0.25 mg l^{-1} and above. Fish exposed to the highest SWCNT concentration also showed what appeared to be aneurisms or swelling of blood vessels on the ventral surface of the cerebellum that were not observed in the controls or solvent controls (Fig. 8). This injury in SWCNT fish was present in every brain where blood vessels could be viewed (4 out of 6 fish at the highest dose). Examination of other blood vessels that were deeper in the ventral region of the brain were normal in the same fish (e.g. in the ventral region around the mid brain). This surface injury to the cerebellum was also observed in 1 out of 4 fish where blood vessels were preserved in the 0.25 mg l^{-1} SWCNT, but not the 0.1 mg l^{-1} CNT treatment. We subsequently also attempted to examine other delicate structures on the surface of the brain, such as the pineal gland, which were normal in the control fish, but there were insufficient sections showing the pineal structure in the SWCNT exposed-fish to make any firm observation.

Histology of the intestine is shown (Fig. 9). Gross observations during dissection showed clear black deposits in the gut lumen which indicated that the fish had been drinking the SWCNT contaminated water. Subsequent histological examination of the intestine showed no effects of the solvent control, but

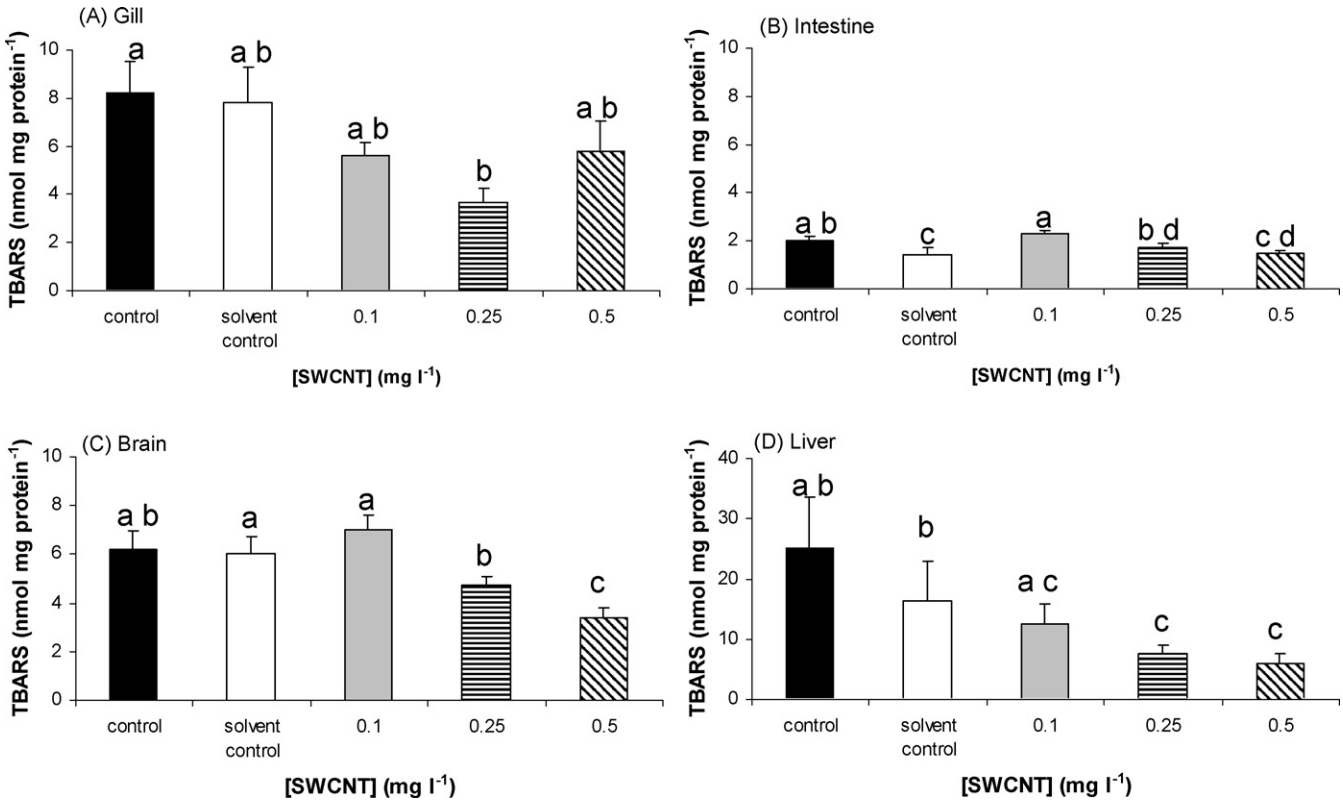

Fig. 6. Thiobarbituric acid reactive substances (TBARS) in crude homogenates from (A) gill; (B) intestine; (C) whole brain and (D) liver of rainbow trout after 10 days exposure to control (black bar), solvent control (white bar), 0.1 (grey bar), 0.25 (horizontal bars) or 0.5 mg l^{-1} SWCNT (diagonal bars). Different letters indicate significant differences between treatments within each tissue (ANOVA or Kruskal–Wallis test, $P < 0.05$). Data are means ± S.E.M., $n = 5$–7 fish/treatment. Note the different y-axis scale for the liver data (panel D).

some clear intestinal pathology associated with SWCNT exposure (Fig. 9). All fish from the 0.1 mg l^{-1} SWCNT treatment showed some areas of fusion of intestinal villi, and areas of inflammation and erosion or total atrophy of the mucosa. Precipitated SWCNT can be seen in the gut lumen (Fig. 9C, arrow). The injuries were observed to a lesser extent at 0.5 mg l^{-1} (in 4 out of 6 of the fish examined) and 1.0 mg l^{-1} SWCNT (half the fish examined). There was no evidence of major bleeding from the blood vessels of the sub mucosa, but the tissue layer with associated nerve plexus appeared more granular in the SWCNT treated specimens than the controls.

4. Discussion

4.1. Dispersion of carbon nanotubes and effects of the solvent

Several authors have already reported that dispersion of carbon nanotubes is difficult to achieve without the use of a dispersing agent or solvent (e.g. Warheit et al., 2004; Ham et al., 2005). Sonication or prolonged stirring can also help to improve dispersion of nanoparticles (Lovern and Klaper, 2006), but the addition of some solvent is needed to maintain dispersion, especially if experiments are to last more than a few days. In some of the first toxicology experiments with CNTs researchers tended to select solvents on the basis of their chemical properties and ability to disperse CNTs in solution. Some of the best dispersants from the view point of chemistry are substances such as *N*,*N*'-dimethylformamide, furan derivatives, and chloroform (for review see Ham et al., 2005). Perhaps for this reason, tetrahydrofuran was the dispersant of choice for some of the pioneering fish and invertebrate studies (e.g. Oberdörster, 2004; Lovern and Klaper, 2006). However the well known toxicity of some of the best dispersants (e.g. chloroforms and amides) raises concerns about the use of solvents, but if experiments are performed without solvents, carbon-based nanoparticles tend to aggregate and produce different ecotoxicological effects (Oberdörster et al., 2006). This presents an intractable dilemma, and our approach was a compromise which used a combination of solvent and sonication to disperse the SWCNT, but the solvent (SDS) was chosen after considering its toxicity to fish as well as its ability to disperse CNTs (Ham et al., 2005; Abel, 1976). We achieved good dispersion of SWCNT in solution (see methods and Fig. 1), and for the majority of end points used in the experiment the SDS had little or no effect. For example there was no solvent effect on ventilation rate (Fig. 2), gill morphology in terms of incidence of swollen tips of lamellae, mucus production, gill hyperplasia. There were no overall effects of SDS compared to the freshwater only controls (Table 1, Figs. 5–8). Apart from some unexpected aggressive behaviour in one fish tank (also observed with SWCNT) and some loss of Cu and Zn from the gills (Fig. 4), the choice of SDS seems reasonable. Interestingly, the completely separate solvent control experiment we conducted as a precaution in the experimental

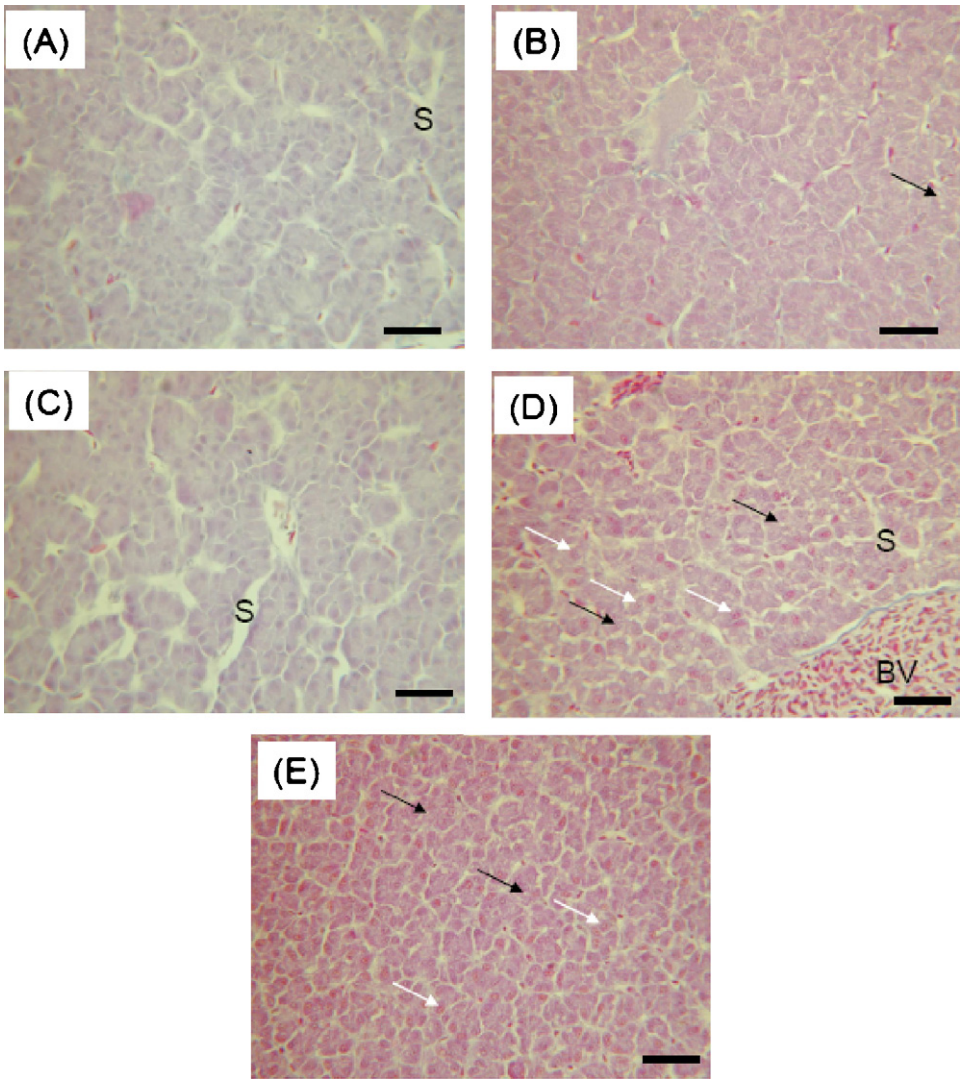

Fig. 7. Liver morphology in trout after 10 days of exposure to (A) control, (B) solvent control, (C) 0.1, (D) 0.25, and (E) 0.5 mg l^{-1} SWCNT. The livers of control fish show normal histology with sinusoid space (*S*) present. Livers from the solvent controls also show normal histology with rare foci of lipidosis (black arrow) and slightly less sinusoid space compared to the freshwater control. Livers from fish exposed to 0.1 mg l^{-1} SWCNT showed normal histology and sinusoid space, although nuclear definition was poor in some cells. Livers of fish exposed to 0.25 mg l^{-1} SWCNT showed pathology, with more frequent foci of lipidosis (black arrow) and many cells showing nuclear fragments which appear to apoptotic bodies (white arrows) and some of the cells are clearly dividing (white arrows). This effect was particularly noted near hepatic blood vessels (*BV*). Similar observations were made in livers from fish exposed to 0.5 mg l^{-1} SWCNT, with some cells showing foci of lipidosis or nuclear bodies. Although this was less frequent than the effects of the 0.25 mg l^{-1} SWCNT treatment. Scale bar = 50 μm, sections were 8 μm thickness and stained with Mallory's trichrome.

design showed no effect on mortality, ventilation rate, haematology, plasma ions etc (data not shown). So, overall only one fish tank out of 12 fish tanks containing solvent showed any solvent effect. It is therefore difficult to attribute the aggressive behaviour and subsequent few mortalities to the solvent.

The choice of solvent is also a concern for the satisfactory development of regulatory test methods for new nanomaterials. Our experience is that a solvent is essential for SWCNTs, and given the concerns about the effects of too much solvent causing deformity or swelling of nanoparticles (Ham et al., 2005), it would seem wise to keep the ratio of solvent: SWCNT roughly the same at each exposure concentration (as we have done). This does mean that the practise of adding "extra" solvent to keep solvent concentrations the same in the one-tank one-dose (pseudo-replicated) design of regulatory fish toxicity tests may need to be reconsidered; or at least some verification that spiking the lower test concentrations with more solvent does not cause deformity of the nanoparticles or changes in their chemical behaviour.

4.2. Respiratory distress and SWCNT precipitation on gill mucus

Aqueous exposure to SWCNT clearly causes respiratory toxicity to trout, with increases in ventilation rate (Fig. 2), mucus secretion, and enlarged mucocytes on the gills (Fig. 3). Increased mucus secretion by the gills is a common response to aqueous pollutants (Mallat, 1985; Handy and Eddy, 1989) and is a short term defence mechanism designed to prevent the toxicant reaching the sensitive gill epithelium. This defence mechanism was

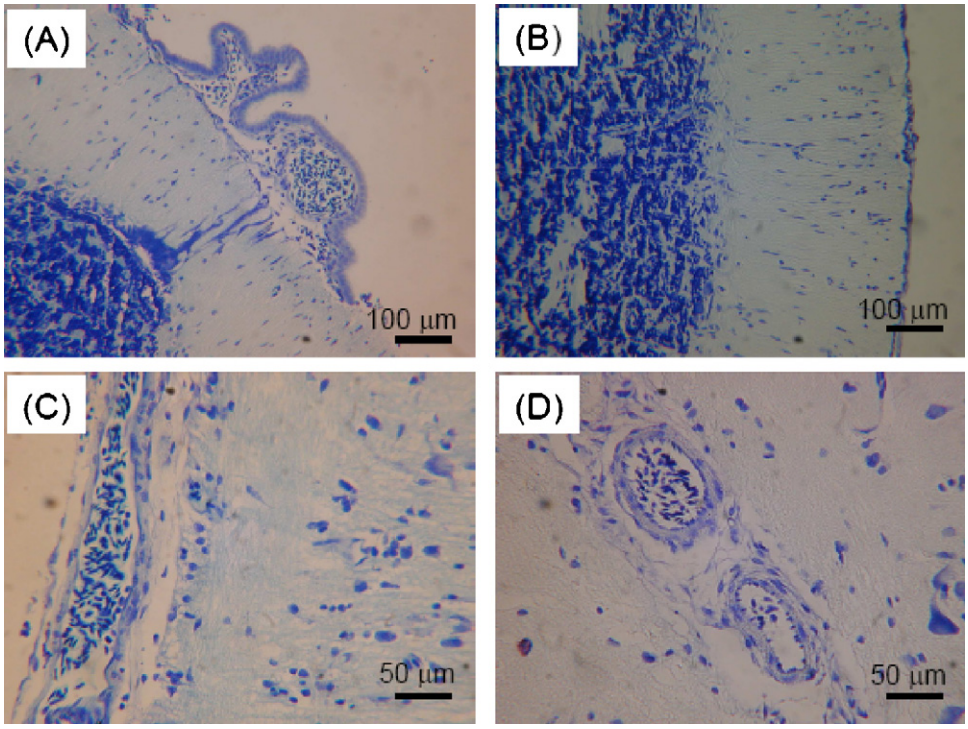

Fig. 8. Brain histology at the end of the experiment. Example of a blood vessel abnormality on the ventral surface of the cerebellum in a fish exposed to 0.5 mg l^{-1} SWCNT (panel A) compared to normal surface architecture on the cerebellum of a solvent control (panel B). Blood vessels in the ventral region of the brain were normal in same fish from the 0.5 mg l^{-1} SWCNT (panel C) and solvent control (panel D). Other minor cellular pathologies are reported in the main text. Scale bar indicates magnification, sections were 8 μm thickness and stained with toluidine blue.

partly successful since SWCNT particles were aggregated in the mucus on the gill surface rather than the gill tissue itself (Figs. 2 and 3). However the protective role of gill mucus secretion cannot be sustained in the long term, and by the end of the experiment gills from fish at the highest SWCNT concentration showed discharged mucocytes. Gill injury had occurred by the end of the experiment (Fig. 3) which would reduce respiratory efficiency (both gas transfer rates and water flow between the lamellae, Malte, 1989) in addition to the effects of the secreted mucus on diffusion (Ultsch and Gros, 1979). It is therefore not surprising to see a dose-dependent increase in ventilation rate (Fig. 2). The types of gill injuries observed (swollen tips of lamellae, enlarged mucocyte, oedema) were not unusual in that numerous chemicals can cause these types of injuries (Mallat, 1985). So for example, it would not be possible to use gill injury to diagnose SWCNT exposure in wild fish. However, we observed a thickening of the primary lamellae during SWCNT exposure which could be indicative of increased cell turnover. Similar observations are made in liver cells (see below). Fish can alter branchial cell turnover and gill dimensions in order to adapt to increased metabolic demands during pollutant exposure (e.g. Handy et al., 1999). Clearly, cellular events in the gill are worthy of further investigation.

4.3. Haematology and ionoregulatory disturbances

The gill is an important osmoregulatory organ in fish, and despite some gill injuries, there was no evidence of major disturbance to salt and water balance or haematology. Fish showed a trend of falling whole blood haemoglobin levels, haematocrits, and red cell counts with increasing SWCNT concentration at each time point (Table 1). However, these effects were within the normal range for trout and haematology was similar to previous reports considering the small juvenile fish used in our study were also not fed. Values in our study (haemoglobin, 3–6 g dl^{-1}; HCT, 21–29%, red cell counts, 0.4–0.7 × 10^6 cells mm^3) are broadly similar to other reports from our laboratory (for fed fish, haemoglobin, 6–7 g dl^{-1}; HCT, 31–43%, red cell counts, 0.6–1.0 × 10^6 cells mm^3; see Handy et al., 1999 and Carriquiriborde et al., 2004). The absence of red cell swelling (no increase in haematocrit), no effects on plasma osmolarity, and the normal plasma Na^+ and K^+ levels at the end of the experiment (Table 1) suggests that fish were able to maintain salt and water balance in the blood. Up-regulation of branchial Na^+K^+-ATPase activity during SWCNT exposure (Fig. 5) probably contributed to this normal plasma electrolyte status, and the secreted mucus (ion rich) would help prevent diffusive electrolyte losses at the damaged gills (Handy, 1989).

The absence of disturbances to tissue Na^+ and K^+ also suggest that SWCNTs are not general ionoregulatory toxicants at the concentrations and exposure durations used in this study. However, there were some dose-dependent losses of Zn and Cu from the gills without Ca^{2+} depletion (Fig. 4), which suggests that SWCNTs may have some specific effects on branchial Cu or Zn transport. The depletion of Cu and Zn was not observed in the other tissues examined (liver, muscle, brain) suggesting this was a specific effect associated with SWCNT exposure in

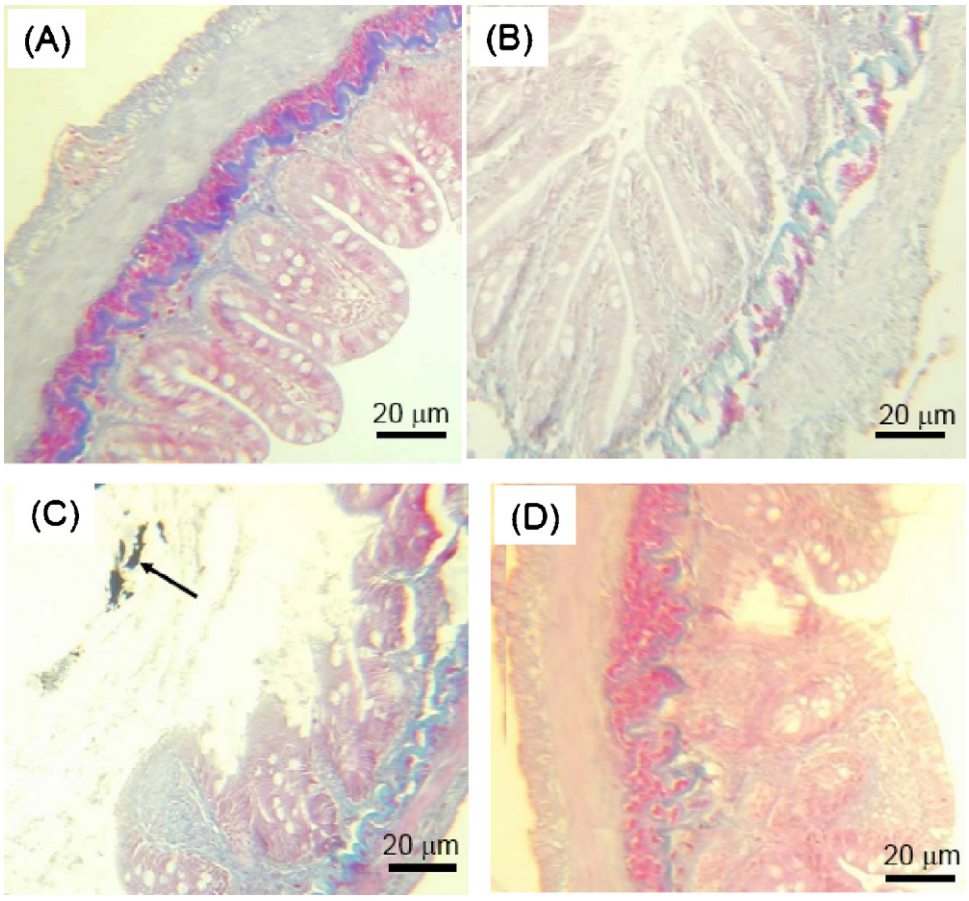

Fig. 9. Histology of the intestine at the end of the experiment. (A) Fresh water control showing normal intestine, (B) solvent control, (C) intestine from a fish in 0.1 mg l^{-1} SWCNT showing erosion of the epithelium and precipitated SWCNT in the gut lumen (arrow), (D) intestine from a fish in 0.5 mg l^{-1} SWCNT showing fusion of intestinal villi. Scale bar = 20 μm, sections were 8 μm thickness and stained with Mallory's trichrome.

the gills. However, we cannot exclude redistribution of existing body Zn or Cu to other tissues during SWCNT exposure, since brain Zn and Cu levels were rising when levels in the gills were decreasing (Fig. 4). Clearly, the effects of SWCNT on Zn and Cu metabolism require further investigation.

4.4. Oxidative stress

The TBARS assay measures the presence of lipid peroxides, and therefore an increase in TBARS gives an approximation of oxidative stress. The values we report (Fig. 6) are broadly similar to previous measurements in trout tissues. For example, TBARS values in our study are between of 2–25 nmol mg^{-1} protein depending on tissue, compared to between 1–10 nmol mg^{-1} protein in Carriquiriborde et al. (2004). Oxidative stress is a concern because mammalian studies have demonstrated lipid peroxidation during carbon nanoparticle exposure (Shvedova et al., 2003; Sayes et al., 2005), and free radical generation is implicated in the accumulation of macrophages and immune responses in tissue damaged by carbon particles (Barlow et al., 2005). For example, Shvedova et al. (2003) found a dose-dependent increase of 30, 33.2 and 45.8% in lipid peroxidation products compared to controls in human keratinocytes exposed to 0.06, 0.12 and 0.24 mg l^{-1} CNT respectively. However, the situation in fish may be different. Oberdörster (2004) showed a decrease in TBARS products in the gill and liver of juvenile largemouth bass exposed to 0.5 mg l^{-1} C_{60} fullerenes for 48 h. We make similar observations in this study, with TBARS decreasing in the gill, liver and brain (Fig. 6). Although unlike the trout, Oberdörster (2004) showed an increase in lipid peroxidation in the brain of largemouth bass that may be attributed to the organic solvent used as well as the fullerenes.

The general fall in TBARS during SWCNT exposure (Fig. 6) is consistent with the absence of fatty change in the liver (Fig. 7), and absence of wide spread vacuolation in the brain of the trout (Fig. 8). It also suggests that SWCNT-induced lipid peroxidation is not the cause of gill injury because gill TBARS did not rise (Fig. 6). Alternatively, induction of other antioxidants might cause TBARS to decrease in tissues. In our study, SWCNT exposure did cause gill and liver total glutathione levels to rise. The rise in total glutathione could be attributed to glutathione synthesis during moderate hypoxia (Lushchak and Bagnyukova, 2006), that might arise from gill injury (Fig. 3) and contribute to the hyper-ventilation response (Fig. 2). The total glutathione values we report (1–2 μmol g^{-1} wet weight of tissue) are similar to previous reports for trout during oxygen-induced stress (1–3 μmol g^{-1} wet weight of tissue, Ritola et al., 2002). Oberdörster (2004) also found that fullerene expo-

sure increased glutathione levels in the liver, but depleted total glutathione from the gills of largemouth bass.

Elevation of reactive oxygen species (ROS) has been implicated in macrophage responses to carbon particles (Barlow et al., 2005) and raised concerns about immune responses in rodent models (see Handy and Shaw, 2007). Carbon nanotubes are known to kill white blood cells. For example, Bottini et al. (2006) showed that 0.4 mg l^{-1} CNT caused apoptosis in human T lymphocytes, with around 50% loss of cell viability. In our study there was no clear dose-dependent effect of SWCNT on total white cell counts (Table 1). However detailed histological investigations of blood smears and spleen prints are still in progress, and we may yet reveal specific effects on parts of the immune system of trout. There was evidence of a time-effect in white cell counts (Table 1), and the decrease in cell counts over time (still in the normal range, see Handy et al., 1999) reflects the unfed status of the fish (e.g. Rios et al., 2005).

4.5. Liver cell injuries

The observation that CNTs can cause apoptosis in mammalian cells (Bottini et al., 2006), is consistent with our observations on trout livers where fish exposed to SWCNT showed condensed nuclear bodies which look like apoptotic bodies (Fig. 7). Trout exposed to SWCNT also showed many more liver cells in nuclear division, especially in proximity to blood vessels (Fig. 7). This suggests that SWCNT do affect the cell cycle, and the location of the injured hepatocytes near blood vessels suggests the factor causing these putative cell cycle effects is being delivered systemically in the blood supply. The toxicological concern is therefore tumour formation in fish livers with a longer exposure period, and this is perhaps no surprise since numerous rodent studies have already reported granulomas in the lung (see Handy and Shaw, 2007). The idea of cell cycle effects is also consistent with observed hyperplasia in the gills (Fig. 3).

4.6. Brain, behaviour and mortality

The reported lipid peroxidation in the brain of largemouth bass during fullerene exposure (Oberdörster, 2004) raises concerns about either direct injury to the brain by nanoparticles, or indirect effects such as oxidative stress or electrolyte disturbances that might result in changes in brain function. In this study there was no biochemical evidence of wide spread oxidative stress in the brain (TBARS unchanged, or decreased, Fig. 6), and the lack of effects on brain Na^+ and K^+ levels (data not shown) or Na^+K^+-ATPase activity (Fig. 5) suggests no major effects on fundamental electrical properties such as resting potentials. The brain did show increasing Zn levels in particular, and increased variability in Ca^{2+}, and a trend of increasing Cu levels during SWCNT exposure. The effect on brain Zn was partly caused by SDS, but also showed dose-dependence with SWCNT concentration (Fig. 4). However, there were no gross pathologies observed within the brain tissue (ignoring the incidence of individual dead cells in the mid-brain) suggesting the divalent ion changes may be physiological and require further investigation. We did not see nanoparticle aggregates in the brain histology, so presumably these are secondary effects on the brain. We also noted that peripheral blood vessels on the brain showed deformity consistent with aneurism (Fig. 8). The vessel walls and dura appeared to be intact, but swollen, so blood supply and oxygenation to the brain might be altered but was not yet completely compromised. This raises the general concern that SWCNT may damage the cardiovascular system of fishes or alter the permeability of the blood-brain barrier. Clearly such vascular lesions associated with a vital organ like the brain will increase the risk of stroke and therefore mortality, or alter behaviour.

Fish showed aggressive behaviour with severe fin nipping during SWCNT exposure. This behaviour started to appear after day 4, and was mainly a concern at the highest SWCNT concentration by day 7. These behaviours got worse on day 9, and now also appeared in one solvent control tank in particular. The aggression caused mortalities on day 9 and we terminated the experiment for ethical reasons the next day. The aggressive behaviour started in the SWCNT tanks (which at the lower doses also had less solvent added), and then in one of the solvent control tanks, so we interpret this mainly as a SWCNT effect, but possibly with some effect of the solvent only at the end of the experiment. The underlying causes of this aggression cannot be firmly established without further biochemical investigations. For example, changes in serotonin levels are associated with motivation and the physiological ability to fight (Campbell et al., 2005). It is intriguing that brain Zn and Cu levels tended to increase in SWCNT-exposed fish, and that fish with higher metal levels are less able to defend themselves in a fight (Campbell et al., 2005).

4.7. Intestine and risk of exposure via the gut

Freshwater fish do drink small amounts of water (a few ml kg^{-1} h^{-1}, Eddy, 1982), and this can increase several fold during stress, with pollutants being known to induce drinking responses (e.g. Best et al., 2003). This raises the possibility of exposure via gut for any aqueous exposure experiment, and we observed aggregates of SWCNTs in the gut lumen of fish from all SWCNT treatments, and injury to the mucosa especially at the lowest SWCNT concentration (Fig. 9). This was associated with an increase in TBARS and Na^+K^+-ATPase at the 0.1 mg l^{-1} SWCNT concentration (Figs. 5 and 6), suggesting at least some effects on the gut. The larger effect at the lowest dose implies either the fish were more willing to drink the lower SWCNT concentrations or it was more bioavailable to the mucosa. The granular appearance of areas of the sub mucosa requires further investigation, but does imply some irritation of the sub mucosal nerve plexus. This is worrying given this nerve plexus is vital to local control of gut function and can be affected by toxicants (Clearwater et al., 2005).

The intestinal histology also has implications for the broader issue of dietary route exposures to nanoparticles. Clearly, we have demonstrated that fish will ingest nanoparticles, and if we also consider the tendency of nanoparticles to aggregate or be adsorbed onto surfaces (Lead and Wilkinson, 2006), then the establishment of nanoparticle exposure via sediment surfaces

and the surface of prey items seems possible. Dietary exposure should therefore be a future concern for risk assessments.

4.8. Conclusions and environmental implications

We have provided the first detailed overview of organ integrity and the physiological effects of SWCNTs in rainbow trout. Overall the data suggests that aqueous SWCNTs are a respiratory toxicant, rather than an ionoregulatory toxicant at the exposure concentrations and durations used here. However, we cannot exclude some subtle disturbances to individual trace elements, and further investigations of especially Zn and Cu are suggested. The absence of pathologies associated with lipid peroxidation in the liver and brain, and the reduction in TBARS in most tissues suggest that the fish were not suffering from overt oxidative stress, contrary to some of the concerns in mammals. Our data also highlights some modes of SWCNT toxicity that have not been identified in fish before, and require further investigation. Examination of the brain and associated blood vessels raises new concerns about subtle neurotoxic or cardiovascular effects of SWCNTs that may alter fish behaviour. The cellular pathologies in the liver are indicative of genotoxicity or cell cycle defects, and raises the concern of carcinogenicity in longer term exposures. Several observations also suggests that SWCNT may be a surface acting toxin on the gills of fish, and mediates a systemic effect via other inflammatory, cell cycle, or vasoactive factors in the blood. The vascular injury on the brain, liver cell pathologies close to blood vessels, but the histological absence of large polymerised CNT fibrous plaques inside any of the tissues examined (observed in mammalian lung, Lam et al., 2004) support this notion. Further investigations of such factors in the blood are needed, along with more detailed electron microscopy of tissues for evidence of CNTs to test this idea.

The environmental implications of acute exposure to these low mg l^{-1} levels of SWCNTs are clear. The fish might successfully tolerate exposure for the first few days, but after 1–2 weeks of continuous exposure it is very unlikely that trout (with the levels of gill injuries and respiratory distress we report) would have the cardiovascular fitness to successfully forage or perform other routine behaviours in the wild. The risk of mortality would therefore be high (Campbell et al., 2005). Predictions for chronic effects are less clear, the aetiology of possible genotoxicity or carcinogenicity in the liver will depend partly on immunity and tissue repair processes. Similarly, we may expect subtle changes in the cell populations in the gills, and perhaps specific trace element disturbances over longer time scales. This will increase metabolic costs and food (energy intake) requirements. Although we now know that trout will ingest water containing SWCNTs, the effects on digestion, nutrient absorption, and energy budgets remain to be investigated.

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