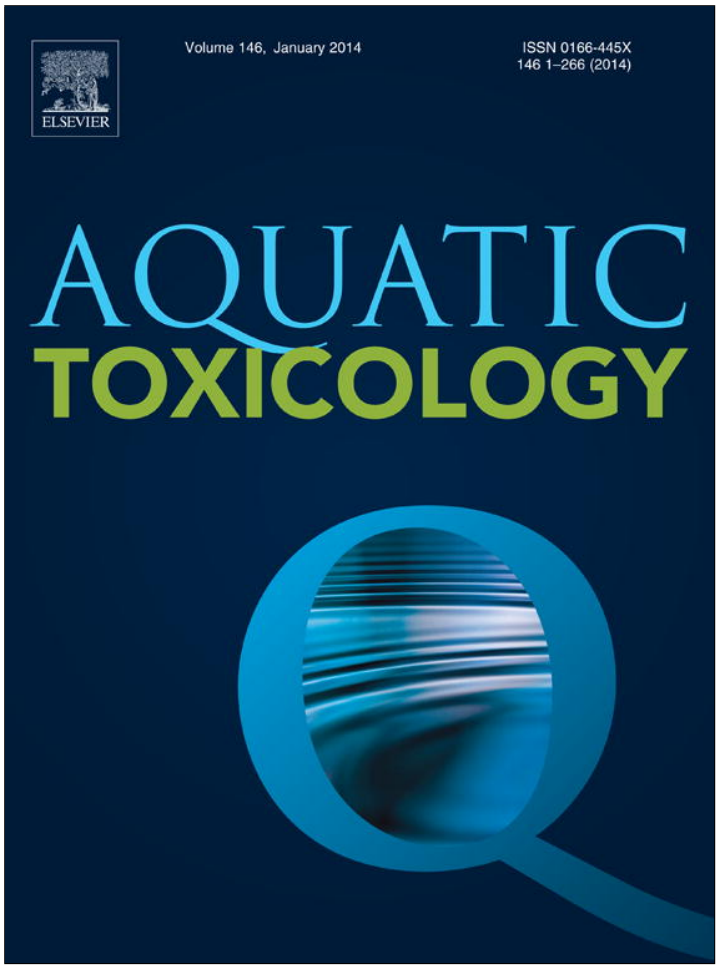

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Minimal effects of waterborne exposure to single-walled carbon nanotubes on behaviour and physiology of juvenile rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Fish behaviours are often considered to be sensitive endpoints of waterborne contaminants, but little attention has been given to engineered nanomaterials. The present study aimed to determine the locomotor and social behaviours of rainbow trout (*Oncorhynchus mykiss*) during waterborne exposure to single-walled carbon nanotubes (SWCNTs), and to ascertain the physiological basis for any observed effects. Dispersed stock suspensions of SWCNTs were prepared by stirring in sodium dodecyl sulphate (SDS), an anionic surfactant, on an equal w/w basis. Trout were exposed to control (no SWCNT or SDS), 0.25 mg L^{-1} SDS (dispersant control), or 0.25 mg L^{-1} of SWCNT for 10 days. Video tracking analysis of spontaneous locomotion of individual fish revealed no significant effects of SWCNT on mean velocity when active, total distance moved, or the distribution of swimming speeds. Hepatic glycogen levels were also unaffected. Fish exposed to SWCNTs retained competitive fitness when compelled to compete in energetically costly aggressive interactions with fish from both control groups. Assessment of the respiratory physiology of the fish revealed no significant changes in ventilation rate or gill injuries. Haematocrit and haemoglobin concentrations in the blood were unaffected by SWCNT exposure; and the absence of changes in the red and white pulp of the spleen excluded a compensatory haematopoietic response to protect the circulation. Despite some minor histological changes in the kidneys of fish exposed to SWCNT compared to controls, plasma ion concentrations and tissue electrolytes were largely unaffected. Direct neurotoxicity of SWCNT was unlikely with the brains showing mostly normal histology, and with no effects on acetylcholinesterase or Na^+/K^+-ATPase activities in whole brain homogenates. The minimal effects of waterborne exposure to SWCNT observed in this study are in contrast to our previous report of SWCNT toxicity in trout, suggesting that details of the dispersion method and co-exposure concentration of the dispersing agent may alter toxicity.

1. Introduction

The 2010 Nobel Prize for physics for the discovery of graphene has renewed interest in carbon-based engineered nanomaterials (NM), and there has been a steady rise in the commercial applications of carbon nanotubes (CNTs); including electronics, industrial polymers and coatings (De Volder et al., 2013). Carbon nanotubes are cylindrical structures with a high aspect ratio and exceptional thermal and electrical conducting properties (Mauter and Elimelech, 2008). There have been several recent reviews which identify the potential for environmental hazards of carbon-based NMs (Handy et al., 2011; Henry et al., 2011; Petersen et al., 2011). Initial models predicted environmental concentrations of CNTs in effluents at around a few ng L^{-1} or less (e.g., 14.8 ng L^{-1}, Gottschalk et al., 2009), but recent studies on fullerene concentrations in effluents suggest μg L^{-1} concentrations may be more likely for carbon-based materials (Farré et al., 2010). With environmental release expected to rise with manufacturing output, studies of the toxicological effects of NMs have often focussed on aquatic organisms, especially fishes (e.g., reviews by Handy et al. (2011), Shaw and Handy (2011)). However, the information on CNTs is relatively sparse compared to knowledge on the ecotoxicity of metallic NMs in fishes (Handy et al., 2011; Shaw and Handy, 2011). Concentrations of single walled carbon nanotubes (SWCNTs) of 1 mg L^{-1} for

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10 days caused gill injury and respiratory distress in trout (Smith et al., 2007); but there remains a concern for sub-lethal effects in fishes exposed to lower CNT concentrations (<1 mg L^{-1}), as well as ability to discern any CNT-specific effects from those of the dispersing agents used (Petersen et al., 2011). In addition, not all the target organs or body systems effects of CNTs have been elucidated (Handy et al., 2011).

Animal behaviours are often sensitive toxicological endpoints and behavioural deficits have been reported in fish exposed to several classes of contaminant including metals and organic compounds (see review by Scott and Sloman (2004)). The effects of NMs on fish behaviours are largely unknown but there is an emerging literature to suggest that some NMs may affect the brain. Notably, the induction or changes in biomarkers of oxidative stress in brains of fish exposed to several NMs (SWCNTs, Fraser et al., 2011; Smith et al., 2007; TiO_2 nanoparticles (NPs), Boyle et al., 2013a; Federici et al., 2007; Hao et al., 2009; Cu NPs, Shaw et al., 2012) has raised particular concern because of the role of oxidative stress in the etiology of some neurodegenerative disorders (e.g., de Oliveira et al., 2012). However, the effects on brain tissue are often subtle, and may vary with the route of exposure. In addition, secondary oxidative stress in the brain from systemic hypoxia following gill injury often cannot be excluded (e.g., TiO_2 NPs, Boyle et al., 2013a). Nonetheless, it is possible that NMs could affect behaviours by impairing neural processes.

Nanomaterials may also impact fish behaviours through changes in energy metabolism. Activity rates (locomotion) form a large component of the tradable energy expenditure in some fishes (Priede, 1977). For example, the proportion of the energy budget for locomotion can be as high as 74% in juvenile salmonids (Boisclair and Sirios, 1993). Fishes may sacrifice locomotion for other components of the energy budget during contaminant exposure, such as tissue repair. This response has previously been observed in rainbow trout fed diets containing elevated Cu where spontaneous swimming activity was decreased to offset the physiological cost of exposure and/or preserve growth rates (Campbell et al., 2002; Handy et al., 1999). Moreover, recent data from our laboratory group suggests that this may also occur in fish exposed to NMs. In individual rainbow trout (*Oncorhynchus mykiss*) exposed to TiO_2 NPs, time spent swimming at energetically expensive high speeds was decreased compared to controls to compensate for gill injury and a developing systemic oxygen debt (Boyle et al., 2013a). However, TiO_2 NP exposure did not affect the outcome of aggressive social encounters with control fish and this suggests that fish retained competitive fitness by selectively decreasing energy expenditure on spontaneous locomotion when unmotivated.

The aim of the present study was to investigate if waterborne exposure to SWCNTs affected the swimming behaviour and the outcome of competitive interactions in juvenile rainbow trout. One hypothesis is that changes in locomotion or behaviour are mediated by brain injury. In rainbow trout exposed to SWCNT, enlargement of blood vessels surrounding the cerebellum has been reported, and fish were also observed to have increased aggression (Smith et al., 2007). However, an alternative hypothesis is that gill injury and respiratory distress contribute to reduction in locomotion and increased aggression in fish exposed to waterborne NPs (Boyle et al., 2013a,b; Smith et al., 2007).

2. Materials and methods

2.1. Experimental design

Juvenile rainbow trout (30.0 ± 5.7 g, mean ± standard deviation (*S.D.*) $n = 112$) were obtained from a local supplier (Torre fishery, Watchet, Somerset) and maintained in a recirculating system with aerated, dechlorinated Plymouth City water (means ± *S.D.*, $n = 6$, in mmol L^{-1}, Ca^{2+}, 0.30 ± 0.02; K^+ 0.02 ± 0.01; Mg^{2+}, 0.04 ± 0.01; Na^+, 0.39 ± 0.05) for four weeks prior to onset of experimentation. Fish were fed twice daily with a commercial trout diet (EWOS, Westfield, UK) until 48 h prior to experimentation. The effects of SWCNT on physiology and behaviour of juvenile rainbow trout were investigated during a 10 day waterborne exposure to either a control [no added SWCNT or sodium dodecyl sulphate (SDS)], 0.25 mg L^{-1} SDS, or 0.25 mg L^{-1} SWCNT dispersed in 0.25 mg L^{-1} SDS in a quadruplicate test design ($n = 4$ replicate aquaria/treatment; equating to 56 fish/treatment). After 10 days fish were subject to behavioural measurements (swimming speed and distance moved by video tracking, or a paired interaction challenge), and tissues were collected to assess trace element homeostasis, haematology, histopathology, and biochemical measurements (see Sections 2.3–2.6). The exposure concentration was chosen to match our previous study on trout by Smith et al. (2007) in which 0.25 mg L^{-1} SWCNT was observed to cause biochemical and haematological disturbances and a subtle indication of a behavioural response (increased gill ventilation rate and aggression) in fish exposed for 10 days. For logistical reasons the exposures were staggered by 48 h in half the tanks to enable all the behavioural measurements to be made. It was only possible to perform behavioural observations in $n = 8$ pairs of trout and record movements of $n = 8$ fish using video tracking software per day (see Sections 2.7 and 2.8).

Following the maintenance period fish were randomly allocated to twelve acid-washed glass aquaria containing 20 L of continuously aerated water (means ± *S.D.* $n = 10$–96 measurements, pH 6.3 ± 0.3; Dissolved oxygen 8.9 ± 0.5 mg L^{-1}; NH_3, <0.001 mg L^{-1}; temperature, 14 ± 1 °C; photoperiod, 14 h light: 10 h dark) and fish were allowed to rest overnight (unfed) prior to the first dosing: the dosing was achieved using a semi-static exposure method modified from Smith et al. (2007) where each tank was dosed with 10 mL of the appropriate stock dispersion (see Section 2.2). Strong aeration in the tanks rapidly spread the material throughout the tank water. An 80% water change was conducted every 12 h, and relevant tanks re-dosed with either 8 mL SWCNT or SDS. To minimise aggression, fish were fed once during the experiment on day 5 at a ration of 1% of total fish body weight. This was done post water change but prior to re-dosing to minimise the risk of ingestion of SWCNT and/or SDS. Fish in all tanks were observed to consume all feed immediately. The entire experiment was under ethical approval and fish were subject to independent health checks during the work.

2.2. SWCNT suspension

The SWCNT in this study were the same as those used by Smith et al. (2007) and Fraser et al. (2011) that were originally obtained from Cheap Tubes Inc. (Brattleboro, VT, USA). As previously described, tubes have a mean outside diameter of 1.1 nm and a length of 5–30 μm (Fraser et al., 2011; Smith et al., 2007). Purity was a minimum of 96.3 wt% with ash comprising <1.5 wt%. The SWCNT stock was prepared in sodium dodecyl sulphate (SDS), an anionic detergent which has been shown to aid dispersion of SWCNT in suspension when compared to other solvents and water alone (Ham et al., 2005). The concentration of SDS chosen (0.5 g L^{-1}) was less than that used by Smith et al. (2007) who attributed minor toxicities in trout to the vehicle control. To prepare the SWCNT suspension, 0.25 g SDS was added to 100 mL distilled water in an acid washed Duran bottle and shaken to dissolve. Then, 0.25 g SWCNT was added with a further 400 mL distilled water and the suspension dispersed overnight with a magnetic stirrer (2400 rpm, Magnetic Stirrer SM1, Stuart Scientific, UK). The resultant suspension was well dispersed with a homogeneous distribution of SWCNT easily obtained by short duration (30 s) vigorous shaking prior to dosing of the experimental aquaria. Stocks were not sonicated because sonication has

been shown to decrease the length of CNTs, likely by fracturing and this may introduce other allotropes of carbon into the suspension (Ruan and Jacobi, 2012). Analysis of the stock suspension (0.5 g L^{-1}) with transmission electron microscopy (TEM, JEOL 12000EXII, Tokyo, Japan) showed the SWCNT to be present as individual dispersed tubes, through to larger agglomerates. Stock suspensions of 0.25 g SDS in 500 mL water were also prepared in an identical manner for dosing of the SDS vehicle control treatment group.

2.3. Haematology and plasma analysis

Haematological analyses were performed according to an original method outlined in Handy and Depledge (1999). For specific details of methods, reagents and modifications see Boyle et al. (2013a). Briefly, two fish per tank (8/treatment) were terminally anaesthetised in 200 mg L^{-1} buffered MS222 (pH 7.0) and weight and total length recorded. Blood was immediately withdrawn by caudal puncture with a heparinised syringe and sub-samples of whole blood were taken for measurement of haematocrit (HCT) and haemoglobin (Hb, Drabkin's reagent, Sigma-Aldrich, UK). Remaining blood was centrifuged (13,000 rpm, 2 min) and osmolality (Osmomat 030, Gonotec, UK), Na^+ and K^+ (Model 420 Flame Photometer, Sherwood Scientific Ltd, UK) were measured in the plasma. After sampling tissues were dissected in the order of gill, intestine, liver, spleen, kidney, muscle and brain for trace element analysis (see Section 2.4).

2.4. Tissue ion analysis

Samples of gill (lamellae from second and third gill arches), brain, liver, spleen, kidney and muscle (immediately beneath dorsal fin) were oven dried to a constant weight and digested at 60 °C for 2 h in 1 mL concentrated HNO_3 (68% nitric acid, trace element grade, Fisher Scientific, UK). Following digestion, samples were diluted to 4 mL with distilled water and analysed in triplicate for Ca^{2+}, Cu, Fe, K^+, Mg^{2+}, Na^+ and Zn by ICP-OES (Varian 725-ES). Matrix matched acidified element standards were measured every 10–15 samples to monitor the instrument for drift and recoveries of analytical standards spiked into tissue digests at known concentrations have previously confirmed good instrument performance (Boyle et al., 2013a,b).

2.5. Biochemical analyses

Biochemical analyses were performed on selected tissues (gill, brain, liver, kidney and spleen) according to methods outlined in Boyle et al. (2013a). A further two fish per tank were terminally anaesthetised and tissues excised (as detailed in Section 2.3), then snap frozen in liquid N_2 and stored at −80 °C until required. Tissues were homogenised on ice (3 × 10 s with 2 min rests at 17,500 rpm, Cat X520D with a T6 shaft, medium speed, Bennett & Co., Weston-super-Mare) in ice cold isotonic buffer (300 mmol L^{-1} sucrose, 20 mmol L^{-1} HEPES, 0.1 mmol L^{-1} EDTA, pH 7.8). Homogenates were centrifuged at 13,000 rpm for 2 min and the supernatants decanted and stored at −80 °C until analysed. Total glutathione (GSH), acetylcholinesterase (AChE) and concentrations of thiobarbituric reactive substances (TBARS) were measured according to Boyle et al. (2013a) and references therein. Briefly, GSH was quantified in triplicate reactions in buffer with final assay concentrations of 76.5 mmol L^{-1} phosphate buffer (pH 7.5), 3.8 mmol L^{-1} EDTA, glutathione reductase (0.12 U mL^{-1}), 0.6 mmol L^{-1} 5′5′-dithiobis-2-nitrobenzoic acid (DTNB), 0.2 mmol L^{-1} ß-Nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH) and absorbance at 412 nm monitored for 20 min on a Versa Max microplate reader (Molecular Devices (UK) Ltd, Wokingham, UK). Activity of acetylcholinesterase (AChE) was measured by recording absorbance at 405 nm for 10 min of a reaction mixture containing 50 μL of brain homogenate, 50 μL of 3 mmol L^{-1} acetylthiocholine iodide and 150 μL of 0.27 mmol L^{-1} DTNB. The concentrations of TBARS were measured in deproteinated samples (50% trichloroacetic acid) in phosphate buffer (100 mmol L^{-1}, pH 7.5) and treated with 1 mmol L^{-1} butylated hydroxytoluene to reduce spontaneous oxidation of samples. Deproteinated samples were incubated with 1.3% thiobarbituric acid in 0.3% NaOH and incubated at 60 °C for 60 min and absorbance at 530 nm was recorded against 0–50 μmol L^{-1} 1,1,3,3-tetraethoxypropane standards (Sigma, UK). Activity of Na^+/K^+-ATPase was assayed at 340 nm, with and without 0.5 mmol L^{-1} ouabain, a Na^+/K^+-ATPase inhibitor, in buffer containing 3 U mL^{-1} lactate dehydrogenase, 3.75 U mL^{-1} pyruvate kinase, 2.1 mmol L^{-1} phosphoenolpyruvate, 0.53 mmol L^{-1} ATP, 0.16 mmol L^{-1} NADH, 37.25 mmol L^{-1} HEPES, 47.25 mmol L^{-1} NaCl, 2.63 mmol L^{-1} $MgCl_2$, 10.5 mmol L^{-1} KCl (pH 7.5) according to the method of McCormick (1993). The concentrations of GSH, TBARS and activities of AChE and Na^+/K^+-ATPase were normalised to total protein in the supernatant using the Bradford's reagent according to the manufacturer's protocol (Sigma-Aldrich, UK). All samples were analysed in triplicate for all assays.

2.6. Histopathology

At day 10 of the exposure two fish per tank were anaesthetised in buffered MS222 and tissues carefully collected into 10% neutral buffered formal saline, dehydrated and embedded in paraffin (Leica TP1020, Leica TPEG1150H, Wetzlar, Germany), after which 7 μm sections were cut for routine histological examination as described in Boyle et al. (2013a). Gills and spleens were stained with Mallory's trichrome. Kidneys and whole brains were stained with haemotoxylin and eosin to show general architecture. All specimens were prepared and stained in batches that included samples from all treatments to minimise staining artefacts between treatments. Slides were observed using an Olympus Vanox-T microscope and photographed on an Olympus digital camera (C-2020 Z), then scored blind to eliminate observer bias. The incidence of gill injuries were quantified by scoring 100 secondary lamellae from each fish for the appearance of pathologies including club tips, fusion of lamellae, aneurysms and epithelial lifting. Fractional areas of tissues in organs were counted manually from randomly selected areas on a section from each fish 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 proportion of red pulp, white pulp and sinusoid space in the spleen. The number of melanomacrophages in each image was also counted. Quantification of morphometrics in the kidney included measuring the total diameter of the renal tubules (proximal and distal) as well as the diameter of the lumen and the width of epithelium in the tubules, from at least 10 random tubules/field of view, and in a representative slide from each fish. The proportion of the total tubule diameter that was lumen was calculated for all tubules. Diameters of the glomerulus and Bowman's space were similarly measured.

2.7. Swimming behaviour

Following the 10 day experimental exposure swimming behaviour of trout was assessed in 1 or 2 randomly selected fish per tank ($n = 5/6$ per treatment) with video tracking software (Ethovision XT *v.* 7.1, Noldus Information Technology, The Netherlands) and as according to Boyle et al. (2013a). Fish were netted from the exposure aquaria and transferred to floor mounted chambers (65 × 40 × 30 cm) filled with 30 L of aquarium water (water parameters were the same as the main experiment, see Section 2.1)

and without SWCNT or SDS in suspension. Fish were then separated from the rest of the aquarium by a curtain to minimise disturbance and left to acclimate to conditions overnight (approximately 24 h). Between 10:00 and 13:00 the following day behaviours were recorded. All recordings were viewed and processed to correct miss-tracks and remove small movements of <0.4 cm between consecutive time points. Ethovision software calculates animal movements from the position of an animal's centre point and observation of tracks indicated fish movements <0.4 cm were often due to the flickering of aquarium lighting as opposed to actual movement. Positional data for fish were then analysed to express fish movements as total distance moved, time inactive, mean velocity when active and time spent at low, medium and high speeds following an approach previously used to assess effects of Cu and TiO_2 NPs on activity levels in trout (Boyle et al., 2013a; Campbell et al., 2002; Handy et al., 1999).

Immediately after tracking had finished, fish were terminally anaesthetised and samples of liver and muscle (red and white) immediately beneath dorsal fin were snap frozen in liquid N_2 and stored at −80 °C until analysed for concentrations of glycogen and triglycerides (livers) and lactate (muscle). Lactic acid was analysed in deproteinated (equal volume of 5% TCA), pH neutralised muscle homogenised in isotonic sucrose buffer (see Section 2.5) according to Gutmann and Wahlefeld (1974). Briefly, 10 μL sample was diluted in 200 μL glycine-hydrazine buffer (0.4 M, pH 9.0) containing 10 μL of 40 mmol L^{-1} NAD and 1 U lactate dehydrogenase and incubated for 2 h at 37 °C. Absorbance at 340 nm was then compared to appropriate 0–8 mmol L^{-1} lactic acid standards. Glycogen in livers of fish was measured according to a methodology outlined by Packard and Packard (2005) using a commercial kit to measure glucose (Sigma, UK) after acid hydrolysis and amyloglucosidase digestion. True triglycerides in livers of fish were analysed in deproteinated (equal volume of 5% TCA), pH neutralised liver homogenised in isotonic sucrose buffer (see Section 2.5) and assayed according to supplier of commercial kit (Sigma, UK).

2.8. Competitive interaction

These measurements broadly followed the methodology of Sloman et al. (2000) as adapted by Boyle et al. (2013a) and manually scored using event-logging software specific to animal behavioural research (Observer XT *v.*7, Noldus Information Technology, The Netherlands). Behavioural interactions were observed in pairs of rainbow trout from the following treatment groups: 1) a SWCNT exposed fish paired with a control fish (n = 8 pairs), and 2) a SWCNT exposed fish paired with a SDS exposed fish (n = 8 pairs). Following the 10 day exposure, fish were lightly anaesthetised to facilitate handling (50 mg L^{-1} MS222, buffered to pH 7.0), weighed and total length measured. Size matched pairs of fish (±2 mm length, ±1 g weight) were then selected and the caudal fin injected with Alcian blue to enable identification of fish from different treatment groups during observations. Fish were revived in strongly aerated aquarium water, monitored to ensure good health (no lasting effects were observed on fish behaviour) and were removed to glass aquaria fitted with an opaque partition and containing 20 L of constantly aerated aquarium water [see Section 2.1 for water parameters (same as for main experiment)]. Neither SWCNT nor SDS was added to the water. Once fish had been allowed to acclimate to the conditions for 24 h, the partitions were removed and interactions between fish observed for a 30 min period commencing from the first aggressive act (bite or charge). Interactions were scored according to the number of aggressive acts by each fish (subsequently identified as the subordinate or dominant fish in the data analysis), the time of retreat of the subordinate fish and the position of the fish in the water column.

Table 1
Haematology and plasma ions in rainbow trout exposed to control (no added SWCNT or SDS), 0.5 mg L^{-1} of SDS or 0.5 mg L^{-1} of SWCNT for 10 days.

	Control	SDS	SWCNT
Haematocrit (%)	26.6 ± 0.5	25.8 ± 0.4	25.6 ± 0.5
Haemoglobin (g dL^{-1})	5.44 ± 0.21	5.60 ± 0.35	6.33 ± 0.60
Mean Corpuscular Haemoglobin (g dL^{-1})	0.21 ± 0.01	0.22 ± 0.02	0.25 ± 0.03
Osmolality (mOsm kg^{-1})	281.3 ± 4.7	283.0 ± 8.3	276.3 ± 10.0
Plasma Na^+ (mmol L^{-1})	127.0 ± 3.2	127.4 ± 6.5	133.0 ± 5.3
Plasma K^+ (mmol L^{-1})	2.87 ± 0.13	3.21 ± 0.24	3.00 ± 0.16

Data are means ± S.E.M. (n = 7/8). Mean corpuscular haemoglobin (MCH) = haemoglobin/haematocrit. Haematology and plasma ions in fish at day 0 (means ± S.E.M., n = 7/8): haematocrit, 29.5 ± 0.3%; haemoglobin, 6.77 ± 0.34 g dL^{-1}; MCH, 0.23 ± 0.01 g dL^{-1}; osmolality, 314.0 ± 5.0 mOsm kg^{-1}; plasma Na^+, 148.0 ± 3.7 mmol L^{-1}; plasma K^+, 3.71 ± 0.14 mmol L^{-1}.

2.9. Data handling and statistical analyses

Statistical analyses were performed using SPSS (version 18.0 for Windows). All data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). Where data were not normally distributed log-transformation was attempted. Where log-transformation failed, non-parametric tests were used. Statistically significant differences between treatment groups were determined by ANOVA with Tukey's test aposteriori. Data of paired contact interactions between fish were analysed with Student's t test or Mann–Whitney U-test as appropriate. A p value of ≤0.05 was considered significant.

3. Results

3.1. Mortality and ventilation rates

No mortalities were observed in fish kept under control conditions or in fish exposed to SWCNT. In two aquaria of fish exposed to SDS there was considerable aggression between fish from the start of the exposure. In both tanks this led to the removal of two fish on welfare grounds and the removal of two aggressors. This had the effect of stabilising the experimental populations and by day 4 the aggression between fish in these tanks, as in controls and fish exposed to SWCNT, was minimal.

Gill ventilation rates were assessed on days 0, 4 and 8 of the exposure. There was no significant difference between treatment groups and ventilation rates did not change over time. At day 8, ventilation rates of control fish were (mean ± *S.D.*, n = 16) 65.4 ± 11.4 min^{-1} compared to 60.0 ± 5.4 min^{-1} and 68.4 ± 8.4 min^{-1} in fish exposed to SDS and SWCNT, respectively.

3.2. Haematology and plasma ions

Exposure to SWCNT did not affect haematology in trout (Table 1). Neither the haematocrit nor the concentration of haemoglobin in blood was significantly different between controls and fish exposed to SWCNT and SDS alone. Plasma ions were similarly unchanged (Table 1). Plasma Na^+ and K^+ concentration and also osmolality were not significantly affected by 10 day exposure to SWCNT when compared to either control.

3.3. Tissue electrolytes

Tissue electrolyte and trace element concentrations were generally unaffected by either SWCNT or SDS exposure, but with several notable exceptions (Fig. 1). Data of Cu concentrations in tissues are not shown in Fig. 1 due to the low concentration of Cu in all tissues (<0.2 μmol g^{-1}, except liver <5.0 μmol g^{-1}). Exposure to SWCNT did not affect Cu concentrations in gill, brain, liver,

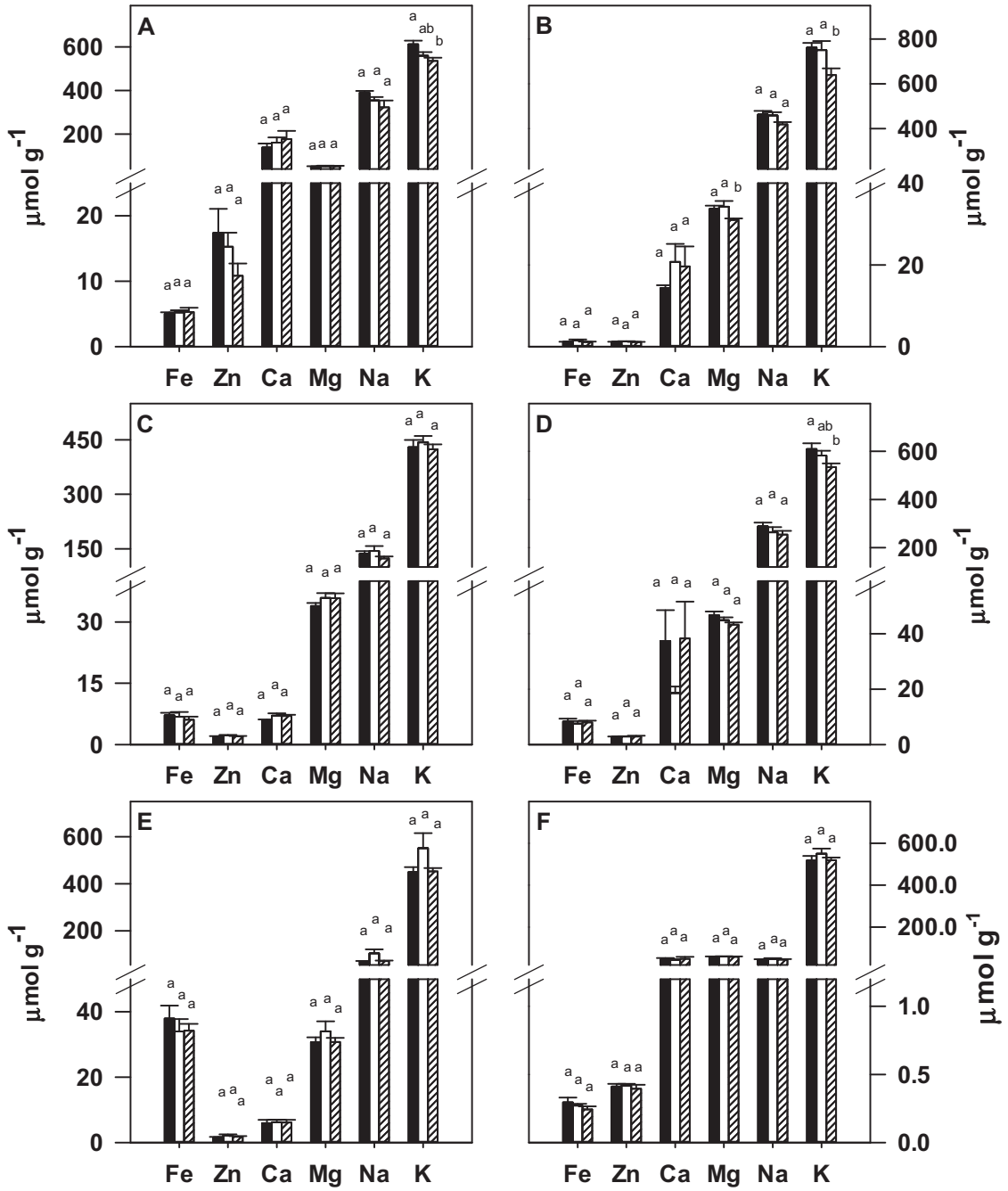

Fig. 1. Concentration (µmol g^{-1} dry weight) of ions in (A) gill, (B) brain, (C) liver, (D) kidney, (E) spleen and (F) muscle of rainbow trout exposed to control (closed bars), 0.25 mg SDS L^{-1} (open bars) and 0.25 mg SWCNT L^{-1} (hatched bars) for 10 days. Data are means ± S.E.M. (n = 6–8). Different lower case letters denote significant difference between treatment groups ($p \leq 0.05$). See text for Cu concentration in tissues.

kidney or muscle (data not shown). However, there was a significantly lower (ANOVA, $p = 0.004$) Cu concentration in spleen of fish exposed to SDS (mean ± S.E.M., $n = 7/8$), 0.03 ± 0.01 µmol g^{-1} dry weight (dw), compared to controls, 0.10 ± 0.01 µmol g^{-1}, but not fish exposed to SWCNT, 0.07 ± 0.01 µmol g^{-1} (ANOVA, $p > 0.05$). Tissue electrolytes were also affected in the gill. Gill K^+ was significantly lower in fish exposed to SWCNT (mean ± S.E.M., $n = 7/8$), 535 ± 14 µmol g^{-1}, compared to controls, 612 ± 17 µmol g^{-1}, but there was no significant difference compared to fish exposed to SDS, 559 ± 17 µmol g^{-1}. Kidney K^+ was also decreased in fish exposed to SWCNT compared to controls but not fish exposed to SDS (Fig. 1). However, exposure to SWCNT did cause perturbations in brain electrolytes that were not seen in fish exposed to SDS alone. There was a significant (ANOVA, $p < 0.019$) 15% decrease in brain K^+ compared to controls and fish exposed to SDS. The concentration of Mg^{2+} was also significantly lower in SWCNT exposed fish, 31.0 ± 0.4 µmol g^{-1} compared to 33.6 ± 0.8 µmol g^{-1} and 34.2 ± 1.4 µmol g^{-1} in controls and SDS exposed trout, respectively (Kruskal–Wallis, $p = 0.028$).

3.4. Biochemical markers of ionoregulatory and neuromuscular function, and oxidative injury

Tissue concentrations of TBARS, GSH and activities of Na^+/K^+-ATPase and AChE are shown in Fig. 2. Overall, there was minimal evidence of oxidative perturbations in organs and no significant

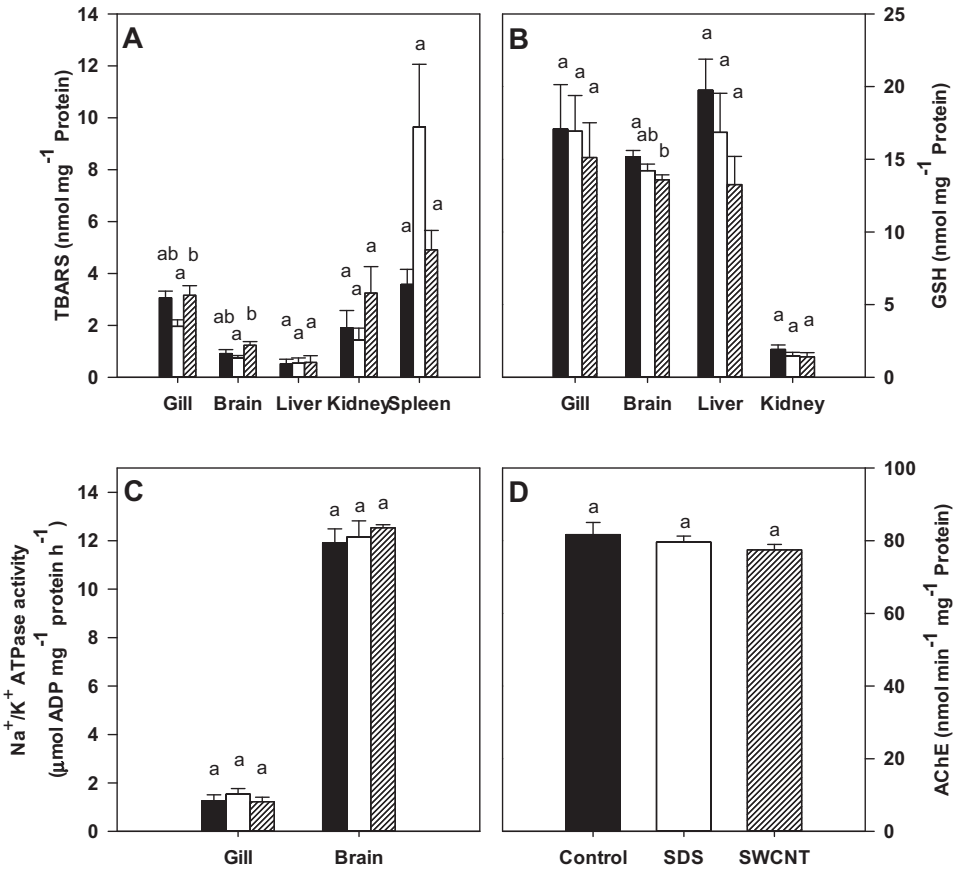

Fig. 2. Concentration of (A) thiobarbituric reactive substances (TBARS; nmol mg^{-1} protein); (B) total glutathione (GSH; nmol mg^{-1} protein); and activity of (C) Na^+/K^+-ATPase (µmol ADP mg^{-1} protein h^{-1}); (D) acetylcholinesterase (AChE; nmol min^{-1} mg^{-1} protein) in tissues of rainbow trout exposed to control (closed bars), 0.25 mg SDS L^{-1} (open bars) and 0.25 mg SWCNT L^{-1} (hatched bars) for 10 days. Data are means ± S.E.M. (n = 6–8). Different lower case letters denote significant difference between treatments ($p \leq 0.05$).

changes in enzyme activities. The concentration of TBARS in the liver, kidney and spleen was not affected by SWCNT exposure (Fig. 2A, ANOVA/Kruskal–Wallis test, $p > 0.05$). There were statistically significant differences in TBARS in gill (ANOVA, $p = 0.044$) and brain (ANOVA, $p = 0.017$) of fish but post-hoc analysis showed these differences to be between fish exposed to SDS and SWCNT and neither group were significantly different from controls. There were no statistically significant differences in GSH in gill, liver or kidneys of fish (ANOVAs, $p > 0.05$). However, there was a significant effect of SWCNT exposure on GSH in brains of fish (ANOVA, $p = 0.043$); GSH was significantly decreased in fish exposed to SWCNT compared to controls, but there was no difference when compared to fish exposed to SDS alone. Measured concentrations of GSH in brains of fish exposed to control, SDS and SWCNT were (means ± S.E.M.): 15.2 ± 0.4, 14.2 ± 0.5 and 13.6 ± 0.3 nmol mg^{-1} protein, respectively. Activities of Na^+/K^+-ATPase and AChE were analysed in gill and brain and are shown in Fig. 2C and D. Overall, activities of these enzymes were unaffected by exposure to SWCNT.

3.5. Histological observations

Waterborne exposure to SWCNT did not cause acute gill pathology in rainbow trout (Fig. 3A–C). At the end of the exposure, fish examined from each of the treatment groups showed normal gill morphology with a few incidences of gill pathologies, including club tips, epithelial lifting and fusion of the secondary lamellae which is consistent with appearance of gills in laboratory maintained fish. The total percentages of secondary lamellae exhibiting pathologies were not significantly different between treatment groups and were (means ± S.E.M., ANOVA, $p > 0.05$): 2.3 ± 0.7, 4.3 ± 1.6 and 6.3 ± 1.6% in controls, SDS and SWCNT fish. Similarly there was no discernible treatment effect of SWCNT detected upon histological examination of the brains of fish. Control fishes (6/6 brains examined) showed normal morphology without vacuole formation, oedema or vascular injury in the tissues, and no evidence of necrotic nerve cell bodies. The brains from SDS solvent controls also showed normal morphology (as above), apart from a rare (background incidence) of one or two necrotic nerve cell bodies in the cerebrum (4/5 fish examined). Brains from fish exposed to SWCNT generally showed normally morphology (5/5 fish examined) that was not discernibly different from the solvent control and showed a similar background incidence of rare necrotic cell bodies (data not shown). Generally all 5/5 examined show normal tissue, except one fish that had evidence of hydropic change in the medulla oblongata, and some irregularity to the cell body layer in the tectum with a small foci of vacuoles. Where blood vessels were observed in the ventral surface of the tectum (mid-brain), these were sometimes dilated, but there was no evidence of haemorrhage. Similarly, on the dorsal surfaces of the brain, blood vessels appeared intact in all animals examined from all treatments.

Kidneys of both control fish (Fig. 3D) and fish exposed to SDS (Fig. 3E) were normal with good definition of the epithelial cells in the kidney tubules, with no evidence of necrosis or haemorrhage from the glomerulus and an intact Bowman's capsule. One control fish (n = 1/8 fish examined) showed some diffuse, mild necrosis of some kidney tubules, but otherwise had normal histology. One fish exposed to SDS showed evidence of a mild peri-venule haemorrhage in a few glomeruli in the renal cortex (n = 1/8 fish examined),

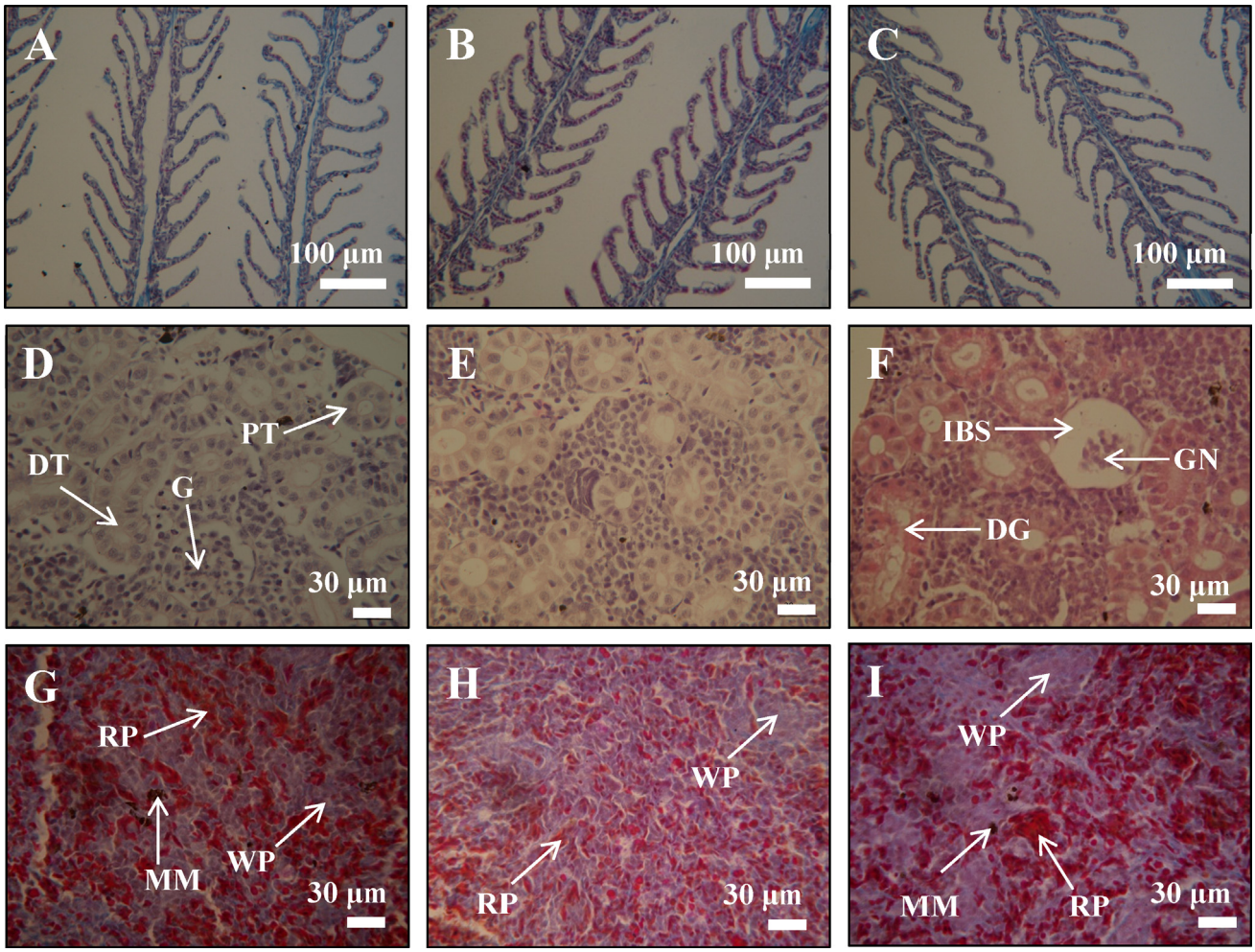

Fig. 3. Representative sample images showing gill ((A)–(C)), kidney ((D)–(F)) and spleen ((G)–(I)) morphology in rainbow trout after exposure to control ((A), (D) and (G)), SDS ((B), (E) and (H)) and SWCNT ((C), (F) and (I)) for 10 days. Gills from fish from all treatment groups were of normal histological appearance. Kidneys from control fish were of normal appearance including glomeruli (G), proximal tubules (PT) and distal tubules (DT). Kidneys from fish exposed to SWCNT (Panel F) exhibited pathologies including: increased Bowman's space (IBS); glomeruli with some necrotic cells (GN); degenerated renal tubules (DRT). Spleens from all treatment groups were normal with no evidence of proportional changes in red pulp (RP) and white pulp (WP) or the numbers of melanomacrophages (MM) observed. Sections were 7 μm thick and stained with Mallory's trichrome (gills, spleens) and haematoxylin and eosin (kidneys). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

but the surrounding tissue was normal. In contrast to both controls, mild pathologies were more apparent in the SWCNT treatment group after 10 days of exposure, but were not consistently observed in a high percentage of the fish examined (Fig. 3F). The injuries sustained included the mild necrotic degeneration of epithelium cells in some of the renal tubules, which was observed in half ($n = 4/8$) of the fish examined. There was also evidence of mild glomerular necrosis ($n = 4/8$), some glomeruli surrounded by an increased Bowman's space ($n = 4/8$ fish) and evidence of increased extracellular space between the renal tubules in the cortex ($n = 8/8$) (Fig. 3F). Blood cells were also present in this space around the tubules of half ($n = 4/8$) of the fish examined, indicating some local blood loss to the extracellular space. However, these minor injuries did not cause any overall change in the mean diameters of the renal tubules or glomerulus in the kidneys of any treatment (no treatment effect on morphometrics, ANOVA, $p > 0.05$). For example, the diameters of the proximal tubules at the end of the experiment were (mean ± *S.D.*) 44 ± 6, 46 ± 7, and 46 ± 7 μm for the control, SDS, and SWCNT treatments, respectively. The apparent increase in the Bowman's space in the SWCNT treatment was also not statistically significant (ANOVA $p > 0.05$) with mean sizes (±*S.D.*) of 3 ± 3, 7 ± 1, and 7 ± 5 μm for the control, SDS and SWCNT, respectively.

The spleens of fish were also examined (Fig. 3G and H). Histological analyses showed no evidence of pathologies in spleens of trout from any treatment group. Few melanomacrophages (≤5 melanomacrophages/field of view of 53,200 μm^2) were observed in spleens of fish from all treatment groups. There were also no significant differences in fractional areas of red and white pulp, or sinusoid space between treatment groups (ANOVAs, $p > 0.05$). Fractional areas of red pulp in controls, SDS and SWCNT exposed fish were (means ± S.E.M., $n = 8$): 39.2 ± 2.6, 41.4 ± 1.5, 44.8 ± 1.9%, respectively. Corresponding fractional areas of white pulp in fish exposed to control, SDS and SWCNT were (means ± S.E.M., $n = 8$): 50.5 ± 2.5, 48.5 ± 1.3, 46.5 ± 2.0%, respectively.

3.6. Spontaneous swimming activity

Analysis of spontaneous swimming behaviour in rainbow trout is shown in Fig. 4. There were no statistically significant effects of exposure to SDS or SWCNT on total distance moved (ANOVA, $p = 0.931$), or the mean velocity of movements when fish were active (ANOVA, $p = 0.902$) compared to controls (Fig. 4A and B). Breakdown of fish movements into speed bins of no, low, medium and high speeds is shown in Fig. 4C and revealed similar profiles

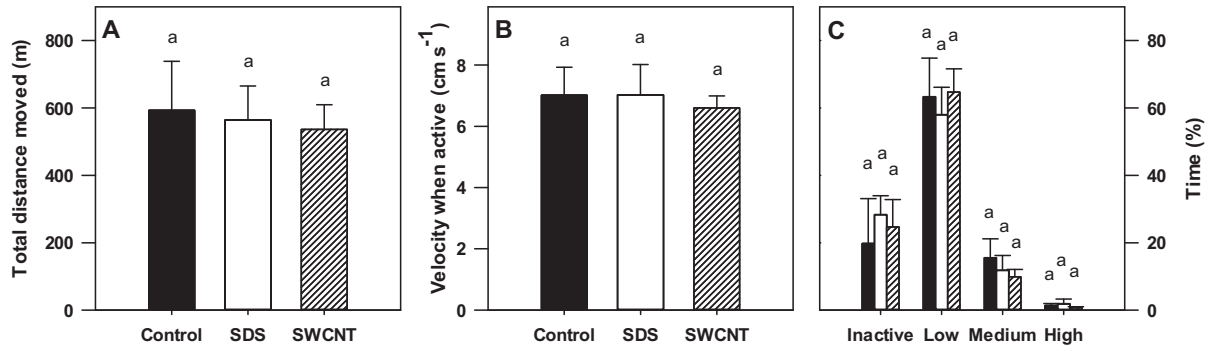

Fig. 4. Swimming behaviour in rainbow trout exposed to control (closed bars), SDS (open bars) and SWCNT (hatched bars) for 10 days. Fish movements (A) total distance moved (m), (B) velocity when active (cm s^{-1}) and (C) time (%) inactive, moving at low (0–10 cm s^{-1}), medium (10–20 cm s^{-1}) and high (>20 cm s^{-1}) speed were analysed for 3 h during daylight hours. Data are means ± S.E.M. (n = 5/6). Different lower case letters denote significant difference between treatments ($p \leq 0.05$).

of activity of fish from all treatment groups with no statistically significant effects of either SDS or SWCNT (ANOVAs, $p > 0.05$). These were characterised by periods of inactivity (<0.4 cm s^{-1}), extended periods of low activity (0.4–10 cm s^{-1}, >50% time) with less time spent moving at medium (10–20 cm s^{-1}) and high speeds (20 + cm s^{-1}). For example, percentage of the 3 h assessment period controls spent at rest, low, medium and high speeds was (means ± S.E.M.) 19.8 ± 13.3, 63.3 ± 11.4, 15.5 ± 5.7 and 1.4 ± 0.6%, respectively.

Analysis of liver glycogen and liver triglycerides in fish assayed immediately after video-tracking of fish movements indicated no significant difference between controls and fish exposed to SDS and SWCNT (Fig. 5, ANOVAs, $p > 0.05$). There was a statistically significant difference in muscle lactate concentration between fish exposed to SDS and SWCNT but neither treatment group was significantly different to controls. Measured muscle lactate concentrations in controls, SDS and SWCNT fish were (means ± S.E.M.): 1.41 ± 0.06, 1.16 ± 0.09 and 1.46 ± 0.07 μmol g^{-1} tissue, respectively.

3.7. Competitive interactions

Analysis of competitive interactions between fish showed no statistically significant effects of exposure to SWCNT on competitive ability compared to controls. Interactions between fish from all treatment groups were similar and were characterised by initial aggressive behaviours, followed by the rapid retreat of the subordinate fish to the surface and continued aggression from the dominant fish throughout the 30 min observation period. In all bar one (removed from subsequent data analysis) encounters the dominant fish was easily identifiable from the number of aggressive acts and the position in the water column. Statistical analysis of grouped data from all fish revealed a statistically significant difference (Mann–Whitney U-test, $p = 0.002$) in time spent at the surface of the water between dominant and subordinate fish (means ± S.E.M.), 14 ± 4 and 276 ± 73 s, respectively, during the 30 min time period.

Where fish exposed to SWCNT were paired with control fish the outcome was not predicted by exposure with both controls and exposed fish identified as dominant in 4/8 encounters. The number of aggressive acts in 30 min was also not affected by exposure (means ± S.E.M., Mann–Whitney U-test, $n = 4$, $p = 0.686$), 40 ± 14 aggressive acts and 87 ± 46 aggressive acts, in dominant control fish and SWCNT fish, respectively. The time of retreat of the subordinate fish was also not significantly different (means ± S.E.M., $n = 4$, Student's t test, $p = 0.7$), 25 ± 13 s and 33 ± 33 s in subordinate control and SWCNT exposed fish, respectively.

Treatment was also not a factor in interactions where a SWCNT-exposed fish was paired with a SDS vehicle control exposed fish. In 4/7 interactions the SWCNT-exposed fish was identified as the dominant fish. The number of aggressive acts in 30 min was also not significantly different (means ± S.E.M., Student's t test, $n = 3/4$, $p = 0.077$), 77 ± 32 aggressive acts and 176 ± 22 aggressive acts, in dominant fish that had been exposed to SDS and SWCNT, respectively. Time of retreat of the subordinate fish was also similar between the two groups although statistical analysis was not possible due to $n = 2$ competitive interactions commencing several hours after the barriers were removed and whilst it was possible to score the number of aggressive acts by the dominant fish over a 30 min period it was not possible to accurately record the time of retreat of the subordinate fish.

4. Discussion

The present study has demonstrated that exposure to 0.25 mg L^{-1} SWCNT for 10 days did not affect the locomotion of rainbow trout or the ability of fish to compete for social status. Brain biochemistry and morphology was generally unperturbed with no evidence of effects attributable solely to SWCNT exposure. The respiratory physiology of fish exposed to SWCNT also appeared intact with no disturbances to gill morphology or haematology of trout. There was also no evidence of compensatory changes in the red pulp of the spleen, indicating that it was not necessary to enhance recruitment of red blood cells into the circulation. Together these data indicate that exposure to 0.25 mg L^{-1} SWCNT for 10 days caused negligible toxicity in rainbow trout. Considering fish behaviours are often reported sensitive targets of pollutant exposure, these data also suggest the risk presented by 0.25 mg L^{-1} SWCNT to fish in shorter term laboratory exposures may be minimal.

Reports of the toxicity of SWCNTs to fishes, and for CNTs in general, vary depending on the exposure method, type of nanotube, and experimental model. Waterborne exposure to SWCNT in trout caused respiratory distress and systemic oxidative injury (Smith et al., 2007) while dietary exposure of trout to the same material had little effect (Fraser et al., 2011). Isolated fish macrophage cells show elevated cytokine releases in response to CNTs (Klaper et al., 2010), while epithelioma papillosum cyprini cells show a limited stress response (Taylor et al., 2012). The present study adds to the reports of no significant effects in fishes with little evidence of toxicities detected in rainbow trout. Histological and biochemical assessment of the gills, the principle target organ for waterborne contaminants, including the SWCNT used in this study, indicated a normal epithelia with no overt treatment-dependent pathologies, oxidative injury, or effects on gill electrolyte concentrations. There was also no evidence of elevated ventilation rates in the SWCNT-exposed fish relative to the controls, indicating that any systemic hypoxia (if present all at) had not reached the threshold for

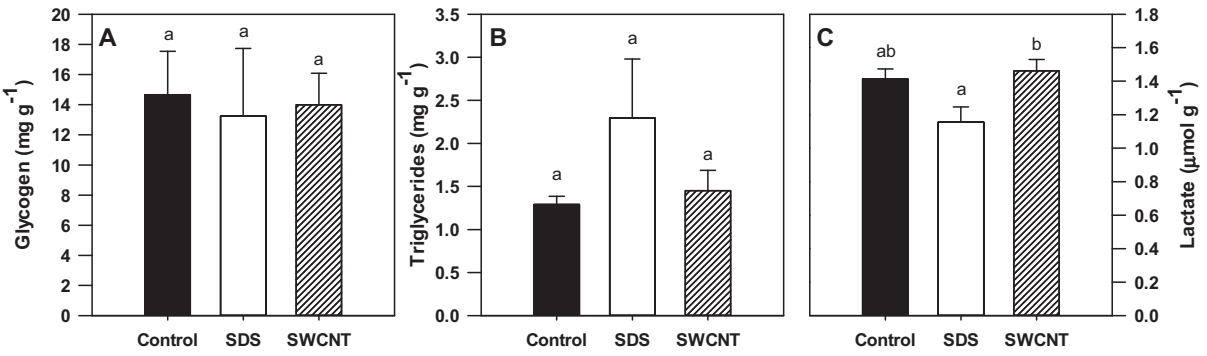

Fig. 5. Concentration of (A) liver glycogen (mg g^{-1}); (B) liver triglycerides (mg g^{-1}); and C) muscle lactate (µmol g^{-1}); in rainbow trout exposed to control (closed bars), 0.25 mg SDS L^{-1} (open bars) and 0.25 mg SWCNT L^{-1} (hatched bars) for 10 days. Data are means ± S.E.M. (n = 5/6). Different lower case letters denote significant difference between treatments ($p \leq 0.05$).

respiratory compensation. This finding is slightly different to Smith et al. (2007) who found a small, but statistically significant increase in the ventilation rates of trout exposed to the same concentration and type of SWCNT for 10 days.

The observations of negligible toxicity of SWCNTs at the gill in the present study also extended to observations on the internal tissues. Plasma ion concentrations and haematology were also unaffected (Table 1) and were within previously reported values for juvenile rainbow trout held in Plymouth City tap water (e.g., Boyle et al., 2013a; Smith et al., 2007). This appears not to be a physiological response mediated via the spleen to maintain blood cell homeostasis because fractional areas of red and white pulp in spleens were similar between fish from all treatment groups, indicating the spleen was simply working normally. The kidney of freshwater-adapted rainbow trout also has a role in maintaining blood volume by producing dilute urine to compensate for the general osmotic influx of body water. Some minor pathologies were occasionally observed in kidney of fish exposed to SWCNT compared to control groups. In the absence of any evidence of haemodilution in the SWCNT treatment (Table 1), it likely that these minor injuries are not of physiological significance in the short term. Tissue electrolyte concentrations in the kidney were also unaffected (Fig. 1). Indeed, with averages of the morphometric measurements in the kidney being normal, there were plenty of healthy nephrons available to meet physiological needs. Scown et al. (2009) showed no effect on glomerular filtration rate or creatinine clearance from trout kidney following injections of TiO_2 NPs, also implying normal renal functions. Similarly, Boyle et al. (2013a) found no effects on the electrolyte content of the kidney following waterborne exposure to 1 mg L^{-1} TiO_2 for 14 days; but did find evidence of oxidative injury (elevated TBARS) likely associated with the systemic effects of gill injury (hypoxia). Interestingly, in the present study, in the absence of respiratory distress, elevation of renal TBARS was also absent (Fig. 2). Currently the only other detailed report of renal pathology in rainbow trout is for waterborne exposures to 100 µg L^{-1} Cu NPs for 10 days (Al-Bairuty et al., 2013); which found some necrosis of the renal tubules and minor but statistically significant increases in the Bowman's space. The SWCNT exposure here also produced some minor changes in the renal tubules, but at a much higher NM concentration (1 mg L^{-1}) than in Al-Bairuty et al. (2013); tentatively suggesting that the threshold for Cu NPs effects on trout kidney may be lower than that for SWCNTs.

The brains of fish exposed to SWCNT exhibited some small K^+ and Mg^{2+} depletions (Fig. 1). However, these elemental perturbations are unlikely to cause global changes in the brain functions of trout; the activity of both Na^+/K^+-ATPase and AChE in brains were unaffected by SWCNT exposures (Fig. 2). The activity of AChE in brain has also been shown to be tightly correlated to movements in fish, including velocity of movements of rainbow trout that were measured with video tracking software (Brewer et al., 2001). In the present study, there was no effect on brain AChE activity (Fig. 2) and no effect on locomotion (Fig. 4). There were also no clinically important changes in brain histology. Overall, the data from the present study suggest that neuromuscular impairment and direct neurotoxicity from SWCNT were absent. A lack of apparent effects on neuronal functioning in trout is also consistent with recent data showing no impairment of compound action potentials in crab nerves exposed to SWCNTs ex vivo (Windeatt and Handy, 2013).

Nevertheless, despite limited toxicity observed in trout, previous studies have demonstrated that fish may offset the energetic costs associated with maintaining tissue integrity and cell homeostasis during contaminant exposure by decreasing expenditure elsewhere e.g., by reducing locomotion (Boyle et al., 2013a; Handy et al., 1999). However, direct analyses of energy reserves in the fish, especially hepatic glycogen stores (Fig. 5), and assessment of spontaneous swimming activity of sexually immature juvenile rainbow trout (Fig. 4) would appear to rule out this explanation for the minimal toxicity of SWCNT observed in this experiment. Moreover, when fish were encouraged to expend energy in energetically expensive competitive interactions, there was no effect of SWCNT on the duration or outcome of the interaction.

The lack of effects of the SWCNT in the present study raises the possibility that the SWCNTs were not bioavailable to the trout, even though the semi-static exposure regime ensured renewal of the test media and direct contact of the fish with the SWCNT. The toxicity of some chemicals through adsorption onto the gill surface, without internal accumulation, is relatively well known (e.g., for aluminium, Handy and Eddy, 1989); and has been suggested for waterborne exposure to TiO_2 NPs in trout (Boyle et al., 2013a; Federici et al., 2007). This mode of toxicity requires some gill injury, or respiratory irritation, to cause indirect deleterious effects on the internal organs via systemic hypoxia, inflammation, and/or oxidative stress. Detection methods for the reliable quantification of SWCNTs in fish tissues are not currently available, but the clear aggregation of SWCNTs on the gill with branchial pathology that we have previously documented (Smith et al., 2007) was not observed in the present study, despite using similar water and the same concentration of SWCNT. The implication of this observation is that small deviations in the experimental approaches used by investigators for toxicity testing may alter how the SWCNT associates with the gill, and its subsequent toxicity. Understanding of what these differences are will provide important insights into the physicochemical properties of SWCNTs that are important to toxicity.

The preparation methods for suspensions of CNTs are likely to influence toxic effects, and are a major contributing factor to the

inconsistencies reported in the literature for carbon-based NMs (see C_{60} review by Henry et al., 2011 for discussion of the factors). Nanotubes rapidly agglomerate in water and the use of detergents and solvents combined with mechanical (e.g., sonication and/or stirring) methods of dispersion is commonplace for preparing suspensions of hydrophobic carbon nanomaterials, including SWCNTs (Ham et al., 2005). In the present study, sufficient dispersion of the stock suspension (suspensions included both individual and agglomerated SWCNTs) was achieved with stirring in SDS to arrive at a final concentration of 0.25 mg L^{-1} SDS in exposure tanks. Fish exposed to this concentration of SDS in the vehicle-control treatment group exhibited no significant physiological effects compared to controls. This agrees with previous reports of the toxicity of SDS in salmonids (Abel, 1976). However, this concentration of SDS is also much lower than previously used by Smith et al. (2007) who observed minor effects in trout exposed to 3 mg L^{-1} SDS.

In the present study, sonication to aid dispersion of SWCNTs in suspension was also intentionally avoided, contrary to the study of Smith et al. (2007). Sonication has been shown to fracture CNTs (Ruan and Jacobi, 2012) and cause the release of impurities adsorbed to the tube surface (Toh et al., 2012). Together, these effects may alter the biological responses of organisms during exposures. One concern is that trace metals, carried over from tube synthesis, are released during sonication. However, this is not the explanation for the differences in the present study or that of Smith et al. (2007), as the latter reported no measurable metal concentrations in the stocks (below detection limits). Recent data has also indicated that sonication of dispersants in the presence of SWCNTs can generate toxic degradation products that do not form when dispersants alone are sonicated (Wang et al., 2012). There is thus the possibility that vehicle-only treatment groups [e.g., SDS treatment group of Smith et al. (2007)] will not fully control for effects observed in organisms when the SWCNT had been sonicated with the dispersing agent. However, even this vehicle-sonication theory is problematic. Smith et al. (2007) generally found more toxic effects when the SDS concentration was the lowest during SWCNT exposure, and highlighted the importance of keeping the SDS: SWCNT ratio constant during experiments to avoid deforming the tubes with excess vehicle. When these observations are considered together, it appears that the higher SDS concentrations in combination with sonication, is the most likely reason for differences between Smith et al. (2007) and the present study.

In conclusion, waterborne exposure to 0.25 mg L^{-1} SWCNT for 10 days had no effects on the locomotor and competitive behaviours of rainbow trout. The absence of effects on locomotion is more easily explained by the absence of toxicity, rather than any underlying neurological compensation or physiological strategy to preserve animal behaviour. The differences in findings between the present study and our previous experiment on waterborne SWCNT exposures in trout highlight that changes in dispersion protocols, and concentrations of solvents in co-exposure with SWCNT, may alter the toxicity of the material.

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