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Subtle alterations in swimming speed distributions of rainbow trout exposed to titanium dioxide nanoparticles are associated with gill rather than brain injury

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ABSTRACT

The effects of engineered nanomaterials on fish behaviours are poorly understood. The present study aimed to determine the locomotor behaviours of trout during waterborne exposure to titanium dioxide nanoparticles (TiO_2 NPs) as well as inform on the underlying physiological mechanisms involved. Trout were exposed to either control (without TiO_2), 1 mg l^{-1} TiO_2 NPs or 1 mg l^{-1} bulk TiO_2 for 14 days. Titanium dioxide exposure resulted in 31 (bulk) and 22 fold (nano) increases in the Ti concentrations of gill tissue compared to controls, but there were no measurable increases of Ti in the internal organs including the brain. Gill pathologies were observed in both TiO_2 treatments. Locomotor behaviours were quantified using video tracking software and the proportion of time spent swimming at high speed (>20 cm s^{-1}) was significantly decreased in fish exposed to TiO_2 NPs, compared to controls, but not fish exposed to bulk TiO_2. The shift in swimming speed distribution in the TiO_2 NP-exposed fish was associated with decreased area of red pulp in the spleen, increases in haematocrit and whole blood haemoglobin, all consistent with a compensation for respiratory hypoxia without the accumulation of plasma lactate. Fish exposed to TiO_2 NPs also retained competitive abilities when paired with controls in aggressive social encounters. The duration of competitive contests, the level of aggression and contest outcome were not affected by NP exposure. Neurological injury did not explain the changes in locomotor behaviour, although there was some apparent enlargement of the blood vessels on the brain. Whole brain homogenates showed a statistically significant increase in oxidative stress defences such as the total glutathione pool, but without loss of Na^+K^+-ATPase or acetylcholinesterase activities.

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1. Introduction

The ecotoxicity of engineered nanomaterials (ENMs) has been the subject of intense research in the last five years (reviews, Moore, 2006; Handy et al., 2008; Klaine et al., 2008; Kahru and Dubourguier, 2010; Klaine et al., 2012). Concerns have been raised about the effects of nano metals on fishes (Shaw and Handy, 2011), and also on the sublethal effects of ENMs on the different body systems of fish (Handy et al., 2011). In general, sparingly soluble metal or metal oxide ENMs are acutely toxic to fishes at high mg l^{-1} concentrations, with sublethal effects on body systems occurring at concentrations around 1 mg l^{-1} or less (Handy et al., 2011).

Sublethal effects associated with exposure to TiO_2 nanoparticles (TiO_2 NPs) in the laboratory are documented for some body systems in fishes. These include damage to the gill epithelium and some loss of sinusoid space with foci of lipidosis in the liver of rainbow trout (*Oncorhynchus mykiss*) during 14 day exposures to 1 mg l^{-1} TiO_2 NPs (Federici et al., 2007). Alterations of the proportions of red and white pulp in the spleen have also been observed in trout, with concerns for the function of the haematopoietic system (Handy et al., 2011). Renal function appears to be largely unaffected by injections of TiO_2 NPs in trout (Scown et al., 2009). However, the effects of ENMs on the central nervous system of fishes are poorly understood (see discussion in Handy et al., 2011). The brain is known for its lack of tolerance to hypoxia and sensitivity to oxidative stress. In the brain of carp (*Cyprinus carpio*) TiO_2 NPs caused dose-dependent increases in thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation (Hao et al., 2009). Changes in TBARS were also reported in whole brain homogenates from rainbow trout exposed to 1 mg l^{-1} TiO_2 NPs (Federici et al., 2007). There are also concerns for the electrical properties of the nervous system, with

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inhibition of Na^+/K^+-ATPase in trout brain following chronic dietary exposure to TiO_2 NPs (Ramsden et al., 2009). However, electrophysiological studies on isolated crab nerve preparations indicate the ability of peripheral nerve to generate action potentials may not be affected (Windeatt and Handy, 2012). Some subtle brain pathologies are also reported in fish (Federici et al., 2007) and mammals (Wang et al., 2007) during exposures to TiO_2 NPs. Together, these studies raise the possibility that the brain may be a sensitive target for the effects of TiO_2 NPs with implications for neural functions, and consequently, animal behaviours.

The idea that contaminants can alter the behaviour of fishes is well known, and effects on behaviour can occur at much lower exposure concentrations than expected for traditional toxicological end points (see Scott and Sloman, 2004 for a review). Behaviour is therefore regarded as a sensitive indicator of pollutant exposure in fishes. It is also ecologically relevant in terms of bioenergetics and the survival of fish populations. In active fishes such as trout, individuals may spend around two thirds of their daily energy budgets on locomotion (Priede, 1985) as part of essential behaviours like foraging, evading predators and defending territories. In addition, many fishes live in schools and social interactions between conspecifics often determine their position in hierarchies, and consequently their access to food. In salmonids, these interactions can be aggressive and are characterised by increased metabolic rate and mobilisation of energy reserves; often revealing fitness disparities between fish (Sloman et al., 2000; review by Sloman and Armstrong, 2002). Metal exposures can have profound effects on fish behaviour. For example, trout exposed to dietary copper will reduce locomotion in order to preserve growth, body maintenance, and tissue repair (Handy et al., 1999) and may even lose daily circadian rhythms of activity (Campbell et al., 2002). Metals also alter the ability of fish to form social groups (e.g. Cd, Sloman et al., 2003a, 2003b; Cu, Campbell et al., 2005). However, the behavioural effects of ENMs are largely unknown.

The aims of this study were firstly to determine whether or not TiO_2 NPs caused changes in the locomotor behaviour or competitive abilities of trout, and if so, whether or not this was associated with underlying changes in the animal's physiology. Neurotoxicity is only one of several possible explanations for changes in animal behaviour and our approach was therefore to take an overview by measuring Ti metal concentrations in the tissues, adverse effects on several physiological processes (osmoregulation, haematology, defences to oxidative stress), as well as organ pathologies including the brain. Finally, the effects of ordinary titania on fish behaviours have not been described, and a bulk TiO_2 treatment was included to revisit the assumption that bulk titania is not hazardous to fishes.

2. Materials and methods

2.1. Experimental design

Juvenile rainbow trout (24.0 ± 3.7 g, mean ± standard deviation (S.D.), $n = 32$) were obtained from a local supplier (Torre fishery, Watchet, Somerset, UK) and maintained in aerated, dechlorinated, recirculating Plymouth tap water (see below for ionic composition) for three weeks prior to onset of experimentation. The background concentration of Ti in the water was below the detection limit (<1.3 $\mu g\,l^{-1}$, $n = 6$ samples, see Section 2.4). Stock fish were fed twice daily with a commercial trout diet (EWOS, Westfield, UK) until 48 h prior to experimentation. Fish were randomly allocated to experimental glass tanks containing 20 l of Plymouth tap water. The continuously aerated water had the following composition (means ± S.D. $n = 36$–156 measurements, in mmol l^{-1}): Ca^{2+}, 0.52 ± 0.07; K^+, 0.06 ± 0.01; Mg^{2+}, 0.17 ± 0.01; Na^+, 0.75 ± 0.11; pH 7.2 ± 0.2; dissolved oxygen 8.3 ± 0.5 mg l^{-1}; NH_3, 0.008 ± 0.003 mg l^{-1}; temperature, $15 \pm 1\,^{\circ}C$. Fish were held in a 14 h light: 10 h dark photoperiod (350–400 lux lighting to comply with ethical approval) and were allowed to rest overnight (unfed) prior to the first dosing.

Fish were exposed to either a control (without TiO_2), 1 mg l^{-1} TiO_2 NPs, or 1 mg l^{-1} bulk TiO_2 in a quadruplicate test design ($n = 4$ replicate aquaria/treatment; equating to 14 fish/tank or 56 fish/treatment) for 14 days. The exposure concentrations were chosen to enable direct comparison with our previous studies on trout (Federici et al., 2007). After 14 days tissues were collected immediately from 8 fish (2/tank) from each treatment to assess Ti metal concentrations in the tissues, trace element homeostasis, haematology, histopathology, as well as for biochemistry (Sections 2.3, 2.4, 2.5 and 2.6). At 14 days, additional randomly selected fish from each treatment were also subject to behavioural measurements. These were either an assessment of spontaneous swimming speeds by video tracking, or competitive interactions studies with pairs of fish from the different treatment group to (Sections 2.7 and 2.8). No fish was used in both behavioural assays. Plasma and tissue samples were also collected from fish that were video tracked for biochemical assays (Section 2.5). The exposures in half the tanks were staggered by 48 h for logistical reasons to enable all the behavioural measurements to be made, as 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 (Sections 2.7 and 2.8).

The dosing was achieved using the semi-static exposure method of Federici et al. (2007), with minor modifications. Briefly, each tank was dosed with 2 ml of the appropriate stock dispersion (Section 2.2) with the aeration rapidly spreading the material in the tank water. An 80% water change was conducted every 12 h, and the relevant tanks re-dosed with either 1.6 ml of bulk TiO_2 or TiO_2 NP as appropriate. To minimise the normal aggressive behaviours of hungry trout (essential to animal husbandry), fish were fed twice during the experiment (days 8 and 12) with a 1.5% body weight maintenance ration. This was done post water change but prior to re-dosing to minimise the risk of ingestion of TiO_2 during feeding. All food was consumed immediately in all tanks on both occasions. The entire experiment was under ethical approval and fish were subject to independent health checks during the work.

2.2. Titanium dioxide stock suspensions

Nano and bulk forms of TiO_2 were obtained from DeGussa AG ("Aeroxide" P25, supplied by Lawrence Industries, Tamworth, UK) and ACROS (New Jersey, USA) respectively. The P25 material was the same batch used by Federici et al. (2007) and according to the manufacturer's revised information was 25% rutile: 75% anatase, >99% purity as TiO_2 (maximum stated impurity, 1% Si) with a mean particle size of 21 nm and a specific surface area of $50 \pm 15\,m^2\,g^{-1}$. The manufacturer's data on the bulk TiO_2 was limited to purity information (98–100.5% pure TiO_2). However, our scoring of the different proportions of the crystal structures from transmission electron micrographs (TEM, JEOL 12000EXII, Tokyo, Japan) showed mineral composition was (means ± S.D., $n = 10$ micrographs) $25 \pm 5\%$ rutile and $75 \pm 5\%$ anatase.

Stock dispersions (500 ml) of 10 g l^{-1} bulk TiO_2 or TiO_2 NPs (nominal concentrations) were prepared in ultrapure water (Milli-Q®, Millipore Corporation) and stored in acid-washed Nalgene bottles. Stocks were stirred overnight (2400 rpm, Magnetic Stirrer SM1, Stuart Scientific, UK) and then subsequently the entire 500 ml was sonicated (500 ml, 4 °C, 35 kHz frequency, Fisherbrand FB 11010, Germany) for 1 h. The sonication step was repeated each day immediately prior to dosing the experimental tanks to ensure reasonably dispersed material was added to the tanks.

Measurement of primary particle sizes was done manually from TEM images of 1 g l^{-1} dilution of the stocks above (to enable visualisation of the particles). The primary particle sizes were (means ± S.D., $n = 100$) 134 ± 42 nm and 24 ± 10 nm of bulk TiO_2 and TiO_2 NPs respectively. Dispersion of the 10 g l^{-1} bulk TiO_2 and TiO_2 NPs stocks above were confirmed by nanoparticle tracking analysis of 10 mg l^{-1} dilutions of the 10 g l^{-1} stocks that were prepared in ultrapure water immediately after sonication (Nanosight LM 10, Nanosight, Salisbury, UK). Mean aggregate sizes of TiO_2 were (means ± S.D., $n = 3$): 211 ± 3 nm and 96 ± 40 nm for bulk TiO_2 and TiO_2 NPs respectively. Particle size distributions of bulk TiO_2 and TiO_2 NPs were significantly different ($p = 0.023$, $n = 3$, two sample Kolmogorov–Smirnov test).

2.3. Haematology and plasma analysis

Haematological analyses were performed according to Handy and Depledge (1999). Briefly, two fish per tank (8/treatment) were terminally anaesthetised in buffered MS222, weighed and the total length recorded. Blood was immediately withdrawn by caudal puncture with a heparinised syringe and sub-samples of whole blood were collected in haematocrit tubes for measurement of packed red blood cell volume after centrifugation (13000 rpm, 2 min, Heraeus pico 17 microcentrifuge), and the percent haematocrit (HCT) recorded. Then, 20 μl of whole blood was diluted to 5 ml with Drabkin's reagent (Sigma–Aldrich, UK) to measure haemoglobin (Hb). A further 20 μl of whole blood was fixed in Dacie's fluid (0.1 M tri sodium citrate, 2.5 mmol l^{-1} cresyl blue, in 1% formalin) for red blood cell counts. The remaining blood was centrifuged (MSE microcentaur, 13,000 rpm, 2 min) and osmolarity (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 (Section 2.4).

2.4. Tissue ion analysis

The methodology for Ti determination from TiO_2 particles involved a modified acid digestion and sample preparation with Triton-X100 that eliminates the need for strong hydrofluoric acid digestion to dissolve Ti, and enables the detection of Ti directly from dispersed particles by inductively coupled plasma optical emission spectrophotometry (ICP-OES, Ramsden et al., in review, patent pending). Briefly, samples (approximately 0.1–0.5 g) of gill (second and third gill arches), brain, liver, spleen, kidney, muscle (immediately beneath dorsal fin) of the fish, and part of mid-intestine were oven dried to a constant weight and digested (60 °C) for 2 h in 1 ml of concentrated HNO_3 (68% nitric acid, trace element grade, Fisher Scientific, UK). Following digestion, samples were diluted to 4 ml with Triton-X100 (Sigma, UK) and ultrapure water, to give a final Triton-X100 concentration of 2% in the acidified sample digest. Samples were stirred or vortexed while being drawn into the instrument and analysed in triplicate for Ca^{2+}, Cu, Fe, K^+, Mg^{2+}, Na^+, Ti, and Zn by ICP-OES (Varian 725-ES). Matrix matched element standards (acidified and in 2% Triton-X100) were measured every 10–15 samples to monitor the instrument for drift. Recovery of metals from tissue digests were calculated for kidney (0.07–0.12 g dry weight, dw) and gill (0.02–0.06 g dw) excised from non-experimental stock trout, digested as above and spiked with element standards and either bulk TiO_2 or TiO_2 NPs. Calculated mean recoveries were not significantly different between tissues. Calculated combined recoveries for both tissues were >90% for dissolved metals ($n = 12$ spiked samples, data not shown). Recovery of bulk TiO_2 and TiO_2 NPs from tissue digests were 84.3 ± 2.7% and 93.9 ± 1.5%, respectively (data are means ± S.D., $n = 6$). The procedural detection limit for Ti in acid digests of tissue samples ($n = 10$ procedural blanks) was 4.54 μg l^{-1}, and for a typical tissue weight this equates to around 0.36 μg g^{-1} dry weight of Ti metal. All tissue metal concentrations are reported g^{-1} dry weight.

For analysis of Ti concentrations in stock suspensions aliquots were diluted with Milli-Q water and analysed directly by ICP-OES and compared to acidified (2% HNO_3) standards of dissolved Ti metal made in deionised water. Similarly, water samples taken from the exposure tanks were also analysed for Ca^{2+}, K^+, Mg^{2+}, Na^+ and Ti directly by ICP-OES. The instrument detection limit for Ti for analysis of water samples and stock suspensions (no Triton X-100 in the standards, nor acidified) was 1.3 μg l^{-1}, calculated from 3 × S.D. of $n = 10$ measurements of the blank.

2.5. Biochemical analyses

Biochemical analyses were performed on selected tissues (gill, brain and kidney) according to methods outlined in Smith et al. (2007) with minor modification. A further two fish per tank were terminally anaesthetised and tissues excised (Section 2.3), then snap frozen in liquid N_2 and stored at −80 °C until required. Tissues (30–150 mg) were thawed on ice and then homogenised (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 5 volumes of 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) was quantified in 20 μl of tissue supernatant in triplicate reactions in 300 μl 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), 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 Na^+/K^+-ATPase was measured in 20 μl of supernatants according to an original protocol by Silva et al. (1977) and as described in Smith et al. (2007). Briefly, Na^+/K^+-ATPase activity was measured as the release of inorganic phosphate from Na_2ATP after a 20 min incubation with/without the Na^+K^+-ATPase inhibitor, 1 mmol l^{-1} ouabain. Following addition of a colour reagent (9.6% $FeSO_4$, 1.15% $(NH_4)_6Mo_7O_{24}$) absorbance at 660 nm was recorded against 0–2 mmol l^{-1} phosphate standards. The concentrations of thiobarbituric reactive substances (TBARS) were measured according to an original protocol in Camejo et al. (1998). In triplicate reactions 40 μl of supernatants were diluted in 140 μl of phosphate buffer (100 mmol l^{-1}, pH 7.5), treated with 10 μl of 1 mmol l^{-1} butylated hydroxytoluene to reduce spontaneous oxidation of samples and de-proteinated with 50 μl 50% trichloroacetic acid (TCA). Samples were centrifuged 13,000 rpm for 2 min and 150 μl supernatant transferred to microplate with 75 μl of 1.3% thiobarbituric acid in 0.3% NaOH and incubated at 60 °C for 60 min. Absorbance at 530 nm was recorded against 0–50 μmol l^{-1} 1,1,3,3-tetraethoxypropane standards (Sigma, UK). For all assays, appropriate negative controls substituting isotonic buffer for tissue supernatants were included in every run. Data from our laboratory demonstrates TiO_2 NPs do not interfere with GSH, TBARS and Na^+/K^+-ATPase assays used (data not shown). The concentration of GSH, TBARS and activity of Na^+/K^+-ATPase was normalised to total protein in the supernatant using the Bradford's reagent according to the manufacturer's protocol (Product B6916, Sigma–Aldrich, UK).

2.6. Histopathology

At day 14 of the exposure two fish per tank were anaesthetised in buffered MS222 and organs carefully collected into buffered formal saline and processed to wax (Leica TP1020, Leica TPEG1150H, Wetzlar, Germany), then 7 μm sections were cut for routine histological examination as described in Smith et al. (2007), with minor modifications. After fixation, the second gill arch of each fish was decalcified (Rapid Decalcifier, CellPath plc, UK) prior to processing and sectioning. Gills were stained with Mallory's trichrome. Spleen, a piece of skeletal muscle from the flank of the fish, liver, posterior kidney and whole brain were fixed, processed, sectioned, and then stained with haemotoxylin and eosin to show general architecture. Images were recorded using an Olympus Vanox – T microscope and an Olympus digital camera (C-2020 Z). In gills, the incidence of lesions was scored on 80–100 secondary lamellae from two randomly selected primary filaments from the middle of the second gill arch on each fish. The total number of secondary lamellae with lesions, and the percentage incidence of each type of lesion were recorded. Sagittal sections of whole brains were made and total dimensions (height) of the tissue layers in the mesencephalon and cerebellum of the brain were measured manually, in triplicate from three random images from each fish. 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 and white pulp and sinusoid space in the spleen, sinusoid space in the liver and fractional fibre areas in skeletal muscle. Mean numbers of melanomacrophages in spleen, liver and kidney were counted in 53,200, 53,200 and 212,400 μm^2 of tissue (i.e. per field of view) respectively at ×400 magnification.

2.7. Swimming behaviour

Following the 14 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). Fish were netted from the exposure aquaria and transferred to chambers (65 cm × 40 cm × 30 cm) filled with 30 l of aquarium water (for water quality parameters see Section 2.1) and with video cameras directly overhead. All behavioural measurements were carried out in clean water to avoid particle interference with video recordings. Chambers were separated from the rest of the aquarium by a curtain to minimise disturbance and left to acclimate to conditions overnight (16 h). Between 10:00 and 13:00 the following day behaviours were recorded. The software captured the position of the fish in each arena expressed as *x*, *y* coordinates, together with time, at a data capture rate of 6.25 locations s^{-1}. During subsequent analysis of sample tracks all recordings were viewed and processed to correct miss-tracks and remove noise which may otherwise overestimate distance moved by removing small movements of <0.4 cm between consecutive time points. 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 (0.4–10 cm s^{-1}), medium (10–20 cm s^{-1}) and high speeds (20+ cm s^{-1}) following an approach previously used to assess effects of Cu on activity levels in trout (Handy et al., 1999; Campbell et al., 2002).

Immediately after tracking had finished some biochemistry was also performed on the plasma and brain of fish. Fish were terminally anaesthetised and plasma isolated from blood (see Section 2.3), then the whole brain was excised and homogenised in isotonic sucrose buffer and then centrifuged to isolate the supernatant (see Section 2.5). Activity of acetylcholinesterase (AChE) was measured according to an original protocol by Ellman et al. (1961) adapted for the microtitre plate by recording absorbance at 405 nm for 10 min of a reaction mixture containing 50 μl of brain supernatant, 50 μl of 3 mmol l^{-1} acetylthiocholine iodide and 150 μl of 0.27 mmol l^{-1} DTNB. Appropriate sample blanks (isotonic buffer without tissue homogenate) were included in every run. Activity of AChE was expressed relative to protein concentration in each homogenate, measured with Bradford's reagent. Plasma glucose concentrations were analysed according to the manufacturer's protocol (Product G3293, Sigma–Aldrich, UK). Lactic acid was analysed in deproteinated (equal volume of 5% TCA), pH neutralised serum according to Gutmann and Wahlefeld (1974). Briefly, 10 μl serum was diluted in 200 μl glycine-hydrazine buffer (0.4 M, pH 9) 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. Appropriate sample blanks (isotonic buffer without tissue homogenate) were included in every run.

2.8. Competitive interaction

These measurements broadly followed the methodology of Sloman et al. (2000). Behavioural interactions were observed in pairs of rainbow trout from the following treatment groups: (1) aTiO_2 NP exposed fish paired with a control fish ($n = 8$ pairs), and (2) a TiO_2 NP exposed fish paired with a bulk TiO_2 exposed fish ($n = 8$ pairs). Following the 14 day exposure, fish were lightly anaesthetised to facilitate handling (50 mg l^{-1} MS222, buffered to ~pH 7), 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 during observations, a process which did not have lasting effects on behaviour. Fish were revived in strongly aerated aquarium water and the two individuals of each pair were placed in a 20 l, aerated, glass aquarium on either side of an opaque partition. Again, no nanoparticles were added to the water to enable behavioural observations. Once fish had been allowed to rest and acclimate to the conditions for 24 h, the opaque 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 during data analysis) and the time of retreat of the subordinate fish (recorded as the time after which no further aggressive behaviours were observed). Subordinate fish typically retreated to the surface of the water and maintained this position in the water column. Dominant fish were easily identified in interactions from the timeline of aggressive behaviours observed and there were typically large differences in the observed numbers of aggressive behaviours between fish.

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 applied. Statistically significant differences between treatment groups were determined by ANOVA with Tukey's test *aposteriori*. Where log-transformation failed, the Kruskal–Wallis test or the Mann–Whitney *U*-test with Bonferroni correction was applied as appropriate. A p value of ≤ 0.05 was considered significant. Data from competitive interactions between pairs of fish were analysed with Student's t test or Mann–Whitney *U*-test as appropriate.

Table 1
Tissue concentration of Ti in rainbow trout exposed to control (no added TiO_2), 1 mg l^{-1} of bulk TiO_2 or 1 mg l^{-1} TiO_2 NPs for 14 days.

Tissue	Control	Bulk TiO_2	TiO_2 NPs
Gill	1.4 ± 0.5[a]	43.3 ± 12.1[b]	31.3 ± 8.1[b]
Intestine	4.8 ± 2.5	11.4 ± 3.8	8.3 ± 3.8
Liver	1.9 ± 0.4	0.8 ± 0.2	2.3 ± 0.2
Kidney	3.6 ± 1.2	2.3 ± 0.6	4.4 ± 1.8
Spleen	8.2 ± 3.3	5.4 ± 1.6	11.1 ± 4.7
Brain	0.9 ± 0.3	2.0 ± 0.8	1.9 ± 0.6

Tissue concentrations are Ti metal expressed as μg g^{-1} dry weight of tissue. Data are means ± S.E.M. (n = 7/8). Tissue concentration of Ti (μg g^{-1} dry weight) in initial fish at day 0 were (means ± S.E.M., n = 7/8): 1.0 ± 0.2 Gill; 1.8 ± 0.6 Intestine; 0.2 ± 0.1 Liver; 0.8 ± 0.4 Kidney; 1.6 ± 0.5 Spleen; 5.9 ± 1.9 Brain. Values with different letters within rows (treatment-effect) were significantly different (ANOVA, $p \leq 0.05$). Rows with no labels were not significantly different from each other.

3. Results

3.1. Confirmation of waterborne TiO_2 exposure and tissue Ti accumulation

Waterborne exposure to TiO_2 in experimental aquaria was confirmed by ICP-OES of water samples. Measured concentrations of Ti metal in water samples taken 5 minutes after dosing were (means ± S.D., n = 6); <1.3 μg l^{-1}, 0.67 ± 0.02 mg l^{-1} and 0.64 ± 0.01 mg l^{-1} for control, bulk TiO_2 and TiO_2 NPs respectively, corresponding to 1.11 ± 0.03 mg l^{-1} and 1.07 ± 0.02 mg l^{-1} concentrations of TiO_2 in the bulk and nano treatments. The exposures were sub-lethal as no mortalities occurred in any of the treatment groups. Titanium metal concentrations in the tissues are shown in Table 1. Overall, there was some detectable background Ti in the control fish, but apart from the gills, there were no increases in Ti concentration in the internal organs of the fish in the TiO_2 treatments and no significant difference between fish exposed to bulk or nano TiO_2 (no "material-type" effect). At the end of the exposure (day 14) the gills of fish exposed to bulk or TiO_2 NPs contained (means ± standard error (S.E.M.), n = 8) 43.3 ± 12.1 and 31.3 ± 8.1 μg g^{-1} respectively, compared to the controls (1.4 ± 0.5 μg g^{-1}); a 31 and 22 fold increase in Ti metal in/on the gill tissue compared to the controls (ANOVA, $p < 0.001$). There was no indication of increasing Ti concentration in liver, spleen, kidney and brain in the fish from the TiO_2 treatments compared to controls at day 14 (ANOVA, $p > 0.05$).

3.2. Haematology and plasma ions

Fish exposed to TiO_2 NPs, but not the bulk material, exhibited some changes in haematocrits and whole blood haemoglobin compared to control fish by the end of the exposure (Table 2). For example, in fish from the TiO_2 NP treatment haematocrit was 34% compared to 26 and 28% in the bulk TiO_2 and control fish. A corresponding increase of whole blood haemoglobin concentration was also apparent in fish exposed to TiO_2 NPs (6.9 g dl^{-1}) compared to controls (5.3 g dl^{-1}), a 23% increase in haemoglobin concentration, but there was no significant increase in mean corpuscular haemoglobin concentration. The absence of changes in these parameters in the fish from the bulk TiO_2 treatment compared to fish exposed to TiO_2 NPs (Table 2) also indicated a material-type effect. There were no treatment-dependent changes in plasma ions (Table 2). Plasma osmolarity, K^+ and Na^+ were not significantly different from controls following exposure to bulk TiO_2 or TiO_2 NPs.

3.3. Tissue electrolytes

Tissue electrolyte and trace element profiles were generally unaffected by either bulk or TiO_2 NP exposure, apart from some perturbations to electrolytes in the brain and gill (Fig. 1). Quality assurance evaluation indicated that usable data were not obtained for spleen Ca^{2+}. The concentrations of Cu are not shown in Fig. 1 due to the low concentration of this essential element in tissues. Copper concentrations in gill, liver, kidney, spleen and muscle were not affected by exposure to both forms of TiO_2 (data not shown). However, the Cu concentration was significantly higher (ANOVA, p = 0.013) in brains of fish exposed to TiO_2 NPs (means ± S.E.M., n = 7/8), 0.11 ± 0.01 μmol g^{-1}, compared to fish exposed to bulk TiO_2, 0.07 ± 0.01 μmol g^{-1} although neither NPs nor bulk exposure caused a significant change in Cu concentration compared to controls, 0.08 ± 0.01 μmol g^{-1} (ANOVA, $p > 0.05$). Exposure to TiO_2 NPs also caused disturbances in other brain electrolytes with evidence of a material-type effect (Fig. 1). Notably, brain K^+ was significantly elevated in fish exposed to NPs, 839 ± 118 μmol g^{-1} compared to 544 ± 50 μmol g^{-1} and 505 ± 60 μmol g^{-1} in controls and fish exposed to the bulk TiO_2, respectively (Fig. 1, ANOVA, p = 0.011). Whole brain Na^+ was elevated in the TiO_2 NP treatment only compared to both the bulk, but not the controls. In the case of Ca^{2+}, exposure to TiO_2 NPs elevated Ca^{2+} compared to the control but there was no material-type effect. For the trace metals, Zn concentrations in the brains of fish exposed to TiO_2 NPs (1.48 ± 0.22 μmol g^{-1}) were 65% higher (ANOVA, p = 0.011) than either the controls (0.91 ± 0.08 μmol g^{-1}) or trout exposed to bulk TiO_2 (0.89 ± 0.09 μmol g^{-1}). Brain Fe concentrations showed a small but statistically significant (Kruskal–Wallis, p = 0.019) decrease in the bulk TiO_2 treatment compared to either the controls or TiO_2 NP treatment.

The concentration of Mg^{2+} in the gill of trout exposed to 1 mg l^{-1} TiO_2 NPs (49.4 ± 1.3 μmol g^{-1}) was significantly lower than gills from the controls (55.8 ± 2.2 μmol g^{-1}) or from the bulk material treatment (54.4 ± 1.7 μmol g^{-1}, ANOVA, p = 0.041). Consequently, there was a material-type effect associated with TiO_2 NPs. The concentrations of the other ions in the gill were not altered by either bulk or TiO_2 NP exposure (Fig. 1).

3.4. Biochemical markers of oxidative injury and ionoregulatory dysfunction

Tissue concentrations of total glutathione, TBARS and Na^+/K^+-ATPase activity are shown in Fig. 2. Overall, there was some evidence of oxidative disturbances in the brain and kidney, but no effects on the gill. The concentration of total GSH in the gill and kidney was not affected by TiO_2 NPs exposure (Fig. 2, ANOVA, $p > 0.05$). However there was a statistically significant increase in GSH in brains of fish exposed to TiO_2 NPs or bulk TiO_2 compared to controls. There was also evidence for a material type-effect. The concentration of GSH was significantly higher in brains in fish exposed to NPs compared to bulk. The GSH concentration in brains of fish exposed to TiO_2 NPs was 22.5 ± 1.0 nmol mg^{-1} protein compared to 18.7 ± 0.2 nmol mg^{-1} protein and 20.4 ± 0.6 nmol GSH mg^{-1} protein in brains of controls and fish exposed to bulk TiO_2, respectively (Kruskal–Wallis, p = 0.001).

There was also an increase of TBARS in the brains of fish exposed to TiO_2 NPs only, indicating a material-type effect in this organ (Fig. 2). TBARS in the brains of fish exposed to TiO_2 NPs was elevated to 1.7 ± 0.2 nmol mg^{-1} protein, compared to controls, 1.0 ± 0.2 nmol mg^{-1} protein and bulk TiO_2 exposed fish 0.7 ± 0.1 nmol mg^{-1} protein (ANOVA, $p < 0.001$). The concentration of TBARS in kidney was also significantly increased in fish exposed to TiO_2 NPs or bulk TiO_2 compared to the controls (Kruskal–Wallis,

Table 2
Haematology and plasma ions in rainbow trout exposed to control (no added TiO_2), 1 mg l^{-1} of bulk TiO_2 or 1 mg l^{-1} of TiO_2 NPs for 14 days.

Parameter	Control	Bulk TiO_2	TiO_2 NPs
Haematocrit (%)	27.6 ± 1.7[a]	26.4 ± 0.6[a]	33.5 ± 1.8[b]
Haemoglobin (g dl^{-1})	5.26 ± 0.56[a]	5.16 ± 0.18[a]	6.93 ± 0.35[b]
Red blood cells (cell × 10^6 mm^3)	0.62 ± 0.04[ab]	0.54 ± 0.03[b]	0.72 ± 0.03[a]
Mean corpuscular haemoglobin (pg $cell^{-1}$)	84.1 ± 8.9	93.9 ± 4.8	94.0 ± 4.1
Osmolarity (mOsm kg^{-1})	282 ± 4	279 ± 5	288 ± 6
Plasma Na^+ (mmol l^{-1})	119.4 ± 1.2	122.8 ± 2.5	123.2 ± 3.4
Plasma K^+ (mmol l^{-1})	3.19 ± 0.12	3.51 ± 0.18	3.49 ± 0.23

Data are means ± S.E.M. (n = 7/8). Haematology and plasma ions in initial fish at day 0 were (means ± S.E.M., n = 7/8): HCT, 27.9 ± 2.0%; Haemoglobin, 5.53 ± 0.40 g dl^{-1}; Red blood cells, 0.56 ± 0.03 cell × 10^6 mm^3; Osmolarity, 287 ± 4 mOsm kg^{-1}; Plasma Na^+, 130.8 ± 1.4 mmol l^{-1}; Plasma K^+, 3.68 ± 0.26 mmol l^{-1}. Values with different letters within rows (treatment-effect) were significantly different (ANOVA or Kruskal–Wallis, $p \leq 0.05$). Rows with no labels were not significantly different from each other.

p = 0.001, Fig. 2), but with no difference between bulk and nano TiO_2.

Activity of Na^+/K^+-ATPase in gill, brain and kidney was not significantly different in fish exposed to TiO_2 NPs compared to TiO_2 bulk or the control fish (ANOVA, $p > 0.05$, Fig. 2). For example, activity of Na^+/K^+-ATPase in the brains of the fish exposed to TiO_2 NPs was 5.1 ± 1.6 μmol Pi mg^{-1} protein h^{-1}, compared to controls 5.5 ± 1.0 μmol Pi mg^{-1} protein h^{-1} and fish exposed to bulk TiO_2 5.8 ± 0.9 μmol Pi mg^{-1} protein h^{-1}.

3.5. Histological observations

Waterborne exposure to both forms of TiO_2 caused gill pathology. At the end of the exposure, all eight fish examined from each of

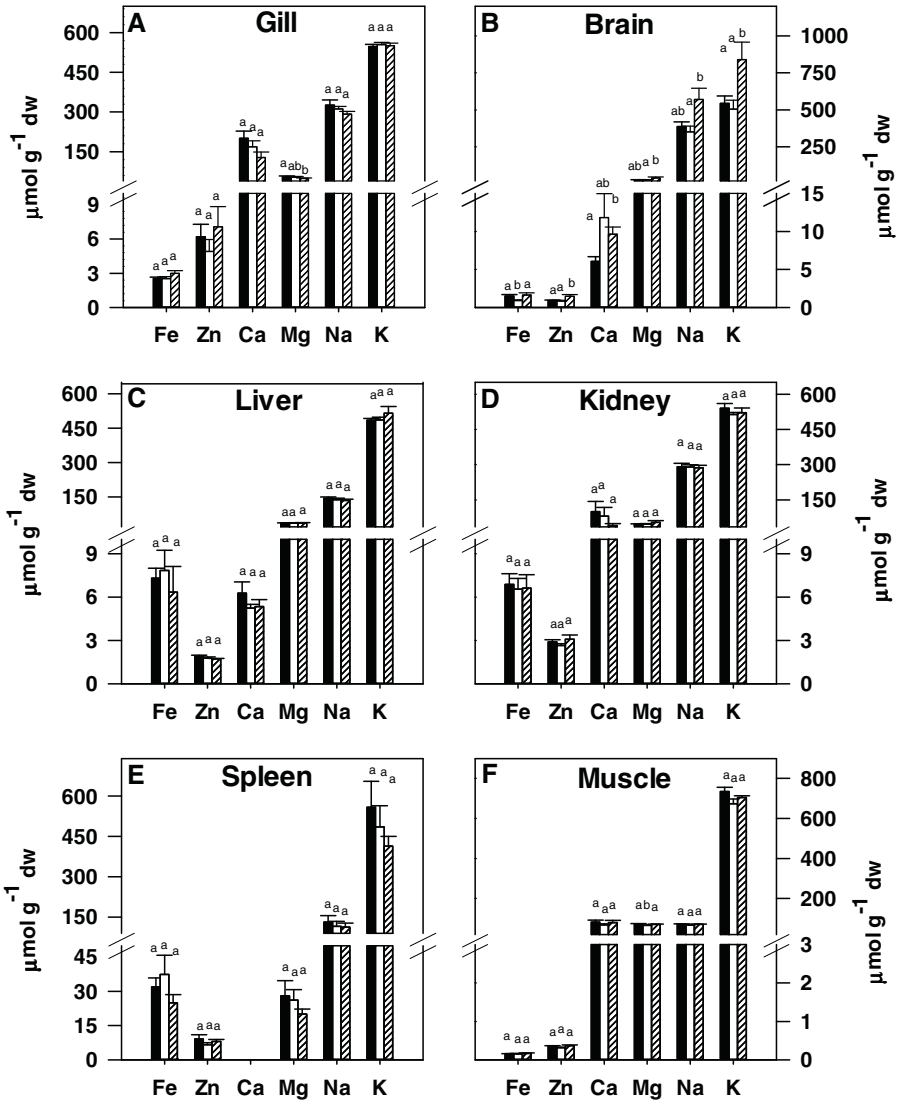

Fig. 1. Concentration (μmol g^{-1} dry weight) of ions in (A) gill, (B) brain, (C) liver, (D) kidney, (E) spleen and (F) muscle of rainbow trout exposed to control (closed bars), 1 mg bulk TiO_2 l^{-1} (open bars) and 1 mg TiO_2 NPs l^{-1} (hatched bars) for 14 days. Data are means ± S.E.M. (n = 6–8). Different lower case letters denote significant difference between treatment groups ($p \leq 0.05$). See text for Cu concentration in tissues.

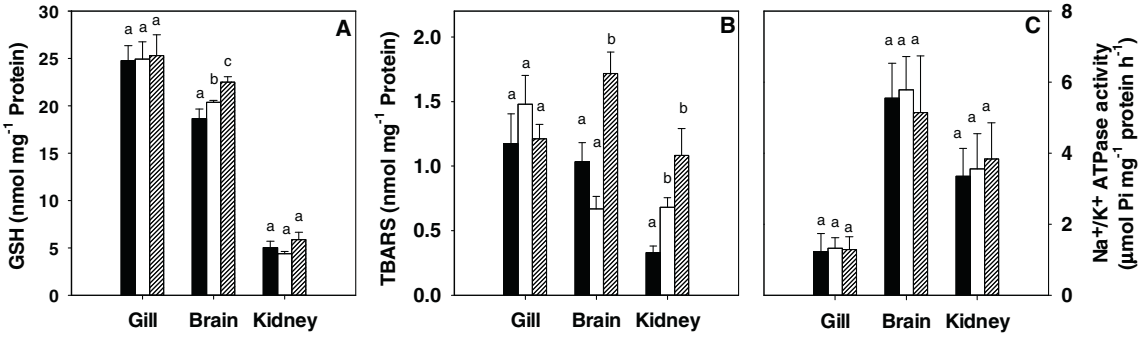

Fig. 2. Concentration of (A) total glutathione (GSH; nmol mg^{-1} protein), (B) thiobarbituric reactive substances (TBARS; nmol mg^{-1} protein) and (C) activity of Na^+/K^+-ATPase (μmol P_i mg^{-1} protein h^{-1}) in tissues of rainbow trout exposed to control (closed bars), 1 mg bulk TiO_2 l^{-1} (open bars) and 1 mg TiO_2 NPs l^{-1} (hatched bars) for 14 days. Data are means ± S.E.M. (n = 7/8). Different lower case letters denote significant difference between treatments ($p \leq 0.05$).

the bulk and nano TiO_2 treatments showed pathology. The injuries included hyperplasia of the primary and secondary lamellae, evidence of oedema and club tips in the secondary lamellae, as well as swollen mucocytes in the gill epithelium (Fig. 3). A quantitative analysis of the incidence of gill injuries confirmed these observations with a statistically significant increase in the incidence of hyperplasia (means ± S.E.M., n = 8) in the TiO_2 NP treatment (7.4 ± 0.6%), and the bulk TiO_2 treatment (6.5 ± 0.6%) compared to the unexposed control (0.7 ± 0.3%) at day 14 (ANOVA, $p < 0.001$, Fig. 3). The incidence of club tips on the lamellae increased from (means ± S.E.M., n = 8) 2.2 ± 0.7% in the controls to 11.1 ± 1.0 and 18.1 ± 1.4% in the bulk and nano TiO_2 treatments respectively, and with a statistically significant material-type effect (greater in fish exposed to TiO_2 NPs, ANOVAs, $p < 0.001$). Similarly, the incidence of swollen mucocytes increased from (means ± S.E.M., n = 8) 2.7 ± 0.7% in the controls to 17.9 ± 2.0 and 11.9 ± 2.0% in the bulk and nano TiO_2 treatments respectively, also with a statistically significant material-type effect (higher incidence in fish exposed to bulk TiO_2, ANOVAs, $p < 0.001$). There were also statistically significant increases in the incidence of aneurisms on the lamellae, fusion of lamellae and necrotic cells in the gill epithelium with both the bulk and nano treatments, but these all remained below a 5% incidence rate (data not shown).

There were some subtle perturbations in the structure of spleen in fish exposed to either 1 mg l^{-1} TiO_2 NPs or the bulk material at day 14 (Fig. 4). There was generally no material-type effect in the spleen structure with both bulk and nano forms causing similar changes in the spleen. The fractional volume of red pulp was significantly decreased in trout exposed to bulk and NPs with a concomitant increase in sinusoid space when compared to controls (Fig. 4, ANOVAs, p = 0.001). Notably, there was also a statistically significant increase in number of melanomacrophages

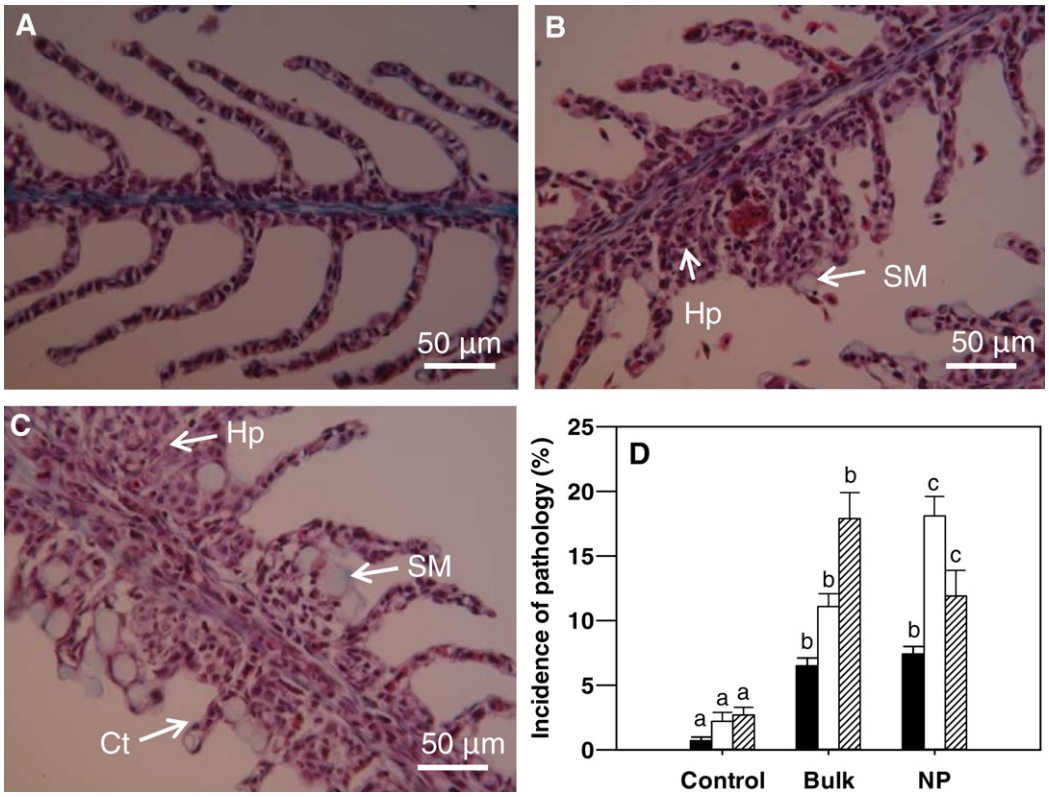

Fig. 3. Gill morphology in rainbow trout after 14 days of waterborne exposure to (A) control, (B) 1 mg l^{-1} bulk TiO_2 and (C) 1 mg l^{-1} TiO_2 NPs. Scale bar indicates magnification (×400). Sections were 7 µm thick and stained with Mallory's trichrome. Panel (D) % secondary gill lamellae showing hyperplasia (Hp, closed bars), clubbed tips (Ct, open bars) and swollen mucocytes (SM, hatched bars). Data are means ± S.E.M. (n = 8). Different lower case letters denote significant difference between treatments ($p \leq 0.05$).

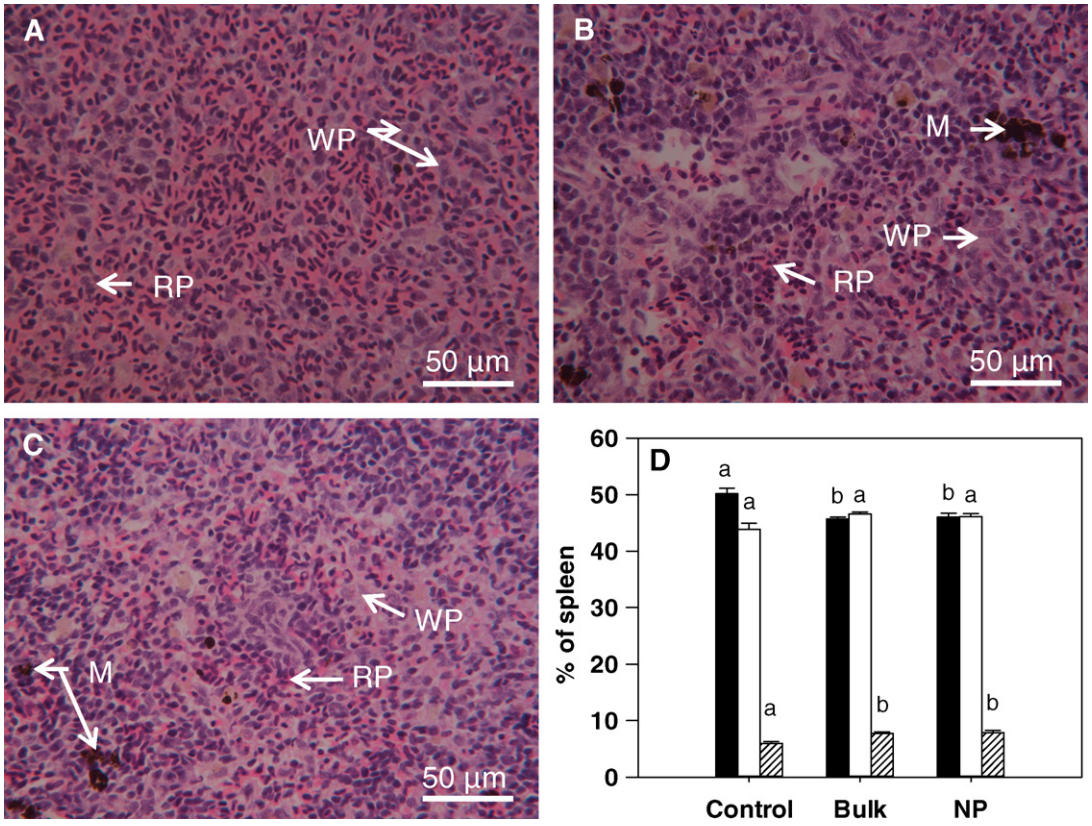

Fig. 4. Spleen morphology in rainbow trout after 14 days of waterborne exposure to (A) control, (B) 1 mg l^{-1} bulk TiO_2 and (C) 1 mg l^{-1} TiO_2 NPs. Scale bar indicates magnification (×400). Sections were 7 μm thick and stained with haematoxylin and eosin. Increased deposition of melanomacrophages (M) and decreased red pulp (RP) were observed in fish exposed to TiO_2 bulk and TiO_2 NPs ($p \leq 0.05$). There was no significant change in the proportion of white pulp (WP). Panel (D) fractional volume of red pulp (closed bars), white pulp (open bars) and sinusoid space (hatched bars). Data are means ± S.E.M. ($n = 8$). Different lower case letters denote significant difference between treatments ($p \leq 0.05$).

(means ± S.E.M, counts/field of view of 53,200 μm^2, $n = 8$) from 58.8 ± 3.2 (control) to 138.2 ± 5.8 (bulk) and 195.0 ± 9.0 (TiO_2 NPs) (ANOVA, $p < 0.001$). This melanomacrophage infiltrate was greater in the nano than bulk treatment (statistically significant material-type effect).

Liver and kidney were also examined. The livers of fish exposed to either bulk or nano TiO_2 showed very minor changes compared to the controls with some mild lipidosis, but there were increases of melanomacrophage deposits. For the latter, the number of deposits (means ± S.E.M, counts/field of view of 53,200 μm^2, $n = 8$) increased from 6.5 ± 1.3 (control) to 17.0 ± 0.7 (bulk) and 18.0 ± 3.0 (TiO_2 NPs). The differences from the control were statistically significant, but with no material-type effect (ANOVA, $p < 0.05$). The liver also showed a small increase in the proportion of sinusoid space of a few percent in both TiO_2 treatments compared to the controls (data not shown). In the kidney, both bulk and nano TiO_2 treatments showed some injuries including mild necrosis of a few individual renal tubules with separation of the epithelium, and enlargement of the Bowman's space; but with no clear material-type effect in these injuries at day 14 (data not shown). The kidney also showed melanomacrophage activity, with a statistically significant increase in number of melanomacrophages (means ± S.E.M, counts/field of view of 212,400 μm^2, $n = 8$) from 230.3 ± 18.0 (control) to 295.3 ± 15.2 (bulk) and 295.5 ± 13.2 (TiO_2 NPs) (ANOVA, $p < 0.001$). There was no material-type effect in the melanomacrophage infiltrate in the kidney.

Excitable tissues were also examined including the skeletal muscle and the brain. The muscle of control fish showed normal morphology and the muscle for both bulk and nano treatments showed increased extracellular space around the muscle fibre bundles. This was reflected in a small but statistically significant decrease in the proportional area of the muscle as fibre bundles by day 14 (means ± S.E.M, % area, $n = 8$) from 71.4 ± 1.3 (control) to 60.4 ± 2.6 (bulk) and 61.0 ± 2.2 (TiO_2 NPs). The differences from the control were statistically significant, but with no material-type effect (ANOVA, $p < 0.05$).

The brains from the control fish were normal, and the gross morphology of brains of trout exposed to either bulk or nano TiO_2 was mainly normal, although very subtle structural changes in the brain were observed. The mesencephalon was normal in fish from all treatments at day 14 (data not shown) with the exception of a contraction in the St. periventricular layer in trout exposed to TiO_2 NPs only (% thickness of the mesencephalon layers, means ± S.E.M., $n = 8$); 31.2 ± 1.2 (control), 31.9 ± 1.0 (bulk) and 27.0 ± 1.2 (TiO_2 NPs). This change, although small, was statistically significant ANOVA, $p = 0.005$). In the cerebellum some subtle changes in the proportions of the tissue layers were also observed, with a slight but statistically significant (ANOVA, $p = 0.004$) thickening of the outer capsule layer of the brain in fish exposed to the bulk TiO_2 only; with the relative thickness of the capsule layer being 1.9 ± 0.2, 2.8 ± 0.5 and 1.7 ± 0.2% in control, bulk and NP exposed fish respectively. Notably, enlarged blood vessels were also observed on the surface of the cerebellum in fish exposed to both nano and bulk TiO_2 (Fig. 5).

3.6. Spontaneous swimming activity

Analysis of spontaneous swimming in rainbow trout (Fig. 6) showed no statistically significant effects of either bulk or nano

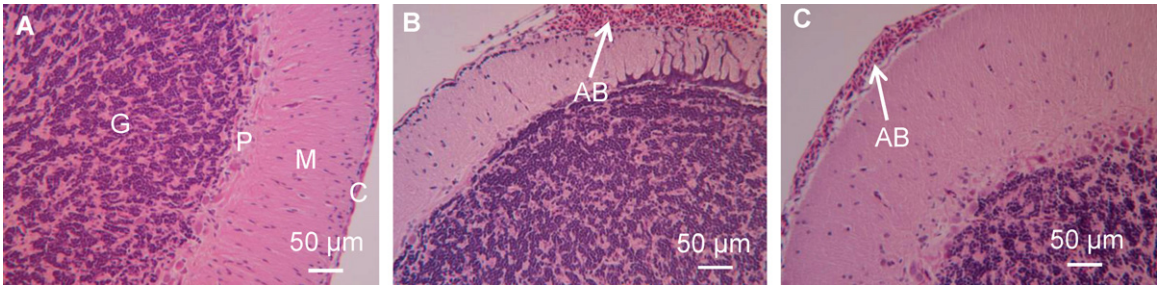

Fig. 5. Cerebellum morphology in rainbow trout after 14 days of waterborne exposure to (A) control, (B) 1 mg l^{-1} bulk TiO_2 and (C) 1 mg l^{-1} TiO_2 NPs. Abnormal blood vessels (AB) were observed at the surface of the cerebellum in fish exposed to nano and bulk TiO_2. Scale bar indicates magnification (×400). Sagittal sections were 7 μm thick and stained with haematoxylin and eosin.

TiO_2 compared to the controls on the total distance moved by fish during the 3 h tracking period (ANOVA, $p = 0.193$), or the mean velocity of fish movements when they were active (ANOVA, $p = 0.307$). However, when activity levels were analysed for time spent moving at different speeds a significant shift to decreased time spent moving at high speed was observed in fish exposed to TiO_2 NPs compared to controls but there was no evidence of a material-type effect. Activity profiles of trout from all treatment groups were characterised by extended inactive periods (<0.4 cm s^{-1}) and moving at low speed (0.4–10 cm s^{-1}) with less time spent moving at medium (10–20 cm s^{-1}) and high (>20 cm s^{-1}) speeds (Fig. 6). There was no significant difference in time spent being inactive (ANOVA, $p = 0.283$), or time moving at low (ANOVA, $p = 0.468$) and medium speeds (ANOVA, $p = 0.262$) in fish exposed to TiO_2 NPs compared to controls (Fig. 6). However, time spent moving at high velocity (>20 cm s^{-1}) was significantly lower in fish exposed to TiO_2 NPs compared to control fish (ANOVA, $p = 0.036$, Fig. 6). Time spent moving at high speed in fish exposed to bulk TiO_2 was not significantly different from controls or fish exposed to TiO_2 NPs (ANOVA, $p > 0.05$).

Analysis of AChE activity in brain of fish assayed immediately after tracking fish movements indicated no significant difference between fish exposed to controls, bulk TiO_2 and TiO_2 NPs (ANOVA, $p = 0.078$). Measured AChE activity levels were (means ± S.E.M., $n = 4$–6); 107.7 ± 3.7, 123.8 ± 8.2, 108.7 ± 3.0 nmol l^{-1} min^{-1} mg^{-1} protein for controls, bulk TiO_2 and TiO_2 NP exposed fish, respectively. Plasma glucose and serum lactate concentrations were also not significantly different in fish from different treatment groups (ANOVAs, $p > 0.05$). Glucose concentrations were 3.66 ± 0.27, 3.16 ± 0.18 and 3.66 ± 0.3 mmol l^{-1} and lactate concentrations 4.1 ± 0.8, 3.0 ± 0.9 and 1.9 ± 0.6 mmol l^{-1} in fish exposed to control, bulk TiO_2 and TiO_2 NPs, respectively.

3.7. Behavioural observations of competitive interactions

Behavioural observations of competitive interactions of trout indicated no statistically significant effect of exposure to TiO_2 NPs on competitive ability compared to controls or bulk TiO_2. Interactions between fish were usually characterised by rapid engagement, the first aggressive act typically occurring 1–4 min following removal of the partition, escalation of the contest and withdrawal of the subordinate fish from the interaction within the 30 min period of observation. Dominant and subordinate fish were identifiable in all contests with the exception of two pairs of fish which did not interact during the 3 h period allocated for behavioural observations; one pair each from (a) control paired with NP exposed fish and (b) bulk exposed paired with a NP exposed fish. These fish were removed from subsequent analysis leaving $n = 14$ remaining pairs of fish. Where TiO_2 NP exposed fish were paired with control fish the outcome of the interaction was not affected by exposure: in 3/7 interactions the TiO_2 NP-exposed fish emerged as the dominant fish in the interaction. The number of aggressive acts in 30 min was also not affected by exposure (means ± S.E.M., Student's t test, $n = 3/4$, $p = 0.57$), 83 ± 41 and 123 ± 55 aggressive acts in control fish and dominant TiO_2 NP-exposed fish, respectively. Time of retreat of the subordinate fish was also not significantly different (means ± S.E.M., $n = 3/4$, Student's t test, $p = 0.19$), 15 ± 7 and 5 ± 2 min in subordinate control and NP exposed fish respectively. Where a TiO_2 NP-exposed fish was paired with a bulk material-exposed fish, treatment was also not a factor in outcome of interaction; in 4/7 interactions the TiO_2 NP-exposed fish emerged as the dominant fish in the interaction. The number of aggressive acts in 30 min was also not affected by the type of material used for exposure (means ± S.E.M., Student's t test, $n = 3/4$, $p = 0.46$), 71 ± 34 and 44 ± 13 aggressive acts in dominant fish that had been exposed to bulk TiO_2 and TiO_2 NPs, respectively.

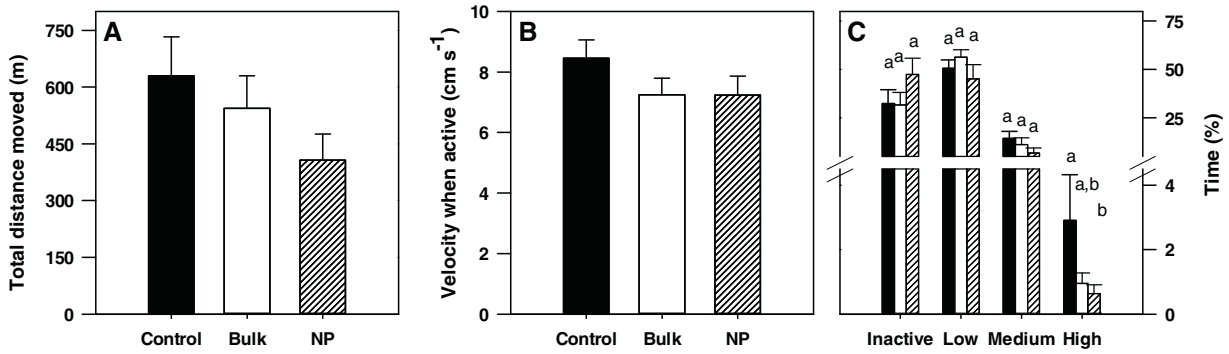

Fig. 6. Swimming behaviour in rainbow trout exposed to control (closed bars), 1 mg bulk TiO_2 l^{-1} (open bars) and 1 mg TiO_2 NPs l^{-1} (hatched bars) for 14 days. Fish movements (A) total distance moved (m), (B) velocity when active (cm s^{-1}) and (C) time (%) moving at low (0–10 cm s^{-1}), medium (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. ($n = 4$–6). Different lower case letters denote significant difference between treatments ($p \leq 0.05$).

Time of retreat of the subordinate fish was also not significantly different (means ± S.E.M., Mann–Whitney *U*-test, $n = 3/4$, $p = 0.29$), 8 ± 4 and 9 ± 2 min in subordinate bulk and TiO_2 NP-exposed fish respectively.

4. Discussion

The present study has demonstrated that exposure to 1 mg l^{-1} TiO_2 NPs for 14 days can cause changes in the locomotor activity of rainbow trout which involve the exposed-fish spending proportionally less time at high swimming speeds compared to controls. Notably, there was no clear material-type effect on locomotor activities, but both materials caused gill injury. The decrease in the proportion of time spent at high swimming speeds is most easily explained by respiratory hypoxia rather than neurotoxicity, with increases of HCT, Hb and changes in the proportion of red pulp in the spleen indicating some physiological compensation; albeit with some subtle differences between the bulk and nano TiO_2 in this response. The animals remained able to compete in aggressive interactions, and exposure to either bulk or nano TiO_2 had no effect on the outcome of these bouts. Overall, the data shows that trout will moderate their swimming speed in response to waterborne TiO_2 exposure, but retain the physiological capacity for short periods of energetically demanding activities such as defending themselves in a dominance contest.

4.1. Toxicity from waterborne exposure to TiO_2

Waterborne exposure to either bulk or nano TiO_2 was characterised by some Ti accumulation on/in the gill tissue, but with no measurable increases in the Ti concentrations in the internal organs (Table 1). Our previous study on rainbow trout (Federici et al., 2007) also found no treatment-dependent increases in Ti metal concentrations in the internal organs. However unlike the present study, Federici et al. (2007) did not detect measurable increases in the gill tissue. This difference is partly due to the lower background Ti concentrations in the fish used here (Ti concentrations vary with the source and feeding history of the fishes, see discussion in Ramsden et al., 2009); but mainly due to improvements in the ICP-MS method in the present study which enable differences in the gill to be detected that could not be measured before. Nonetheless, the Ti concentrations on/in the gills (Table 1) remain at μg g^{-1} concentrations for an exposure at mg l^{-1} levels, indicating only a fraction of the exposure dose became associated with gill tissue. The absence of increases in the Ti concentrations of the internal organs, and only modest increases in/on the gill tissue of trout, from waterborne TiO_2 exposure is consistent with Johnston et al. (2010) who conducted Coherent Anti-Stokes Raman Scattering (CARS) microscopy on the gill of rainbow trout exposed to 5 mg l^{-1} TiO_2 NPs for 10 days. Johnston et al. (2010) showed that (for TiO_2 NPs at least) most of the TiO_2 aggregates on the surface of the gill epithelium, with the occasional aggregate appearing within the secondary lamellae. Nonetheless, exposure in this study was confirmed by measurable Ti concentrations in the exposure tanks, and similar to Federici et al. (2007), histological examination of the gills of fish confirmed respiratory irritation in trout exposed to bulk and nano TiO_2. Gills showed an increased incidence of swollen mucocytes that was greatest for the bulk material (Fig. 3). The mucus defences were partially successful with a lower incidence of clubbed tips in the bulk compared to nano TiO_2 treatment. The hyperplasia in the gill epithelium of fish exposed to TiO_2 NPs or bulk TiO_2 compared to controls (Fig. 3) was similar to the findings of Federici et al. (2007). Gill injury has also been reported in other species of fish e.g. *Cyprinus carpio*, exposed to waterborne TiO_2 NPs (Hao et al., 2009).

The gill is an important organ for both osmoregulation and respiratory gas exchange. In the present study the general absence of disturbances to plasma ions and osmolarity (Table 2), the normal gill tissue Na^+ and K^+ concentrations (Fig. 1A), as well as the absence of inhibition of branchial (or renal) Na^+/K^+-ATPase (Fig. 2C) indicates that the gill injury was not sufficient to cause major osmoregulatory disturbances within 14 days (similar to the conclusions of Federici et al., 2007). However, thickening of the gill epithelium due to hyperplasia and oedema (Fig. 3) will increase the diffusion distance for gas exchange; and even small increases can have profound effects on the efficiency of oxygen transfer across the gill (Malte, 1992). Gill injury from metal exposures is known for being associated with a decrease in arterial oxygen tension (P_aO_2), which may be recoverable depending on the extent of the injury (e.g., Zn, Lappivaara et al., 1995). Decreases in P_aO_2 in fish exposed to Ag NPs have also been reported (Bilberg et al., 2010). It therefore seems probable that the gill injury reported here (Fig. 3) would cause some hypoxia.

A 5% increase in HCT values, and a subtle but statistically significant increase of 1.7 g dl^{-1} in the haemoglobin concentration of blood was observed in the TiO_2 NP treatment compared to controls (Table 2); partly supporting the idea of compensation for a systemic hypoxia. A trend of increasing haemoglobin (not statistically significant) was noted by Federici et al. (2007). However, the variations in haematology in both studies are within physiological limits. For example, temporal changes in the control HCTs in Federici et al. (2007) was around 6%, exceeding the increase observed here. Interestingly, despite similarities in the incidence of hyperplasia in the gill caused by exposure to TiO_2 NPs or bulk TiO_2 (Fig. 3), and the subtly increased HCT values (Table 2); this effect was only apparent in trout exposed to TiO_2 NPs and not the bulk TiO_2. However, both materials resulted in comparable proportional decreases in the area of red pulp in the spleen (Fig. 4), consistent with the release of red cells from the spleen stores into the circulation in response to hypoxia (see Handy et al., 2011 for discussion on the spleen). However, the spleen of the NP-exposed fish showed more melanomacrophage infiltrate than the controls or bulk material treatment. These deposits reflect the normal activity of the spleen in processing damaged blood cells, indicating the spleen was working harder to maintain the normal counts of circulating blood cells, especially in the NP-exposed fish.

Regardless of the material-type, the haematological response appears to have been successful in terms of decreasing any hypoxia-driven oxidative stress. Hypoxia is known to cause oxidative stress in the internal tissues of fish, (Luschak and Bagnyukova, 2007), and exposure to Ag NPs appears to reduce the hypoxia tolerance of the Eurasian perch *Perca fluviatilis* (Bilberg et al., 2010). In the present study biomarkers of oxidative stress in the gill (total glutathione, TBARS, Fig. 2) did not change, presumably because this external epithelia was bathed in well oxygenated water throughout the experiment. In Federici et al. (2007) the branchial glutathione only increased by 1 μmol g^{-1} tissue (probably not physiologically important), and the TBARS increased by about 3 nmol mg $protein^{-1}$ due to TiO_2 NP exposure. These are arguably only subtle differences in the biochemical responses of the gills between the two studies, and in the absence of glutathione depletion in the gills, both studies suggest that oxidative stress was not the likely cause of gill pathology. However, the situation was different for the internal organs, with both the kidney and brain showing increases in TBARS (Fig. 2) without measurable increases in brain Ti concentration (Table 1). Federici et al. (2007) also observed elevations of TBARS in the brain without changes in Ti concentration of the organ. This is suggestive of an indirect effect of TiO_2 NPs on the brain via systemic hypoxia. The brain, being a critical organ to protect from hypoxia, also showed an increased total glutathione pool; with the

biggest changes in the TiO_2 NP-exposed fish (Fig. 2) consistent with the material-type effect in the haematological response above.

The absence of changes in oxidative stress parameters in the external epithelia (no change in branchial total glutathione or TBARS, Fig. 2), and the moderately low lux of the laboratory lighting used in the present study, suggests that ultraviolet (UV) activation of reactive oxygen species generation by the TiO_2 NPs was not likely in the experiment. Using the same TiO_2 NPs, Reeves et al. (2008) found that relatively high UVA dose rates of 2.5 kJ m^{-2} were needed to produce oxidative DNA damage in fish skin cell lines (GFSk-S1 cells). The modest changes in oxidative stress parameters in the internal organs (Fig. 2) are therefore best explained by some systemic hypoxia associated with gill injury. Ecologically, rainbow trout prefer shade and areas of low light in rivers and one might therefore expect this fish to avoid the worst of any theoretical UV-dependent reactive oxygen releases from TiO_2 in the wild.

4.2. Effects of TiO_2 on behaviour of trout

Rainbow trout can spend two thirds or more of their daily energy expenditure on swimming, and consequently, changes in locomotor activity could have profound effects on their overall energy budget and probability of survival (Priede, 1985). From a biomechanics perspective, high speed swimming is (exponentially) much more energetically expensive than low or moderate speeds, and fish therefore rarely attain maximum velocities at the limit of aerobic metabolic scope (Young et al., 1972; Priede, 1977). This is reflected in the normal locomotor activity pattern reported here, with fish spending most of their time at low or moderate speeds, and only with the occasional short burst of high speed swimming (Fig. 6C). This observation is also consistent with our previous recordings for juvenile rainbow trout (Handy et al., 1999; Campbell et al., 2002).

In fish exposed to TiO_2 NPs only, the proportion of time spent swimming at high speed (>20 cm s^{-1}) was significantly decreased compared to the unexposed controls, although there was no overall difference in mean swimming speed distributions between bulk and nano TiO_2 (Fig. 6C). Consequently, the mean velocity of fish when active was unchanged (Fig. 6B). This effect of metals has previously been reported in fish exposed to dietary Cu where mean swimming speeds and total distanced moved show limited or no overall change, but the fish had altered swimming speed distributions in favour of lower speeds (Handy et al., 1999; Campbell et al., 2002). This phenomenon has been explained in terms of a metabolic trade off in the energy budget of the animal, where Cu-exposed fish redirect energy from locomotion to tissue repair in order to maintain normal growth and physiology during chronic Cu exposure (Handy et al., 1999). In the case of waterborne TiO_2 exposure, the situation is analogous in that the fish have damaged gills (Fig. 3) and are evidently expending energy to maintain the circulating red blood cells (Table 2); and probably on osmoregulation to keep all the tissue electrolytes generally normal (Fig. 1) in the face of a leaky (damaged) gill epithelium. The overall strategy of TiO_2-exposed fish appeared to be effective because the fish had normal blood glucose concentrations, and did not show elevations of plasma lactate (i.e., no build-up of an oxygen debt) at the end of the experiment.

However, the question arises as to whether the TiO_2-exposed fish "voluntarily" adopt this behavioural strategy of less time at high swimming speeds, or are simply forced to do it because they are physiologically incapable of bursts of heavy exercise. The latter seems unlikely because there was no effect of TiO_2 on competitive ability. The duration of each contest, the level of aggression and the outcome were not affected by TiO_2 NP exposure. Previous studies on exercise physiology have also demonstrated that fish can sustain considerable loss of respiratory function before swimming ability is compromised. For example, Duthie and Hughes (1987) report >30% reduction in functional gill area was required to reduce maximum sustained speed in exercised fish.

4.3. Neurotoxicity and behaviour

An alternative hypothesis to gill injury (systemic hypoxia risk) driving the haematological and behavioural changes observed in the present study is direct neurotoxicity of TiO_2 NPs. However, several lines of evidence suggest this is unlikely, including no detectable increases of Ti in the brain tissue, although secondary neurotoxicity associated with hypoxia remains possible. The levels of pathologies observed in brains were minimal, and unlikely to compromise the role of the cerebellum (hind brain) in coordinating motor functions. The presence of enlarged blood vessels on the surface of the cerebellum (Fig. 5) may not be pathological, but simply part of a prolonged vasodilation response to ensure oxygen supply to the brain to compensate for the gill damage. The contraction of the St. periventricular layer in the mesencephalon was most likely due to a simple fluid shift, from the tissue layer into the underlying ventricle. This observation and the elevation of the main electrolytes in the brain tissue of the TiO_2 NP-treated fish (Fig. 1) suggest some dehydration. If this explanation is correct, there is also a theoretically possible that an electrolyte and fluid imbalance in the brain could also affect motor outputs (locomotion). In clinical terms, these sorts of injuries might cause some discomfort or delay in processing of stimuli (i.e., a fish with a "headache", but still functioning behaviourally).

The data supporting electrical properties (Na^+/K^+-ATPase, ratio of Na^+: K^+ in the tissue) and activity of AChE in the brains of fish exposed to TiO_2 NPs were normal. Loss of sympathetic tone (i.e., pathology of motor neurons in the peripheral nervous system) also seems unlikely with no evidence of muscle atrophy. The spleen also retained its ability to change the proportions of the red and white pulp; a function that can only be achieved with intact sympathetic control of the organ. Windeatt and Handy (2012) also recently showed no effects on action potential generation with direct applications of 1 mg l^{-1} TiO_2 NPs onto isolated crab nerve preparations in vitro.

4.4. Conclusions

In conclusion, this study demonstrates that juvenile rainbow trout exposed to 1 mg l^{-1} TiO_2 NPs for 14 days spend proportionally less time at high swimming speeds. The cause of this change in swimming speed distribution is more easily explained by damage to the gill epithelium with a subsequent hypoxia rather than direct neurotoxicity of TiO_2. Indirect toxicity through hypoxia is supported by a decrease in the red pulp in the spleen and a compensatory increase in HCT. Subtle changes in brain histology with enlarged vasculature are also consistent with systemic hypoxia. While there was no overall material-type effect on animal behaviour, there were some differences between bulk and nano TiO_2 on the organ pathologies, as well as the underlying haematological responses of the fish; with the NP-exposed fish generally working harder to compensate for the disturbances. The reasons for these material-type effects in the cardiovascular physiology of fishes require further investigation. However when challenged, the animals retained the physiological ability to compete in aggressive encounters. From an ecological perspective, both bulk and nano TiO_2-exposed fish can maintain routine swimming speeds for activities like foraging behaviours, as well as defend themselves from aggressors. However, bursts of high speed swimming may be problematic for the TiO_2-NP exposed fish in particular.

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