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Critical comparison of intravenous injection of TiO_2 nanoparticles with waterborne and dietary exposures concludes minimal environmentally-relevant toxicity in juvenile rainbow trout *Oncorhynchus mykiss*

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ABSTRACT

A critical comparison of studies that have investigated tissue accumulation and toxicity of TiO_2-NPs in fish is necessary to resolve inconsistencies. The present study used identical TiO_2-NPs, toxicological endpoints, and fish (juvenile rainbow trout *Oncorhynchus mykiss*) as previous studies that investigated waterborne and dietary toxicity of TiO_2-NPs, and conducted a critical comparison of results after intravenous caudal-vein injection of 50 μg of TiO_2-NPs and bulk TiO_2. Injected TiO_2-NPs accumulated only in kidney (94% of measured Ti) and to a lesser extent in spleen; and injected bulk TiO_2 was found only in kidney. No toxicity of TiO_2 was observed in kidney, spleen, or other tissues. Critical comparison of these data with previous studies indicates that dietary and waterborne exposures to TiO_2-NPs do not lead to Ti accumulation in internal tissues, and previous reports of minor toxicity are inconsistent or attributable to respiratory distress resulting from gill occlusion during waterborne exposure.

1. Introduction

Concern over the potential for negative environmental effects to occur as a consequence of the production and use of manufactured nanoparticles (NPs) has led to the emergence of new disciplines of nanotoxicology and nanoecotoxicology to investigate these effects (Oberdörster et al., 2005; Kahru and Dubourguier, 2010). However, NPs do not behave as traditional toxicants, and poor initial understanding of methods for testing have led to subsequent revision of NP toxicity and considerable inconsistency in reports of NP toxicity in the literature (e.g. Henry et al., 2011). Among the NPs for which results of toxicity have been inconsistent are TiO_2-NPs. Titanium dioxide NPs have photo-refractive and photo-absorptive properties and have widespread utility in consumer and industrial products as a pigment (Weir et al., 2012) and as an active component in sunscreen (Popov et al., 2005). The growing use of TiO_2-NPs has resulted in release and detection of particulate Ti (presumed TiO_2, including NPs) in effluents released into the environment (Johnson et al., 2011) and predicted environmental concentrations of between 0.01 and 16 μg L^{-1} in European surface waters (Mueller and Nowack, 2008; Gottschalk et al., 2009; Johnson et al., 2011). Accordingly, much focus of toxicological effects of TiO_2-NPs has been in aquatic organisms.

A key determinant of NP toxicity is bioavailability at biological membranes. At the gill epithelium, NPs are predicted to be too large to be transported by membrane-bound ion transporters, and uptake via paracellular pathways is likely retarded by particle aggregation in natural waters (Handy et al., 2008). In agreement with this hypothesis, intracellular aggregates of TiO_2-NPs were detected, but below quantifiable levels in gill and were undetected in internal organs of rainbow trout (*Oncorhynchus mykiss*) exposed to 5 mg L^{-1} TiO_2-NPs (Johnston et al., 2010). Boyle et al. (2013) also report accumulation only at the gill after 14-d exposure to 1 mg L^{-1} TiO_2-NPs in rainbow trout, indicating TiO_2-NPs were not internalised. Conversely, accumulation of TiO_2-NPs was reported in gill, liver, heart and brain in zebrafish (*Danio rerio*) exposed to 1 mg L^{-1} TiO_2-NPs (Chen et al., 2011). The TiO_2 accumulation reported by Chen et al. (2011) may have included TiO_2 contamination from the presence of TiO_2 on external surfaces (including gut lumen) during

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tissue excision. Rapid depuration (half-life 1.9 days) of TiO_2 after whole body accumulation (96.76 ± 3.02 μg g^{-1}) in zebrafish exposed to 1 mg L^{-1} TiO_2 NPs (Zhu et al., 2010) indicated that most TiO_2 adhered to external surfaces and was not internalized, which also suggests that accumulation of TiO_2 reported in zebrafish heart and brain (Chen et al., 2011) was an experimental artefact. Scown et al. (2009) reported that TiO_2 injected into the caudal vein accumulated in kidney but not in other tissues of rainbow trout. Overall, there is no consistent evidence of appreciable absorption or accumulation of TiO_2-NPs in fish; however, irrespective of uptake, sub-lethal toxicity, including effects on internal organs, has been reported in fish.

Toxicity of TiO_2-NPs could result from accumulation of NPs on epithelial surfaces and not be dependent on absorption and distribution to internal target tissues. The few reports of acute toxicity of TiO_2-NPs in fish [e.g. 96 h LC_{50} of 124.5 mg L^{-1} in adult zebrafish (Xiong et al., 2011)] indicate fish are orders of magnitude less sensitive to TiO_2-NPs compared to more traditional chemicals such as dissolved metals (Shaw and Handy, 2011). Sub-lethal effects in fish after exposures to low and sub mg L^{-1} TiO_2-NP concentrations have been reported in most major body systems including reproductive and immune systems (see review by Handy et al., 2011). For example, exposure to 0.1 mg L^{-1} TiO_2-NPs for 40 days caused a decrease in cumulative egg production in zebrafish (Wang et al., 2011). Exposure to TiO_2-NPs was reported also to induce minor biochemical changes in brain of fish after waterborne and dietary TiO_2 exposures (Federici et al., 2007; Hao et al., 2009; Ramsden et al., 2009; Boyle et al., 2013); however, Boyle et al. (2013) attributed these changes to gill injury and resultant systemic hypoxia rather than a neurotoxic mechanism of TiO_2-NPs. Further evidence that effects of TiO_2-NPs in fish can be explained by accumulation of NPs on external epithelial surfaces (e.g. gills) is that after intravenous injection of TiO_2-NPs, no toxicity was reported in rainbow trout (Scown et al., 2009). While some evidence indicates that TiO_2-NPs are not appreciably absorbed or accumulate in fish tissues, and that toxicity is consistent with NP accumulation on external surfaces (e.g. respiratory distress), uncertainty regarding the importance and effects of TiO_2-NP toxicity remains in the literature.

To move understanding of TiO_2-NP toxicity in fish forward, a critical examination of comparable previous reports and targeted further experimentation are required to resolve inconsistencies in the literature. In the present study we investigate the tissue distribution and toxic effects of an intravenous injection of TiO_2-NPs and bulk TiO_2 in rainbow trout. This approach eliminates the issue of TiO_2-NP bioavailability from the external environment, and enables identification of tissues where Ti metal from exposure to TiO_2-NPs may accumulate. Our laboratory is also one of few to have previously published data on both waterborne and dietary exposure to TiO_2-NPs in trout (Federici et al., 2007; Ramsden et al., 2009; Boyle et al., 2013). In the present study, we use the same batch of NPs, the same species and life history stage of fish and employ the same histological, haematological and biochemical endpoints as in our previous studies and conduct a unique comparative evaluation of TiO_2-NP toxicity in fish.

2. Materials and methods

2.1. Experimental animals

Rainbow trout (38.6 ± 8.6 g, Mean ± S.D., $n = 49$) were obtained from a local supplier (Torre fishery, Watchet, Somerset) and maintained in aerated, dechlorinated and recirculating Plymouth tap water (means ± standard deviations (S.D.), $n = 5$; Ca^{2+} 0.342 ± 0.004 mmol L^{-1}; Mg^{2+} 0.046 ± 0.001 mmol L^{-1}; Na^+ 0.422 ± 0.008 mmol L^{-1}; K^+ 0.027 ± 0.003 mmol L^{-1}; pH 6.8 ± 0.3; temperature 16.2 ± 0.5 °C; DO 100 ± 1% saturation; [total ammonia] 0.1 ± 0.1 mg L^{-1}) for 4 weeks prior to experimentation. Twice daily during this acclimation period fish were fed to satiation with a commercial trout diet (EWOS, Westfield, UK) until 48 h prior to injection.

2.2. Titanium dioxide stock suspensions

Nano and bulk particle forms of TiO_2 were obtained from DeGussa AG ("Aeroxide" P25, supplied by Lawrence Industries, Tamworth, UK) and ACROS (New jersey, USA) respectively and were the same dry powders as used by Federici et al. (2007), Ramsden et al. (2009) and Boyle et al. (2013). According to manufacturer's information, TiO_2-NPs have a crystal structure of 25% rutile and 75% anatase TiO_2, with >99% purity (as TiO_2, maximum impurity stated was 1% Si). Transmission electron microscopy (TEM, JEOL 12000EXII, Tokyo, Japan) analysis of crystal structure of bulk TiO_2 showed particles were (means ± S.D., $n = 10$ micrographs analysed) 25.3 ± 4.8% rutile and 74.68 ± 4.78% anatase. Boyle et al. (2013) reported primary particle sizes of (means ± S.D., $n = 100$) 134.06 ± 42.54 nm and 24.53 ± 10.63 nm of TiO_2 bulk and TiO_2-NPs respectively. Stock suspensions of 1 g L^{-1} bulk TiO_2 and 1 g L^{-1} TiO_2-NPs (nominal concentrations) were prepared in Cortland's saline (in mmol L^{-1}; 137 NaCl; 2.7 KCl; 4.3 Na_2HPO_4; 1.4 NaH_2PO_4; pH 7.8, in ultrapure water (MilliQ®, Millipore Company)) and sonicated for 6 h (35 kHz frequency, Fisherbrand FB 11010, Germany) before injection. Actual measured concentrations of Ti (and adjusted to molecular weight of TiO_2) in stock suspensions were (Means ± S.D., $n = 3$): 0.975 ± 0.016 g L^{-1} and 1.08 ± 0.026 g L^{-1} for bulk and NPs respectively. It was not possible to gather reproducible data of particle size distributions in the stock salines due to the rapid aggregation and settling out of the particles in the instrument (Nanosight LM 10, Nanosight, Salisbury, UK).

2.3. Titanium dioxide injection

Trout ($n = 21$ fish per treatment) were anaesthetised in buffered MS222 (50 mg L^{-1}, pH 7.1) and injected in the caudal vein with Cortland's saline (50 μl, control), TiO_2 bulk or TiO_2-NPs (both 50 μg TiO_2 in 50 μl saline) with a 1 ml Terumo insulin syringe with 29 gauge needle (Terumo Medical Corporation, Somerset, NJ, USA). Trout were then resuscitated in oxygen saturated aquarium water and returned to recirculating tanks in Plymouth tap water (as above). None of the fish exhibited signs of poor health (e.g. no loss of equilibrium, laboured swimming or respiratory distress) immediately following injection or for the duration of the exposure. Fish were not fed during the 96 h exposure period. Fish sampled at time 0 were not injected with Cortland's saline.

Additional fish ($n = 3$, per treatment) were also injected intramuscularly, near the posterior insertion of the dorsal fin with Cortland's saline (50 μl, control), TiO_2 bulk or TiO_2-NPs (both 50 μg TiO_2 in 50 μl saline) for subsequent examination of the site of injection by histopathology 96 h post-injection. The objective of this procedure was to enable examination of TiO_2 bulk or TiO_2-NPs in tissue and assess the inflammatory response to the presence of these materials.

2.4. Haematology and plasma analysis

At 0, 24 and 96 h post-injection 7 fish per treatment ($n = 7$ total fish at time 0) were terminally anaesthetised in buffered MS222, weighed, blood immediately withdrawn by caudal puncture with a heparinised syringe and sub-samples of tissues excised and stored on ice for element analysis and snap frozen in liquid N_2 and stored at −80 °C for biochemical analyses (see subsequent sections). Sub-samples of whole blood were taken for measurement of haematocrit (HCT), haemoglobin (Drabkin's reagent, Sigma–Aldrich, UK), and red blood cell counts (Dacie's fluid, 0.1 M tri sodium citrate, 2.5 mmol L^{-1} cresyl blue, in 1% formalin). 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) measured in plasma.

2.5. Tissue ion analysis

The methodology for Ti analysis in fish tissues has evolved since Ti measurements were first reported by Federici et al. (2007). Specifically, the addition of Triton-X100 (Fisher Scientific, UK) to the digestion matrix has been demonstrated to improve recovery of Ti from TiO_2 (Shaw et al., 2013). Tissues were oven dried to constant weight and digested in 1 ml concentrated HNO_3 (Trace element grade, Fisher Scientific, UK) at 80 °C) for 2 h. Following digestion, samples were diluted to 4 ml with Triton-X100 (2% final concentration in digest) diluted in ultrapure water. Acid digests and corresponding acidified element standards matrix matched in 2% Triton-X100 were analysed in triplicate for Ti by ICP-OES (Varian 725-ES). Method detection limit for Ti for samples measured in this study was 4.8 μg L^{-1}.

2.6. Biochemical analyses

Biochemical analyses of Na^+/K^+-ATPase activity, total glutathione (GSH) concentration and concentration of thiobarbituric reactive substances (TBARS) were performed according to the same methods each used by Federici et al. (2007), Ramsden et al. (2009) and Boyle et al. (2013). Tissues excised from fish were defrosted on ice and homogenised in ice cold sucrose buffer (300 mmol L^{-1} sucrose,

20 mmol L^{-1} HEPES, 0.1 mmol L^{-1} EDTA, pH 7.8 (All reagents Sigma–Aldrich, UK)) before being centrifuged at 13,000 rpm for 2 min. Concentration of GSH, TBARS, and activity of Na^+/K^+-ATPase were normalized to total protein concentration in supernatants measured with Bradford's reagent (Sigma–Aldrich, UK).

2.7. Histological analyses

At 96 h post-injection an additional $n = 7$ fish per treatment were terminally anaesthetised as above and tissues excised and processed for routine histological examination by light microscopy. After fixation in 10% neutral buffered formalin, tissues were dehydrated, embedded in paraffin, sectioned (5–6 μm), and stained (haematoxylin and eosin) following methods previously described by Boyle et al. (2013). Histological sections of kidney, liver, brain, and spleen were evaluated by light microscopy in fish that received an intravenous injection with saline (controls), TiO_2-bulk, and TiO_2-NPs. Skeletal muscle surrounding and including the site of intramuscular injection of either saline (control), TiO_2-bulk, or TiO_2-NPs was excised in a further $n = 3$ fish 96 h after injection and processed and examined as described above.

Spleen excised within 15 min of euthanasia of sampled fish were used to make spleen prints as described in Peters and Schwarzer (1985) with minor modification. Spleen prints were stained with May – Grunwald – Giemsa, and scored by the method of Peters and Schwarzer (1985). At least 200 cells were examined (100× objective, total magnification ×1000, an Olympus Vanox-T microscope, Olympus digital camera (C-2020 Z)) and the different cell types (erythrocytes, leukocytes) were enumerated including the number of abnormal erythrocytes.

2.8. Data handling and statistical analyses

Statistical analyses with appropriate parametric and non-parametric tests were performed using SPSS (version 18.0 for Windows). Data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test) and where appropriate data were Log_{10} transformed prior to statistical tests being used on data sets. Comparison of results from the present study with those of previous TiO_2-NP exposures conducted in our laboratory (i.e. Federici et al., 2007; Ramsden et al., 2009; Boyle et al., 2013) was conducted by normalizing results to time-matched control values within same study and expressing data as percentage change.

3. Results and discussion

Previous investigations have reported considerable differences in bioavailability and toxicity of TiO_2-NPs in fish. One potential reason for the differences among studies is the route of exposure and the issue of whether TiO_2-NPs are appreciably absorbed across epithelial membranes. In the present study, TiO_2-NPs injected into blood circulation of fish via caudal vein accumulated predominantly in kidney, with no significant tissue clearance during 96-h exposure (Fig. 1). There was also a small but significant accumulation of TiO_2-NPs in spleen that was not observed in fish injected with bulk TiO_2, indicative of a particle size effect on tissue distribution (Fig. 1). At 96 h, measured Ti concentrations in spleen of controls, bulk TiO_2, and TiO_2-NPs injected fish were (means ± S.E.M, $n = 6/7$): 1.5 ± 0.3, 2.4 ± 0.5, 26.2 ± 6.2 μg g^{-1} dry weight, respectively. No significant accumulation of TiO_2 was observed in any other tissue examined (including brain, liver, skeletal muscle), and identification of kidney as the predominant tissue of accumulation of intravenously injected TiO_2 is consistent with the findings of a previous study with rainbow trout (Scown et al., 2009). However, accumulation in the kidney could be a consequence of the site of injection (caudal vessel); blood flow from the site of injection in caudal vein passes first to kidney and further studies are required to investigate if injection at other points on the circulatory system of trout affects tissue distribution of TiO_2. Results of TiO_2 intravenous injection studies in mice indicate that the main filtration organs (spleen, liver and kidney) are also sites of accumulation of Ti from intravenous injection in mammals (Umbreit et al., 2012).

Comparison of the present study with other studies of rainbow trout exposed via waterborne or dietary routes indicate that accumulation of TiO_2 in internal tissues is minimal and not an important concern for environmentally relevant dietary and waterborne exposures (Table 2). An early study of rainbow trout exposed to waterborne TiO_2-NPs (up to 1 mg L^{-1}) reported no significant accumulation in any tissues [kidney not examined (Federici et al., 2007)], and no significant accumulation of TiO_2-NPs in any internal tissues (including kidney) after waterborne exposure was confirmed in a subsequent study [Table 2 (Boyle et al., 2013)]. TiO_2-NPs accumulated on gills of rainbow trout, but there is no evidence of absorption across epithelial membranes or accumulation in internal tissues of rainbow trout. There was no significant accumulation of Ti in any tissues of rainbow trout after 8-week dietary exposure to up to 100 mg TiO_2-NPs Kg^{-1} food (fish fed to satiation twice daily) by Ramsden et al. (2009). In the Ramsden et al. (2009) study, significantly higher Ti tissue concentrations were reported in brain and liver relative to controls in week four of exposure, and spleen at week two of exposure; however, mean concentrations were low [0.18 μg g^{-1}(liver), 0.29 μg g^{-1} (brain), and 0.57 μg g^{-1} (spleen)], and are likely transient fluctuations in tissues rather than treatment effects. The range of the maximum mean Ti concentrations for tissues from unexposed control rainbow trout from Ramsden et al. (2009) and Boyle et al. (2013) were: 0.74–1.9 μg g^{-1} (liver), 0.38–8.2 μg g^{-1} (spleen), and 0.24–0.9 μg g^{-1} (brain), and the mean Ti concentrations for these tissues in control fish of the present study are within this range. There do not appear to be any comparable studies of TiO_2-NP dietary exposure in mammals with evaluation of Ti accumulation in internal tissues. Collectively, the results obtained in these studies with rainbow trout indicate that TiO_2-NP absorption is minimal and there is no evidence of accumulation of Ti in internal tissues after environmentally relevant dietary and waterborne exposure.

No toxicological effects of injected TiO_2 were observed in any of the tissues examined in the present study. Despite the presence of both nano and bulk TiO_2 in the kidney and spleen, no treatment-dependent effects on any of the biochemical parameters measured were observed. Over the 96-h experiment there were fluctuations in TBARS (Fig. 2), GSH (Fig. 3), and activity of Na^+/K^+-ATPase (Fig. 4) but no effects related to TiO_2 treatments. Similarly, effects on fish haematology were minimal (no effect of TiO_2) and limited to a transient increase in plasma osmolality and Na^+ concentration at 24 h, consistent with intravenous injection of a physiological saline (Table 1). A previous study of rainbow trout intravenously injected with TiO_2-NPs also reported no toxic effects related to the presence of the NP in tissues (Scown et al., 2009).

There were no gross or microscopic (histopathology) lesions related to TiO_2 injection in any tissues examined (Fig. 5). Furthermore, where TiO_2 was injected intramuscularly and particles were clearly visible in tissue, histological examination of the site of injection revealed no lesions related to the presence of TiO_2 (Fig. 6). The gills also showed normal histology with a background incidence of lesions (all <4% incidence, data not shown) indicating that there was no waterborne exposure of the gills or systemic effects of the TiO_2 injections on the gills within 96 h. In the kidney, the proportional areas of the different types of renal tubules varied by less than 5% and there were no TiO_2 related effects. In the spleen, there were minor differences among treatments regarding proportion of red and white pulp (discussed below), but no lesions associated with TiO_2 were observed upon examination of histological sections or spleen prints (Fig. 6).

The role of the teleost kidney in trapping blood-borne immunogenic and non-immunogenic particulate matter, including particles of nano scale, is well established (see review by Dalmo et al., 1997). In fish injected with TiO_2-NPs (present study) the kidney was the principle site of tissue accumulation (Fig. 1) accounting for 94.0 ± 0.7% of total measured Ti in fish tissues after 24 h [calculated from relative total organ masses in trout of similar size reported in Gingerich et al. (1987)] and there was no significant change in

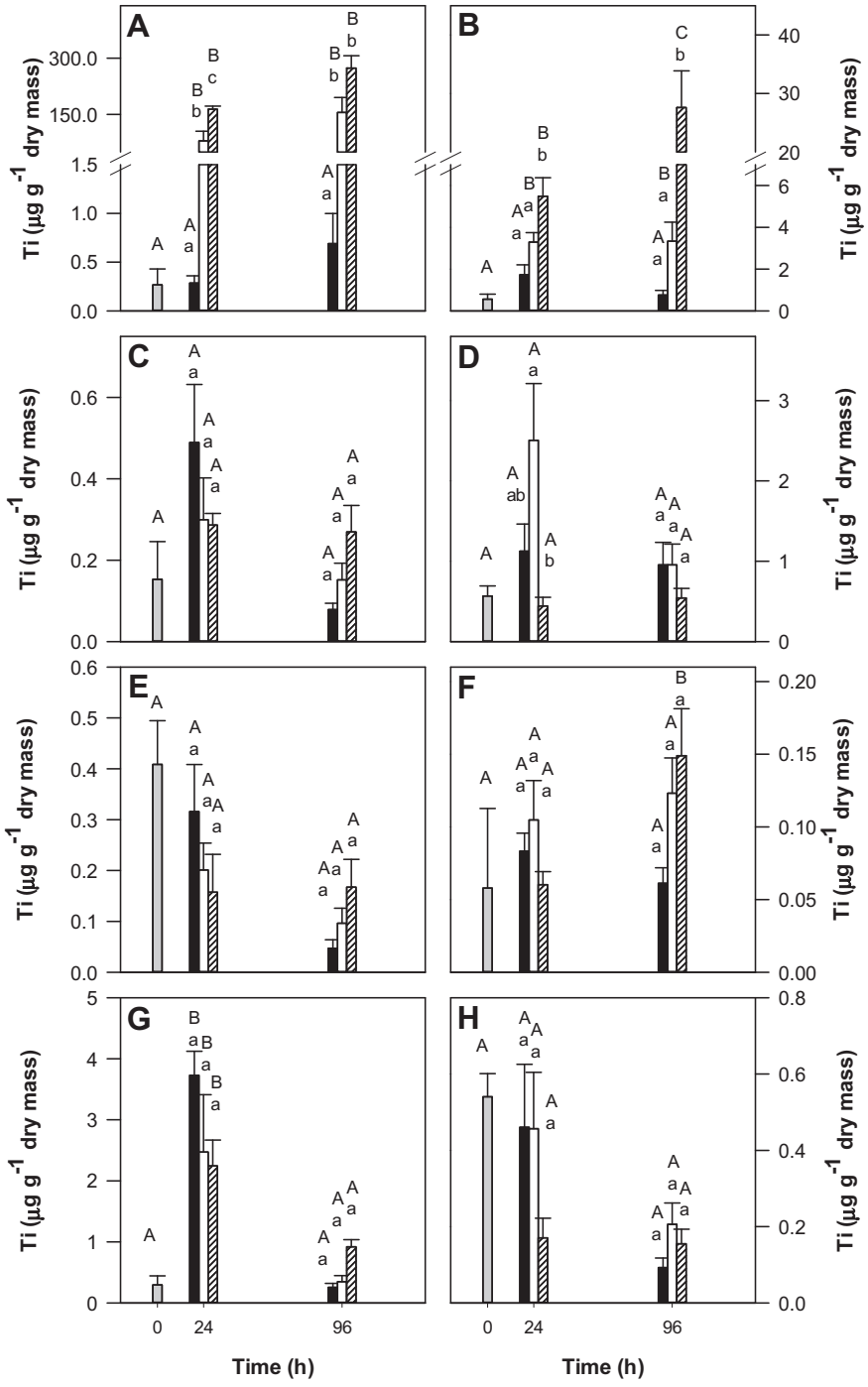

Fig. 1. Concentration of Ti (µg g^{-1} dry mass) in A) kidney, B) spleen, C) Liver, D) Heart, E) Brain, F) Muscle, G) Gill, H) Intestine, in juvenile rainbow trout injected with saline (controls, closed bars) 50 µg bulk TiO_2 in saline (open bars) and 50 µg TiO_2-NPs in saline (hatched bars). Time 0 fish (grey bars) were not injected. Data are means ± S.E.M. ($n = 6/7$). Different lower case letters denote significant difference within time-points. Different upper case letters denote significant difference between time-points ($p \leq 0.05$).

either the proportional distribution of Ti between tissues or measured concentration of Ti in kidney after 96 h. A similar pattern of renal accumulation was also apparent in fish injected with bulk TiO_2 (Fig. 1). The intra- or extracellular localisation of TiO_2 in the kidney in fish is unclear but these data indicate TiO_2 was retained in the kidney and did not enter renal excretion pathways within 96 h. This agrees with the observations of Scown et al. (2009) who report total removal of NPs from blood into hematopoietic cells surrounding the kidney tubules within 6 h and no evidence of excretion of NPs within 90 days in trout injected with TiO_2-NPs.

Titanium dioxide NPs, but not bulk TiO_2, also accumulated in spleen but to a lower tissue concentration compared to kidney (Fig. 1). In fish, the spleen serves as a secondary site of blood clearance selectively retaining particles and other macromolecules in splenic ellipsoids, which is a collective term for macrophages and reticular cells that surround terminal arterioles within the spleen (Espenes et al., 1995). There were some small (statistically significant) changes in the proportional area of the spleen tissue with a relative increase in the percentage of white pulp and decrease in red pulp in TiO_2-NPs injected fish compared to the

Table 1
Haematological parameters and plasma ion concentrations in rainbow trout caudally injected with Cortland's saline (50 μl), bulk TiO_2 (50 μg in 50 μl saline) or TiO_2-NPs (50 μg in 50 μl saline). Time 0 fish were not injected with saline.

	Time (hours)	Control	50 μg bulk TiO_2	50 μg TiO_2 NPs
Haematocrit (%)	0	38.2 ± 2.5		
	24	32.9 ± 2.0	35.7 ± 1.9	34.9 ± 1.0
	96	34.1 ± 1.6	32.7 ± 1.6	32.6 ± 1.7
Haemoglobin	0	7.54 ± 1.10		
(g dL^{-1})	24	6.22 ± 0.87	8.76 ± 1.04	7.09 ± 0.31
	96	7.36 ± 0.36	7.49 ± 0.48	7.32 ± 0.40
Red blood cells	0	0.84 ± 0.06		
(cell × 10^6 mm^3)	24	0.83 ± 0.09	0.77 ± 0.04	0.83 ± 0.02
	96	0.84 ± 0.12	0.75 ± 0.04	0.77 ± 0.04
Osmolality	0	279.7 ± 7.4^{A}		
(mOsm Kg^{-1})	24	304.1 ± 4.9^{a,B}	308.8 ± 4.4^{a,B}	316.2 ± 4.0^{a,B}
	96	293.8 ± 5.2^{a,AB}	299.3 ± 1.8^{a,B}	300.7 ± 4.8^{a,B}
Plasma Na^+	0	133.3 ± 5.1^{A}		
(mmol L^{-1})	24	142.0 ± 5.8^{a,A}	151.9 ± 2.2^{a,B}	156.0 ± 2.2^{a,B}
	96	143.8 ± 1.3^{a,A}	143.7 ± 2.1^{a,B}	137.0 ± 2.9^{a,A}
Plasma K^+	0	4.1 ± 0.4		
(mmol L^{-1})	24	3.8 ± 0.2	4.3 ± 0.1	4.1 ± 0.1
	96	3.7 ± 0.1	4.0 ± 0.4	3.5 ± 0.1

Data are means ± S.E.M. ($n = 5–7$).
Different lower case letters denote significant differences in rows ($p \leq 0.05$). Values with different upper case letters are significantly different within treatment groups, over time.

unexposed control and there was also a significant material type effect compared to the bulk material. The proportional area of the spleen that was white pulp was (means ± S.E.M, $n = 7$): 40.0 ± 0.8, 46.4 ± 0.7, 52.2 ± 0.8% in fish exposed to control, bulk material and NPs, respectively. Conversely, red pulp amounted to (means ± S.E.M. $n = 7$): 52.8 ± 1.8, 46.8 ± 0.9 and 41.4 ± 0.7% of total area in controls, bulk TiO_2 and TiO_2-NPs injected fish, respectively, The sinusoid space remaining around 5–8% in spleens of fish from all treatments. These changes are within the normal working range of trout spleen (not pathological) and appear driven predominantly by an increase in the white pulp content of the spleen [no increase in red blood cell counts or haemoglobin were observed in blood (Table 1)] rather than the greater recruitment of red blood cells into circulation seen previously in response to respiratory stress reported by Boyle et al. (2013) using waterborne TiO_2-NPs exposure. This hypothesis is supported by counts of cell types in spleen prints (Fig. 5). Counts of the haematopoetic cells in the spleen prints at 24 and 96 h, in most cases, showed only a few percentage differences in proportions of each type of cell (data not shown); indicating only minor effects. Nonetheless a transient rise in proportion of neutrophils in the spleen prints at 24 h with a material-type effect (means ± S.E.M., $n = 7$ fish/treatment); 5.0 ± 0.1 (control), 7.8 ± 0.4 (bulk), 11.3 ± 0.7% (nano) was observed which had lessened but was nonetheless significant at 96 h (means ± S.E.M, $n = 7$ fish/treatment); 2.2 ± 0.4 (control), 7.1 ± 0.3 (bulk), 7.0 ± 0.4% (nano). These data indicate the spleen was responsive, including to injection of saline in control fish, but also able to maintain homeostasis. Overall, these observations suggest the injections did not damage the spleen, but the introduction of particulate material into the circulation caused a foreign body response in fish characterised by a significantly elevated proliferation of white blood cells, especially neutrophils, in the spleens of fish exposed to both bulk TiO_2 and TiO_2-NPs.

Given the increase of the total Ti metal concentration in the spleen from TiO_2 injections, and some evidence of physiological responsiveness of the spleen, the question arises as to whether or not the TiO_2 could be trapped as particles by the splenic tissue. One of the criteria demonstrated to influence if splenic ellipsoids retain particulate matter is particle size. In rainbow trout, Espenes et al. (1995) estimate a maximum diameter of particles of 0.15–0.5 μm for entry into splenic ellipsoids. Similarly, in Japanese conger (*Conger myriaster*) injected with latex beads, beads of 0.5 μm diameter were trapped in splenic ellipsoids whilst larger microspheres of 2.0 μm diameter were partitioned to the kidney (Furukawa et al., 2002). Clearly the size classes estimated by Espenes et al. (1995) and demonstrated by Furukawa et al. (2002) encompass both aggregates of the nano form and primary particle sizes of the bulk TiO_2 used in this study. Both forms of the TiO_2 used here formed clearly visible aggregates in Cortland's saline here and uncoated TiO_2-NPs have been demonstrated to aggregate rapidly [estimate 5–15 min (French et al., 2009)] to form micron sized secondary particles in high ionic strength solutions such as physiological saline. It therefore would be probable that aggregates of TiO_2 particles would form in the syringe at the time of injection, and on contact with the high ionic strength of the blood plasma. It was not possible to measure the particle size distributions inside the syringe at the time of injection into trout in this study, however the formation of aggregates of TiO_2 in the syringe and in blood plasma post-injection may explain why bulk material with larger primary particle partitioned exclusively to kidney and only a very small proportion of injected TiO_2-NPs were observed to accumulate in spleen of fish. Additional studies investigating tissue distributions of stable dispersions of TiO_2-NPs and preferably several suspensions of discrete size are needed to verify this hypothesis.

Table 2
Comparative table of statistically significant (relative to time matched controls) Ti tissue concentrations and effects after waterborne, dietary, and intravenous exposure to TiO_2-NPs in rainbow trout.

Study	Exposure[a]	Ti (μg g^{-1} tissue)[b]	Haematology	TBARS	GSH	Na^+/K^+-ATPase
Federici et al. (2007)	1 mg L^{-1}	No effect	No effect	Brain ↑ Gill ↑	Gill ↑ Liver ↓	No effect
Boyle et al. (2013)	1 mg L^{-1}	Gill (31.3)	Hb↑ HCT↑	Brain ↑ Kidney ↑	Kidney ↑ Kidney ↑	No effect
Ramsden et al. (2009)	100 mg Kg^{-1}	Gill (0.119) Liver (0.179) Brain (0.292) Spleen (0.57)	No effect	Gill ↓ intestine ↓	Gill ↓	Brain ↓
Present (intravenous)	50 μg	Kidney (272.9) Spleen (26.2)	No effect	No effect	No effect	No effect

TBARS = Thiobarbituric Acid Reactive Substances.
GSH = Total glutathione.
Arrows (↑↓) denote significant increase (↑) or decrease (↓) in measured endpoint.
[a] Maximum exposure concentration used in each study.
[b] Mean Ti concentration in tissue when significant elevation was observed with time-matched control.

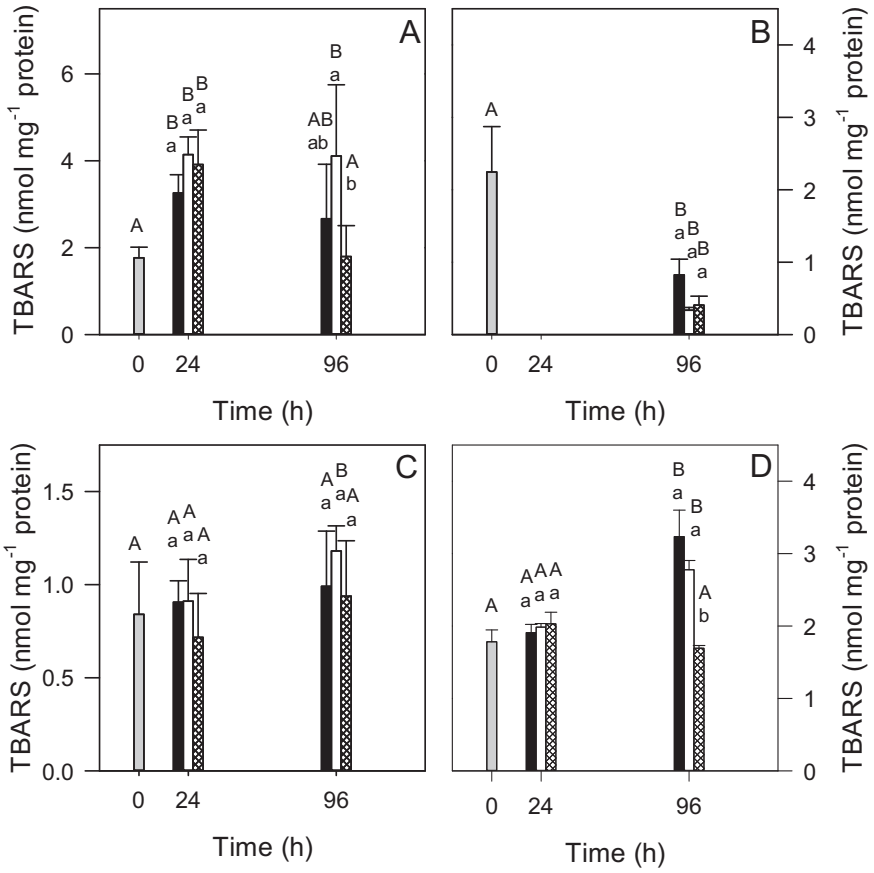

Fig. 2. Concentration of TBARS (nmol mg^{-1} protein) in A) kidney, B) spleen, C) Liver, D) Brain, in juvenile rainbow trout injected with saline (controls, closed bars) 50 μg bulk TiO_2 in saline (open bars) and 50 μg TiO_2-NPs in saline (hatched bars). Time 0 fish (grey bars) were not injected. Data are means ± S.E.M. ($n = 6/7$). Different lower case letters denote significant difference between treatments. Different upper case letters denote significant difference between time-points ($p \leq 0.05$).

Comparison of the present study with previous investigations of TiO_2 toxicity in rainbow trout indicated that minor, but statistically significant indications of toxicity, were not consistent among studies and did not relate to presence of TiO_2 in tissues. For clarity and comparison the main findings of the injection study reported here are summarised together with data from waterborne and dietary exposures to TiO_2-NPs previously conducted by our research group, and used the same stock of TiO_2-NPs, the same species, size, and age of fish (i.e. juvenile rainbow trout), and evaluated the same endpoints of toxicity by the same methodology (Table 2) (i.e. Federici et al., 2007; Ramsden et al., 2009; Boyle et al., 2013). A clear trend in both the present and our other studies is that observations of sub-lethal toxicity are not associated with tissue-specific increases of Ti metal concentrations in the organs where the sub-lethal effect occurred following exposure to TiO_2-NPs (Table 2). Exposure of trout to waterborne and dietary TiO_2-NPs induced haematological and ionoregulatory (Na^+/K^+-ATPase) perturbations and altered expression of GSH and TBARS, biomarkers of oxidative stress, in blood and internal tissues, including kidney, but in the organs where these effects were observed measurable increases of Ti metal concentrations were generally not observed or very transient (Federici et al., 2007; Ramsden et al., 2009; Boyle et al., 2013). Similarly, there is a disconnect with the concept of direct target organ toxicity in this injection study with Ti metal concentrations increasing in the kidney and spleen, but no effect on biochemical parameters or haematology in the body (Table 2). Overall, the implication of these data is that the concept of direct internal target organ toxicity of dissolved metals (e.g. Handy et al., 2005) may not apply to TiO_2 particles (bulk or NPs) because the TiO_2-NPs do not appear to be appreciably absorbed or accumulate in internal tissues after exposure. However, indirect systemic effects (suggested in Boyle et al., 2013) associated with surface acting toxicity of TiO_2 on the external epithelia (gut, gills) are the more likely cause of the reported toxicities.

Despite considerable focus on potential for TiO_2-NPs to generate reactive oxygen species (ROS) and cause cytotoxicity, there is no evidence that this has been a source of toxicity in fish in laboratory studies where NPs have not been photo activated (excluding indirect effects e.g. oxidative stress from respiratory impairment). In both *in vitro* studies with fish cell lines (Reeves et al., 2008; Vevers and Jha, 2008) and in vivo studies with zebrafish larvae (Bar-Ilan et al., 2012, 2013), toxicity has been demonstrated to be photo-dependent with decreased or no effect at concentrations exceeding 50 mg L^{-1} in the absence of light irradiation. Whilst photo-activation of TiO_2-NPs could occur in shallow waters under natural light and this may cause toxicity in sensitive early-life stage fishes (Bar-Ilan et al., 2013) it is unlikely to be relevant to juvenile fishes since light does not penetrate through fish skin into internal organs. In the present study, Ti concentration increased in kidney and spleen in fish injected with TiO_2-NPs, but no indications of oxidative stress responses were observed in these tissues (Figs. 2 and 3). In addition, no toxic effects were reported in kidney from another study of rainbow trout injected with TiO_2-NPs (Scown et al., 2009), and together these studies indicate that internalised TiO_2-NPs on their own do not

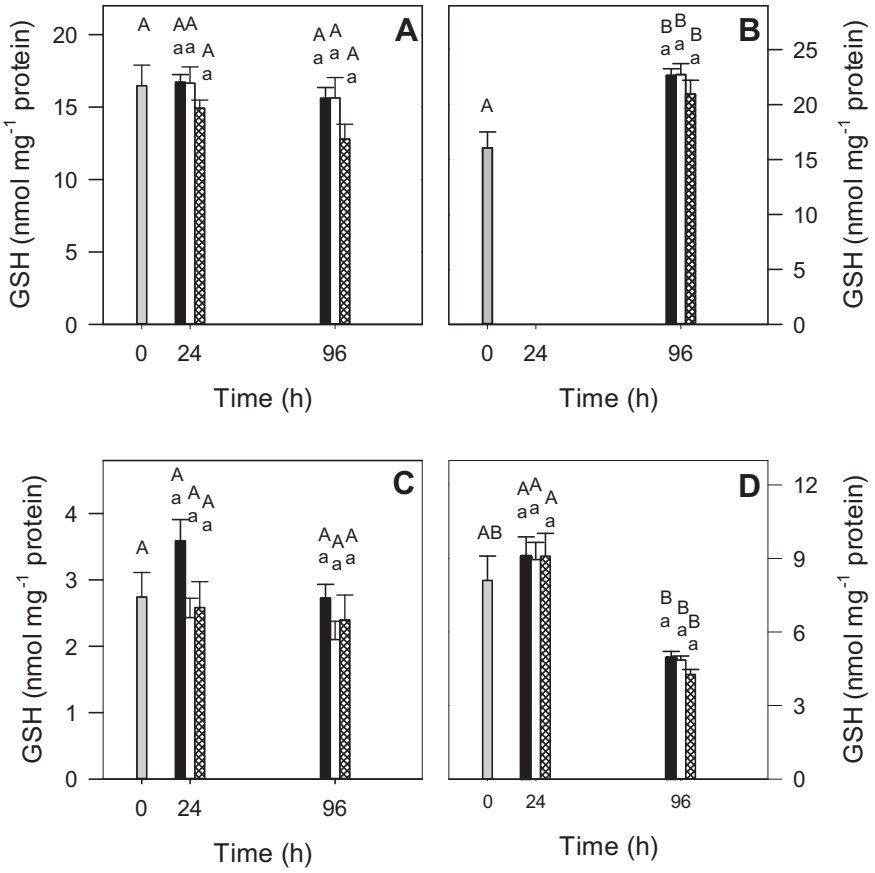

Fig. 3. Concentration of GSH (nmol mg^{-1} protein) in A) kidney and B) spleen in juvenile rainbow trout injected with saline (controls, closed bars) 50 μg bulk TiO_2 in saline (open bars) and 50 μg TiO_2-NPs in saline (hatched bars). Time 0 fish (grey bars) were not injected. Data are means ± S.E.M. ($n = 6/7$). Different lower case letters denote significant difference between treatments. Different upper case letters denote significant difference between time-points ($p \leq 0.05$).

present a significant oxidative stress risk at the concentrations administered.

Indications of oxidative stress have been reported in the brain of rainbow trout exposed to TiO_2-NPs. Elevated brain TBARS, a marker of lipid peroxidation, were observed in trout exposed to TiO_2-NPs in water (Federici et al., 2007; Boyle et al., 2013) but not diet (Ramsden et al., 2009) or intravenous injection of present study (data summarised in Fig. 7A). Boyle et al. (2013) also reported elevated brain GSH (Fig. 7B); however, no increase of Ti metal was detected in the brain and the GSH response was attributed to systemic hypoxia caused by particle occlusion of the gill, gill injury, and respiratory impairment [see Boyle et al. (2013) for extended discussion]. Oxidative stress in response to tissue hypoxia is well documented in animals including fishes (see review by Bickler and Buck, 2007) and may be a general response to waterborne NP exposure e.g. Ag-NPs (Bilberg et al., 2010). Further, hypoxia driven oxidative stress may cause wider impacts on fish physiology e.g. decreased swimming activity (Boyle et al., 2013).

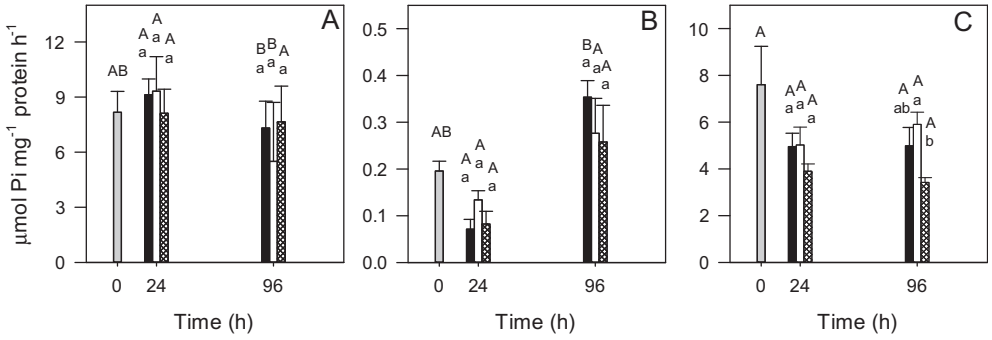

Fig. 4. Activity of Na^+/K^+-ATPase (μmol P_i mg^{-1} protein h^{-1}) in A) kidney, B) Liver and C) Brain in juvenile rainbow trout injected with saline (controls, closed bars) 50 μg bulk TiO_2 in saline (open bars) and 50 μg TiO_2-NPs in saline (hatched bars). Time 0 fish (grey bars) were not injected. Data are means ± S.E.M. ($n = 6/7$). Different lower case letters denote significant difference between treatments. Different upper case letters denote significant difference between time-points ($p \leq 0.05$).

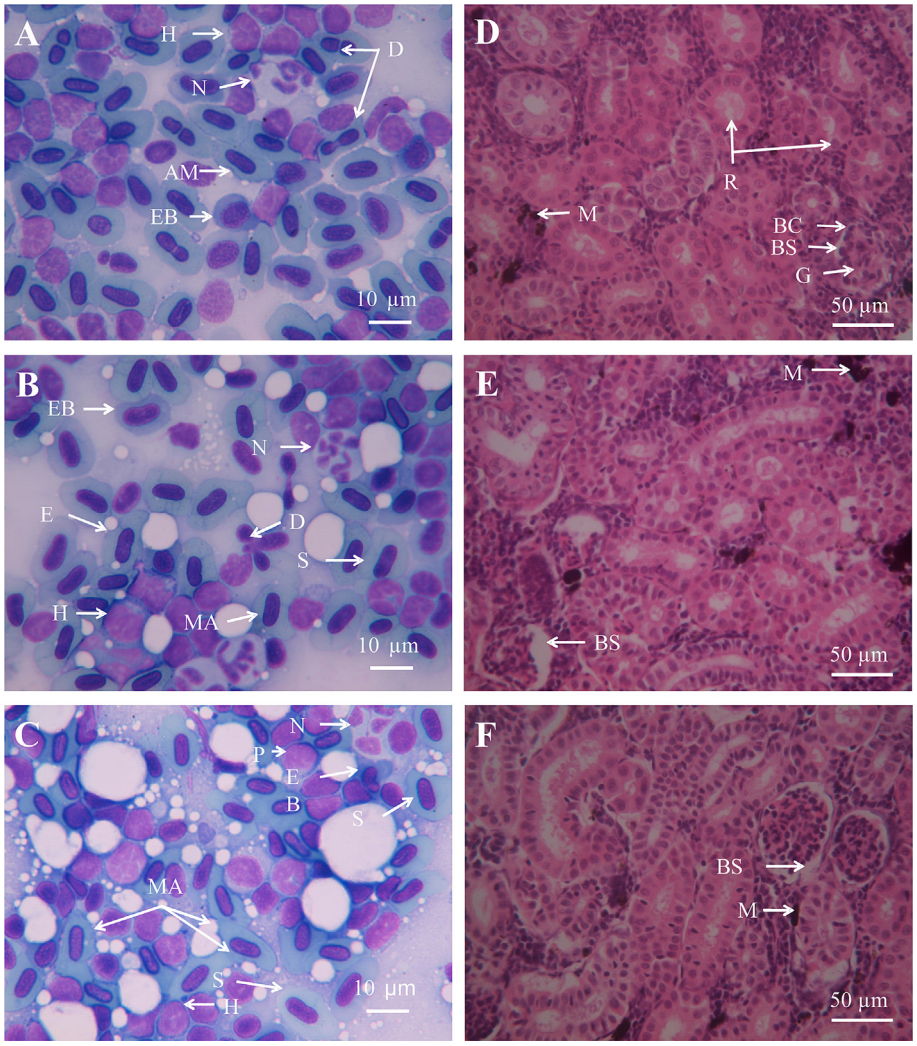

Fig. 5. Haematopoietic tissues in juvenile rainbow trout 96 h after intravascular injection of saline (controls, images A and D) 50 μg bulk (images B and E) and 50 μg nano TiO_2 (images C and F). Spleen prints (images A–C) were fixed with methanol (15 min) and stained with May- Grunwald-Giemsa. Erythrocytes (E), erythroblast (EB), neutrophil (N), haemocytoblast (H), progranulocyte (P), a dividing nucleus (D), membrane abnormalities of erythrocytes (MA), swollen erythrocyte (S), abnormal nucleus (AN). Scale bar indicates magnification (×1000). Kidney sections (images D–F) were 7 μm thick and stained with haematoxylin and eosin. Renal tubule (R), glomerulus (G), Bowman's capsule (BC), Bowman's space (BS) and melanomacrophage deposits (M). Scale bar indicates magnification (×400).

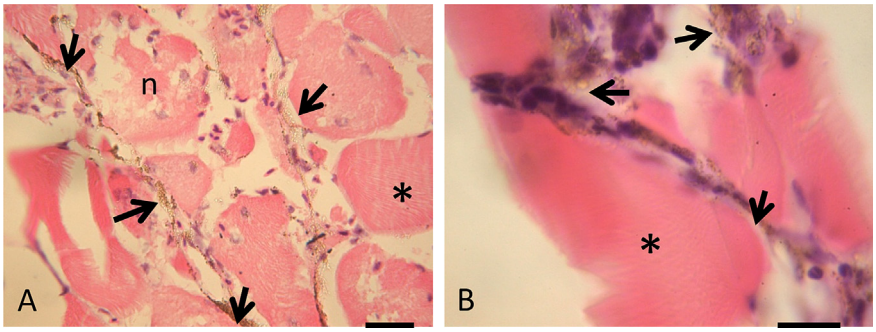

Fig. 6. Histological sections of skeletal muscle of juvenile rainbow trout collected 96 h after intramuscular injection near the anterior insertion of the dorsal fin. A) TiO_2-bulk injections with TiO_2 particulates indicated by arrows. Normal bundles of skeletal muscle fibres (*) compared to bundles that were injured during the injection with evidence of necrotic (n) fibres (400×, bar = 50 μm). B) TiO_2-NP injection with TiO_2-NP particulates (arrows) distributed along the margins of adjacent blood capillaries in the tissue section. No evidence of injury to muscle fibre bundles or inflammation around TiO_2-NP particulates (1000×, bar = 50 μm).

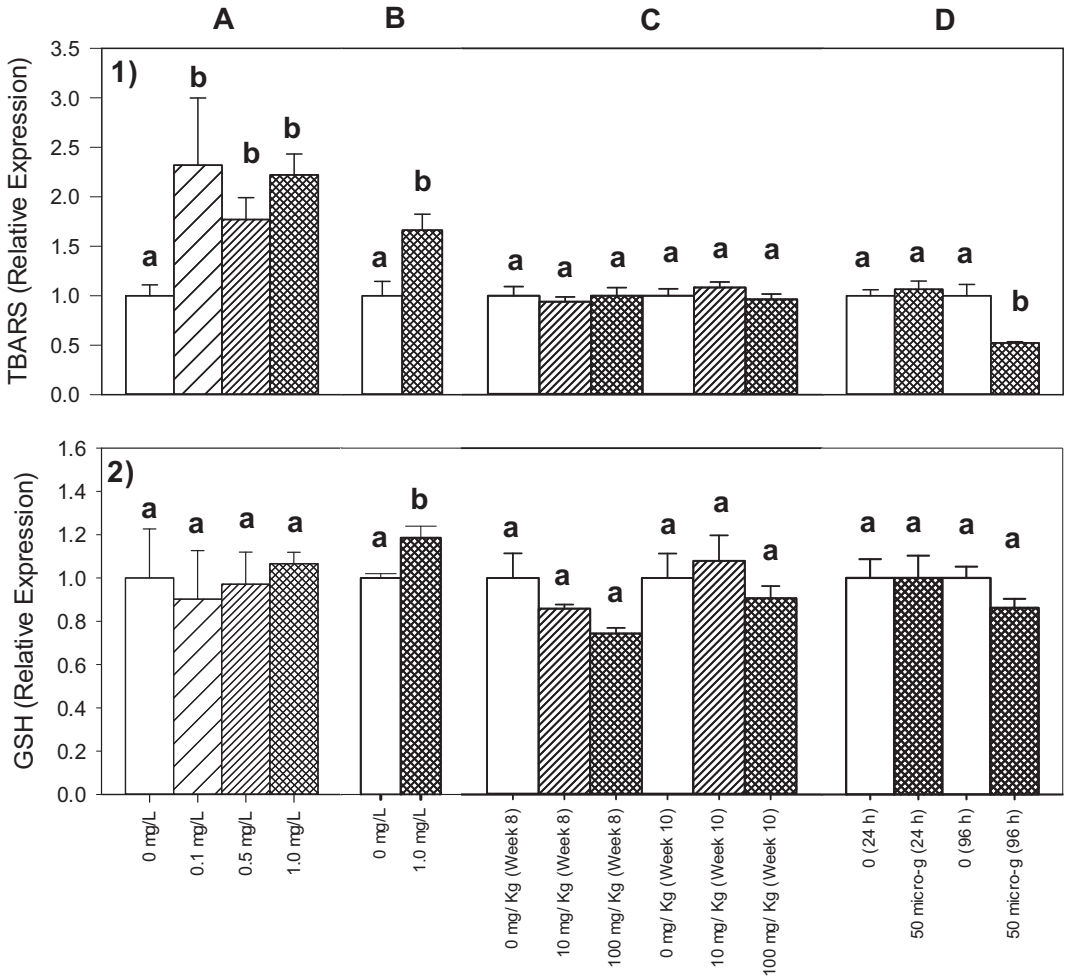

Fig. 7. Comparison of 1) Thiobarbituric acid reactive substances (TBARS) and 2) Total glutathione (GSH) in crude homogenates from the brain of juvenile rainbow trout exposed to TiO_2-NPs in different experiments: A) Federici et al., 2007; B) Boyle et al., 2013; C) Ramsden et al. (2009); D) present study. Results are presented as percent of time matched controls for fish in each experiment, and actual values for each experiment are presented within the respective publications. Data are means ± S.E.M. ($n = 4–8$). Lower case letters denote significant differences between treatment groups where reported in original publication.

Reports of effects of waterborne TiO_2-NPs on fish brain (e.g. Federici et al., 2007), behaviour (e.g. Boyle et al., 2013) and reproduction (e.g. Wang et al., 2011) should frame discussions of toxicity to consider effects as a continuum of a non-specific response to TiO_2-NPs exposures as well as specific mechanisms of toxicity.

In conclusion, injection of nano- and bulk TiO_2 into caudal vasculature of trout resulted in identification of kidney as the principle site of accumulation. Ti metal from the TiO_2-NP but not bulk-TiO_2 injection also accumulated in spleen, indicative of particle size being a determinant of tissue distribution. Despite measurable tissue accumulation there was no indication of toxicity in either organ. Overall this comparative analysis suggests that environmentally relevant dietary and waterborne exposures of juvenile rainbow trout to TiO_2-NPs do not lead to significant accumulation of Ti in internal tissues, and that previously reported relatively minor indications of toxicity (e.g. elevation of TBARS and GSH) are most likely attributed to physical disruption and occlusion of gills during waterborne exposure.

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References

Bar-Ilan, O., Louis, K.M., Yang, S.P., Pedersen, J.A., Hamers, R.J., Peterson, R.E., Heideman, W., 2012. Titanium dioxide nanoparticles produce phototoxicity in the developing zebrafish. Nanotoxicology 6, 670–679.

Bar-Ilan, O., Chuang, C.C., Schwahn, D.J., Yang, S.P., Joshi, S., Pedersen, J.A., Hamers, R.J., Peterson, R.E., Heideman, W., 2013. TiO_2 nanoparticle exposure and illumination during zebrafish development: mortality at parts per billion concentrations. Environmental Science and Technology 47, 4726–4733.

Bickler, P.E., Buck, L.T., 2007. Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. Annual Review in Physiology 69, 145–170.

Bilberg, K.A., Malte, H., Wang, T., Baatrup, E., 2010. Silver nanoparticles and silver nitrate cause respiratory stress in Eurasian perch (*Perca fluviatilis*). Aquatic Toxicology 96, 159–165.

Boyle, D., Al-Bairuty, G.A., Ramsden, C.S., Sloman, K.A., Henry, T.B., Handy, R.D., 2013. Subtle alterations in swimming speed distributions of rainbow trout exposed to titanium dioxide nanoparticles are associated with gill rather than brain injury. Aquatic Toxicology 126, 116–127.

Chen, T.H., Lin, C.Y., Tseng, M.C., 2011. Behavioral effects of titanium dioxide nanoparticles on larval zebrafish (*Danio rerio*). Marine Pollution Bulletin 63, 303–308.

Dalmo, R.A., Ingebrigtsen, K., Bøgwald, J., 1997. Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). Journal of Fish Diseases 20, 241–273.

Espenes, A., Press, C.M., Dannevig, B.H., Landsverk, T., 1995. Immune-complex trapping in the splenic ellipsoids of rainbow trout (*Oncorhynchus mykiss*). Cell Tissue Research 282, 41–48.

Federici, G., Shaw, B.J., Handy, R.D., 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout, (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects. Aquatic Toxicology 84, 415–430.

French, R.A., Jacobson, A.R., Kim, B., Isley, S.L., Penn, R.L., Baveye, P.C., 2009. Influence of ionic strength, pH, and cation valence on aggregation kinetics of titanium dioxide nanoparticles. Environmental Science and Technology 43, 1354–1359.

Furukawa, T., Nakamura, O., Suzuki, Y., Atsuta, S., Nakamura, H., Watanabe, T., 2002. Entrapment and transport of foreign material in the spleen and kidney of Japanese conger *Conger myriaster*. Fisheries Science 68, 1219–1225.

Gingerich, W.H., Pityer, R.A., Rach, J.J., 1987. Estimates of plasma, packed cell and total blood volume in tissues of the rainbow trout (*Salmo gairdneri*). Comparative Biochemistry and Physiology A 87, 251–256.

Gottschalk, F., Sonderer, T., Scholz, R.W., Nowack, B., 2009. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, Fullerenes) for different regions. Environmental Science and Technology 43, 9216–9222.

Handy, R.D., von der Kammer, F., Lead, J.R., Hassellöv, M., Owen, R., Crane, M., 2008. The ecotoxicology and chemistry of manufactured nanoparticles. Ecotoxicology 17, 287–314.

Handy, R.D., Al-Bairuty, G.A., Al-Jubory, A., Ramsden, C.S., Boyle, D., Shaw, B.J., Henry, T.B., 2011. Effects of manufactured nanomaterials on fishes: a target organ and body systems physiology approach. Journal of Fish Biology 79, 821–853.

Handy, R.D., McGeer, J.C., Allen, H.E., Drevnick, P.E., Gorsuch, J.W., Green, A.S., Lundebye-Haldorsen, A.-K., Hook, S.E., Mount, D.R., Stubblefield, W.A., 2005. Toxic effects of dietborne metals: laboratory studies. In: Meyer, J.S., Adams, W.J., Brix, K.V., Luoma, S.N., Mount, D.R., Stubblefield, W.A., Wood, C.M. (Eds.), Toxicity of Dietborne Metals to Aquatic Organisms. SETAC Press, Pensacola, pp. 59–112.

Hao, L., Wang, Z., Xing, B., 2009. Effect of sub-acute exposure to TiO_2 nanoparticles on oxidative stress and histopathological changes in Juvenile Carp (*Cyprinus carpio*). Journal of Environmental Sciences 21, 1459–1466.

Henry, T.B., Petersen, E.J., Compton, R.N., 2011. Aqueous fullerene aggregates (nC60) generate minimal reactive oxygen species and are of low toxicity in fish: a revision of previous reports. Current Opinion in Biotechnology 22, 533–537.

Johnson, A.C., Bowes, M.J., Crossley, A., Jarvie, H.P., Jurkschat, K., Jürgens, M.D., Lawlor, A.J., Park, B., Rowland, P., Spurgeon, D., Svendsen, C., Thompson, I.P., Barnes, R.J., Williams, R.J., Xu, N., 2011. An assessment of the fate, behaviour and environmental risk associated with sunscreen TiO_2 nanoparticles in UK field scenarios. Science of the Total Environment 409, 2503–2510.

Johnston, B.D., Scown, T.M., Moger, J., Cumberland, S.A., Baalousha, M., Linge, K., van Aerle, R., Jarvis, K., Lead, J.R., Tyler, C.R., 2010. Bioavailability of nanoscale metal oxides TiO_2, CeO_2, and ZnO to fish. Environmental Science and Technology 44, 1144–1151.

Kahru, A., Dubourguier, H.-C., 2010. From ecotoxicology to nanoecotoxicology. Toxicology 269, 105–119.

Mueller, N.C., Nowack, B., 2008. Exposure modeling of engineered nanoparticles in the environment. Environmental Science and Technology 42, 4447–4453.

Oberdörster, G., Oberdörster, E., Oberdörster, J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environmental Health Perspectives 113, 823–839.

Peters, G., Schwarzer, R., 1985. Changes in hemopoietic tissue of rainbow trout under influence of stress. Diseases of Aquatic Organisms 1, 1–10.

Popov, A.P., Lademann, J., Priezzhev, A.V., Myllylä, R., 2005. Effect of size of TiO_2 nanoparticles embedded into stratum corneum on ultraviolet-A and ultraviolet-B sun-blocking properties of the skin. Journal of Biomedical Optics 10, 064037.

Ramsden, C.S., Smith, T.J., Shaw, B.J., Handy, R.D., 2009. Dietary exposure to titanium dioxide nanoparticles in rainbow trout, (*Oncorhynchus mykiss*): no effect on growth, but subtle biochemical disturbances in the brain. Ecotoxicology 18, 939–951.

Reeves, J.F., Davies, S.J., Dodd, N.J., Jha, A.N., 2008. Hydroxyl radicals (*OH) are associated with titanium dioxide TiO_2 nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. Mutation Research 640, 113–122.

Scown, T.M., van Aerle, R., Johnston, B.D., Cumberland, S., Lead, J.R., Owen, R., Tyler, C.R., 2009. High doses of intravenously administered titanium dioxide nanoparticles accumulate in the kidneys of rainbow trout but with no observable impairment of renal function. Toxicological Sciences 109, 372–380.

Shaw, B.J., Ramsden, C.S., Turner, A., Handy, R.D., 2013. A simplified method for determining titanium from TiO_2 nanoparticles in fish tissue with a concomitant multi-element analysis. Chemosphere. http://dx.doi.org/10.1016/j.chemosphere.2013.01.065.

Shaw, B.J., Handy, R.D., 2011. Physiological effects of nanoparticles on fish: a comparison of nanometals versus metal ions. Environment International 37, 1083–1097.

Umbreit, T.H., Francke-Carroll, S., Weaver, J.L., Miller, T.J., Goering, P.L., Sadrieh, N., Stratmeyer, M.E., 2012. Tissue distribution and histopathological effects of titanium dioxide nanoparticles after intravenous or subcutaneous injection in mice. Journal of Applied Toxicology 32, 350–357.

Vevers, W.F., Jha, A.N., 2008. Genotoxic and cytotoxic potential of titanium dioxide TiO_2 nanoparticles on fish cells in vitro. Ecotoxicology 17, 410–420.

Wang, J., Zhu, X., Zhang, X., Zhao, Z., Liu, H., George, R., Wilson-Rawls, J., Chang, Y., Chen, Y., 2011. Disruption of zebrafish (*Danio rerio*) reproduction upon chronic exposure to TiO_2 nanoparticles. Chemosphere 83, 461–467.

Weir, A., Westerhoff, P., Fabricius, L., Hristovski, K., von Goetz, N., 2012. Titanium dioxide nanoparticles in food and personal care products. Environmental Science and Technology 46, 2242–2250.

Xiong, D., Fang, T., Yu, L., Sima, X., Zhu, W., 2011. Effects of nano-scale TiO_2, ZnO and their bulk counterparts on zebrafish: acute toxicity, oxidative stress and oxidative damage. Science of the Total Environment 409, 1444–1452.

Zhu, X., Wang, J., Zhang, X., Chang, Y., Chen, Y., 2010. Trophic transfer of TiO_2 nanoparticles from Daphnia to zebrafish in a simplified freshwater food chain. Chemosphere 79, 928–933.