Haematopoietic observation on spleen prints from rainbow trout (Oncorhynchus mykiss) following dietary or waterborne exposure to TiO_2 nanoparticles

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Abstract

Little is known about the immunotoxicity of dietary and waterborne exposure to nanoparticles in aquatic organisms. In this study, cellular pathologies and haematopoietic characteristics were examined in spleen prints of juvenile rainbow trout that were exposed to TiO_2 NPs. Dietary exposures were TiO_2 (10-100 mg kg^{-1} for up to 8 weeks and followed by 2 weeks recoveries), and waterborne exposures were TiO_2 (0.1-1 mg l^{-1} for up to 14 days). At least 200 cells were counted on each slide of spleen from each fish per treatment at each time point (6 fish/treatment). Dietary exposure to TiO_2 showed minor effects on the proportion of haematopoietic cells and the erythrocyte morphology in the spleen. This included small transient changes in the proportion of erythroblast and lymphocytes when compared to controls. An increase in the proportion of erythrocytes with abnormalities (swollen red blood cells and cell with membrane abnormalities) was observed in the spleen following dietary exposure even though the incident rate was only 1-2 % of the red cells (all statistically significant, ANOVA, $P < 0.05$). Waterborne exposure to TiO_2 showed a decrease in the proportion of erythroblasts and an increase in the proportion of some types of immune cells compared to controls (ANOVA, $P < 0.05$). At day 14 of waterborne exposure to TiO_2 NPs showed a few more statistically significant changes than day 4 (ANOVA, $P < 0.05$); including an increase in the proportion of erythrocyte abnormalities (swollen red blood cells, red cells with micronuclei, and cell membrane abnormalities). The incident rate was only 1-4 % of the red cells observed compared to the control (0-1%). It was concluded that dietary exposure of TiO_2 caused minor alteration in the erythropoiesis and immune system while exposure to waterborne TiO_2 caused slightly greater alterations in the erythropoiesis and immune system. The adverse effects via the dietary route are less severe than waterborne exposure on the immune system. The levels of changes that observed are unlikely to be clinically important to fish health.

1 Introduction

Trace metals are known to be toxic to fish via the water [1, 2] and food [3, 4, 5]. There are also some concerns about the immunotoxicity from chemicals in fishes [6]. For example, immunotoxic of chemicals shown to cause adverse health effects by suppressing or stimulating the immune capacity of exposed organisms. Recently, a new area of sciences emerged called nanomaterials which defined as materials with a primary particle size between 1-100 NM [7]. Data on the uptake mechanisms, ecotoxicity, and target organs for nanomaterials are limited [8, 9, 10, and one of the gaps in the data is on the effects of nanomaterials on the immune system of fishes [10, 11]. Nanoparticles are known to cause inflammation reactions and immune responses in mammals [12, 13, 14, 15]. *In vitro* toxicity experiments with human fibroblast cells showed inflammation, oxidative stress, cytokine production, cellular apoptosis (necrosis), changes in gene expression in response to the CNT exposure {MWCNT, [12]; SWCNT, [13]; MWCNT, [15]} . Recently, there have also been concerns that NPs could interfere with the immune system of fishes [11]. The immune system of fishes shares many common features with mammals [*e.g.*, immunoglobulins, albumin and fibrinogen, 16; 14] , although in fish the immune cells are not all organised into discrete organs. One organ of great importance is the spleen, which is considered one of the main haematopoietic organs in fishes [17, 18]. The information on the haematopoietic system such as spleen in fish is scarce. However, the spleen functions as a filter to remove damaged blood cell and any foreign material from the circulation, and to maintain normal haematology of the cells in the cardiovascular system. Environmental pollutants can change the proportion of red and white pulp in the spleen [*e.g.*, in sticklebacks exposed to sewage treatment effluents, 19; in rainbow trout exposed to bulk and TiO_2 NPs, 20]. Handy *et al.* [21] mentioned that an increase in red pulp (mostly sinusoids containing red blood cell) may indicate the spleen was working hard to remove the damaged red blood cells, whilst an increase in white pulp (mostly immune cells) may indicate an immunological stress. The haematopoietic efficiency of the spleen has been quantified in various ways including the spleen prints method [22]. These researchers observed

changes in the haematopoietic tissue (spleen and head kidney) of rainbow trout under influence of stress. Recently, a study by Boyle *et al.* [20] showed that waterborne exposure to 1 mg l^{-1} of bulk or TiO_2 NPs materials for 14 days caused a decrease in the proportion of red pulp that a concomitant with an increase in the proportion of sinusoid space in the spleen of rainbow trout. Boyle *et al.* [20] also found an increase in the number of melanomacrophage deposits in the spleen with both bulk and TiO_2 NP treatments, with more elevation with NP treatment.

The objective of the present study was to evaluate the effects of dietary or waterborne exposure to TiO_2 NPs on the spleen contents (erythrocyte morphology and haematopoietic cells) of rainbow trout by using spleen prints. Some of the exposure data and general toxic effects on these same fish for dietary and waterborne TiO_2 NPs are published elsewhere [23, 24].

2 Materials and Methods

2.1 Experimental design

2.2 Characterisation of TiO_2 nanoparticles

The characterisation of the TiO_2 NPs used here has been reported and both exposures used material from the same batch of powder. The TiO_2 was characterised for the water exposure in Federici *et al.* [23] and in Ramsden *et al.* [24] for the dietary studies. Briefly, for all TiO_2 NPs experiments, dry powder of TiO_2 NPs ("Aeroxide" P25 TiO_2, DeGussa AG, supplied via Lawrence Industries, Tamworth, UK) was used. This was made of (revised manufacture's information); crystal structure of 25 % rutile and 75 % anatase TiO_2, purity was at least 99 % TiO_2 (maximum impurity stated was 1 % Si), and with an average particle size of 21 nm with a specific surface area of 50 ± 15 m^2 g^{-1}. A 10 g l^{-1} stock solution of TiO_2 NPs was made (no solvents) by dispersing the NPs in ultrapure (Millipore) water with sonication (bath type sonicator, 35 kHz frequency, fisherbrand FB 11010, Germany) for 6 h. Chemical analysis of stock solutions revealed no metal impurities and the batch was high (data not shown), with a measured mean primary particle size of 24.1 ± 2.8 nm (mean ± SEM, n = 100 electron microscope images), see [23]. For the dietary experiment, the same 10 g l^{-1} stock solution of TiO_2 NPs was sonicated for 8 h, and then either 1 or 10 ml of the stock was added to 49 and 40 ml of ultrapure water to make a 0.2 or 2.0 g l^{-1} TiO_2 NP dilution that could be sprayed onto the food for 10 and 100 mg kg^{-1} treatments respectively. After that, the diluted TiO_2 solutions were sonicated for further 15 min just before spraying to ensure even delivery of the material through the spray nozzle. One kilogram of commercial feed was placed in a commercial food mixer (Kenwood Catering Professional food mixer XKM810) and gradually sprayed with the appropriate TiO_2 NP solution. The TiO_2 NPs immediately coated the feed, and was then sealed in by spraying the food with a 10 % bovine gelatine (BDH, Poole, UK) solution. The gelatine coat was allowed to dry, after that feed was transferred into airtight containers for storage. The control diet was made exactly the same way, except that the TiO_2 solution was replaced by an equal volume of ultrapure water (see [24] for details).

The spleens examined here for TiO_2 NPs effects were obtained from fish exposed to either dietary or waterborne TiO_2. Exposure of juvenile rainbow trout to dietary TiO_2 NPs is reported in Ramsden *et al.* [24]. Briefly, fish was exposed in triplicate (3 tanks/treatments) a control diet of no added TiO_2 and TiO_2 NP contaminated diets (10 and 100 mg kg^{-1} dry weight feed TiO_2 NPs) for 8 weeks and followed by 2 weeks recovery on normal food. The feeds for fish were prepared by spraying dispersed stock solutions of TiO_2 NPs onto the food pellets. The exposure concentration and periods when spleens were collected are summarised in Table 1. Waterborne exposure to TiO_2 NPs is reported in Federici *et al.* [23]. Briefly, a triplicated semi-static exposure method was used with replacement of the water every 12 hours to ensure the exposure concentration of material was maintained. Sonicated stock solutions of TiO_2 NPs were used for re-dosing each tank. Fish were exposed to various concentrations of TiO_2 NPs up to 1 mg l^{-1} for up to 2 weeks (see Table 1).

2.3 Preparation of spleen prints

Spleen samples were collected from juvenile rainbow trout at the time points in Table A.1 of the exposure to dietary and waterborne of TiO_2 NPs. Spleen prints were collected from the trout, then stained with May-Grünwald Giemsa stain and scored haematopoietic cells and cellular pathologies by using the criteria of Peters &Schwarzer [22].

2.4 Statistical analysis

All data were presented as % mean ± S.E.M and analysed by StatGraphics Plus version 5.1. Multifactor ANOVA followed by Fisher's 95% least squares difference post hoc test was used to test treatment, time, and treatment x time interaction effects. When a statistical significant effect was indicated by this model, a one way ANOVA (or Kruskal-Wallis test for non-parametric data) was used to assess for simple effects. All statistical analysis used the default 5 % rejection level.

3 Results

3.1 Dietary exposure to titanium dioxide nanoparticles

Dietary exposure to TiO_2 NP showed small changes in haematopoietic cells in the spleen after 8 weeks of exposure (Fig. 1). After eight weeks of exposure to 10 and 100 mg kg^{-1} TiO_2 NP, the spleen image showed the presence of different types of immature (haemocytoblast, erythroblast) and mature red blood cells (erythrocytes) compared to controls and recovery phase

(Fig. 1). An immune cells such as neutrophils and macrophages were also observed with both TiO_2 concentrations compared to the control and the recovery phase image (Fig. 1). Quantitative spleen print analysis showed that exposure to both TiO_2 concentrations caused a small transient change in the proportion of erythroblast, but still a statistically significant change (ANOVA, P <0.05), although the proportion of erythrocytes remained the same after 8 weeks of exposure when compared to controls and to the recovery phase (Table 2). The proportion of lymphocytes also showed a small but statistically significant increase (ANOVA, P <0.05) after 8 weeks of exposure to 100 mg kg^{-1} of the TiO_2 NP treatment compared to control groups and the recovery phase (Table.2). After 8 weeks of exposure, the concentration effect exhibited a statistically significant decrease (ANOVA, P <0.05) in the proportion of erythroblast and an increase in the proportion of lymphocytes as well as neutrophils were observed with the highest TiO_2 concentration compared to the lowest concentration of TiO_2 NPs (Table 2).

Erythrocyte morphology in the spleen prints also showed small changes after 8 weeks of exposure to TiO_2 NPs (erythrocytes recognised as having lesions; Fig. 1). These changes included swollen erythrocytes, red blood cells with dividing nuclei, and cells with membrane abnormality. After an additional two weeks of recovery on normal food, less changes were observed in the erythrocyte morphology in the spleen prints with both TiO_2 concentrations (Fig. 1). A quantitative analysis of erythrocyte abnormalities confirmed that dietary exposure to 10 and 100 mg kg^{-1} of the TiO_2 for 8 weeks caused a small but statistically significant increase in the proportion of swollen erythrocytes and cells with membrane abnormality compared to controls (ANOVA, P <0.05; Table 3). For most of the abnormalities, the incident rate was only 1-2 % of the red blood cells observed, regardless of the concentration. High concentration of TiO_2 treatment showed more increased in the proportion of swollen red cells and cells with membrane abnormality compared to the 10 mg kg^{-1} TiO_2 treatment (ANOVA, P <0.05; Table 3). No changes in the proportion of erythrocyte abnormalities were observed after 2 weeks of recovery (Table 3). This level of changes is unlikely to be clinically important to fish health.

3.2 Waterborne exposure to titanium dioxide nanoparticles

Differentiation of the haematopoietic cells in spleen prints after 7 and 14 days of exposure to 0.1 and 1 mg l^{-1} of TiO_2 NP showed the presence of immature cells (erythroblast and haemocytoblast) and mature cells (erythrocytes) compared to control images (Fig. 2). Waterborne exposure to both TiO_2 concentrations also showed the presence of different types of immune cells such as neutrophils, lymphocytes, monocytes, and macrophages compared to the control image (Fig. 2). A quantitative analysis of spleen prints showed a statistically significant transient change (ANOVA, P <0.05) in the proportion of haemocytoblast and erythroblast, although the proportion of erythrocytes remained the same at the end of exposure with both TiO_2 concentrations compared to controls (Fig. 2; Table 4). For the immune immature cells, the proportion of progranulocytes also showed a small increase after 7 days of exposure to 0.1 mg l^{-1} of TiO_2 NP compared to the control (ANOVA, P <0.05). The transient statistically significant changes depending on the TiO_2 concentration were shown in the proportion of lymphocytes at day 14 when compared to controls (ANOVA, P <0.05; Table 4). The proportion of neutrophils and monocytes showed a small increase after 7 days of exposure to TiO_2 compared to controls (all statistically significant, ANOVA, P <0.05; Table 4). The incident rate of immature and immune cell changes was only 1- 4 % of the haematopoietic cells on the spleen. Some measurements were approaching the limits of experimental error. For example, quantitative scoring of the spleen prints at day 14 found no thrombocytes (not observed, mean score of zero, Table 5). Earlier on in the study, however, thrombocytes were counted in the control (*e.g.*, % mean ± S.E.M = 0.9 ± 0.3 on day 7, n = 6). However, the proportion of haemocytoblast and lymphocytes also showed concentration (greater effects with high concentration) and time effects (greater effects at day 14 than 7) (Table 4).

Increasing the presence of erythrocytes abnormality was observed in the spleen prints of trout following waterborne exposure to 0.1 and 1 mg l^{-1} of the TiO_2 NPs at each time points (Fig. 2). These abnormalities included swollen erythrocytes, red cells with micronuclei, and cells with membrane abnormality. A quantitative analysis of the erythrocytes abnormality showed exposure to 0.1 mg l^{-1} TiO_2 at each time point caused a small but statistically significant increase in the proportion of swollen red cells, cells with micronuclei, and cells with membrane abnormality compared to the control (ANOVA, P <0.05; Table 5). Whereas, exposure to 1 mg l^{-1} of TiO_2 NP treatment showed a small increase in the proportion of swollen blood cells (at day 7 only) and cells with membrane abnormality (at day 14 only) compared to controls (all statistically significant, ANOVA, P <0.05). The incident rate of most abnormalities was only 1-4 % of the red blood cells observed, regardless of the time point (Table 5). Concentrations effects within each time point showed a small but statistically significant increase in the proportion of swollen red cells and cells with dividing nuclei at 1 mg l^{-1} TiO_2 treatment when compared to the 0.1 mg l^{-1} TiO_2 NP treatment (ANOVA, P <0.05; Table 5). The proportion of swollen red blood cells and cells with membrane abnormality also showed time effects which include more elevation at day 14 than day 7.

4 Discussion

Information on the effects of metals and metal oxide nanoparticles on the spleen or blood component in the haematopoietic tissues of fish are scarce. In this study, the results show that dietary exposure to TiO_2 NPs caused

less effect on the proportion of haematopoietic cells and the erythrocyte morphology compared to controls. However, responses were very minor compared to controls of the different routes of exposure. Waterborne exposure to the same materials (TiO_2 NPs) showed greater effects on the spleen contents of rainbow trout.

4.1 Effects of dietary TiO_2 NPs exposure on the spleen

Dietary exposure to TiO_2 NPs caused small changes in the haematopoietic cells in the spleen which included an increase in the proportion of erythroblasts, but the number of erythrocytes remained the same. The proportion of erythrocytes with abnormalities also showed small changes. This could indicate the spleen function is not affected by TiO_2, or the amount of TiO_2 that reached the spleen is not adequate to induce greater lesions in the spleen contents. Ramsden *et al.* [24] found that dietary exposure to sub-lethal of TiO_2 NPs gave normal growth level, no changes in the haematology and no evidence of osmotic stress, although Ti was accumulated in the spleen and other organs (gill, gut, liver and brain) with minor organ pathology. The presence of measurable levels of Ti in the spleens indicated that organ as an early target for dietary Ti exposure [24]. The level of Ti accumulation was decreased in the spleen after six and eight weeks of dietary exposure and Ramsden *et al.* [24] suggested that the spleen is able to regulate the excess TiO_2 during the exposure. Ramsden *et al.* [24] also found an increase in the proportion of red pulp after 8 weeks of exposure to 100 mg kg^{-1} of TiO_2 treatment. Therefore, the spleen prints results could reflect that the spleen was working to supply red blood cells into the circulatory system after removal of damaged cells from the circulation. After two weeks of recovery, a decrease in the proportion of erythrocytes with abnormalities (present study) and the proportion of red pulp [24] was observed in the spleen of trout. This suggests that the spleen was working to filter damaged red blood cells to return blood (and spleen function) to normal levels. For the immune response, a small increase in the proportion of immune cells that observed here could suggest the spleen responded by an induced immune cells to counteract these materials. The presence of granular deposits associated with the activity of fixed macrophages in the same samples of the spleen from all TiO_2 treatments that observed in Ramsden *et al.* [24] could suggests normal phagocytic functions. In fishes, the primary target of nanoparticle immunotoxicity is the cell mediated immunity and the phagocytic cells [11]. The increasing immune cells and removing foreign materials by ingesting them through a phagocytosis process has been reported in Dobrovolskaia and Mc Neil [14].

A.4.2 Effects of waterborne TiO_2 NPs exposure on the spleen

Waterborne exposure to TiO_2 NPs caused a small decrease in the proportion of immature red blood cells, but the proportion of mature erythrocytes remained the same after 14 days of exposure. These results suggest that fish suffered from hypoxia (see gill injury; [23]). A study by Federici*et al.* [23] found that waterborne exposure to sub-lethal concentration of TiO_2 NPs caused organ pathologies (gill, liver, and brain), biochemical disturbances, oxidative stress and no major haematological disturbance. The finding of Federici*et al.* [23] may be explained by the results found in the present study on the proportion of immature and mature erythrocytes in the spleen prints that collected from the same experiment. Usually, a confounding factor for any study of the spleen in fishes exposed via the water column is the indirect effects of gill injury and systemic hypoxia on the spleen function. Gill injury could stimulate the conscription of blood cells from the spleen into the circulation. Recent study by Bilberg*et al.* [25] showed that exposure to silver NPs can reduce blood pO_2 in fish.

The transient increased in the proportion of immune cells in the spleen prints of trout following exposure to TiO_2 that observed here could indicate the spleen responding to the NPs by producing immune cells (lymphocytes, neutrophils, and macrophages) as an immune response. A small proportion of thrombocytes that observed here could attribute to the experimental error instead of biological impact.

The current study also found a small increase in the proportion of erythrocytes with abnormalities compared to the control. Observation cells with dividing nuclei in the spleen prints are not considered as a type of erythrocyte abnormalities, but are evidence of stimulation of reticulocytes for dividing to produce new cells. These new cells compensated to the deficiencies of the blood cells in the circulatory system. Whereas, the swollen red cells and cells with membrane abnormality that observed in this study could result due to the osmotic influx of water that lead in the worst case to haemolysis.

Differences between dietary and waterborne exposure

The current study provides details about the effects of dietary and waterborne exposure to TiO_2 NPs on the spleen contents of rainbow trout by using spleen prints method. Over all, the dietary exposure to TiO_2 NPs had minor effects on the spleen contents of trout compared to waterborne exposure (morphology of erythrocytes and haematopoiesis cells). This suggests that dietary exposure to TiO_2 NPs had a lower bioavailability than waterborne NPs. Low oral bioavailability of NPs was found during dietary exposure in Ramsden *et al.* [24] study, because this route of exposure caused minor effects on the haematology parameters (red or white blood cell counts, haematocrits, whole blood haemoglobin) with no impact on growth or nutritional performance. Ramsden *et al.* [24] also found more elevation of Ti levels in the spleen after 2 weeks of exposure than 4 or 6 weeks, and these levels were sharply decreased at week 8 of exposure. These authors suggest that fishes were able to regulate excess TiO_2 in the spleen.Therefore, the minor changes that observed here in the spleen prints contents may be due to the Ramsden *et al.* [24] observation, but the function of spleen did not affect by this route of

exposure to TiO_2 NPs. However, the dietary exposure is less dangerous to the immune system of fishes.

Waterborne exposure to TiO_2 NPs induces lesions in the gills and other internal organs, with no major haematological or blood disturbances [23], as well as small changes in the spleen contents (in the current study). Changes in the spleen prints contents are therefore not necessarily indicative of NM uptake, or direct NM toxicity on the spleen could be occurred due to hypoxia. Therefore, the waterborne exposure is more dangerous to the respiratory health of fishes than dietary exposure. Additionally, the waterborne exposure to TiO_2 showed greater effects on the immune system of fish by producing different types of immune cells to phagocytic the effects of NPs. Overall, the levels of changes observed with dietary and waterborne exposure to TiO_2 are unlikely to be clinically important to fish health.

References

[1] Luoma, S. N. 1983. Bioavailability of trace metals to aquatic organisms—A review. *The Science of the Total Environment,* 28**,** 1-22.

[2] Taylor, C. R.; Shi, S. R. and Cote, R. J. 1996. Antigen retrieval for immunohistochemistry: Status and need for greater standar dization. *Applied Immuno Histochemistry,* 4**,** 144-166.

[3] Handy, R. D. 1996. Dietary exposure to toxic metals in fish. *In:* Taylor, E. W. (ed.) *Toxicology of Aquatic Pollution: Physiological, Cellular and Molecular Approaches. Society for Experimental Biology Seminar series:57,*. UK: Cambridge University Press, Cambridge, 29-60.

[4] Clearwater, S. J.; Farag, A. M. and Meyer, J. S. 2002. Bioavailability and toxicity of dietborne copper and zinc to fish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 132**,** 269-313.

[5] Handy, R. D.; Mcgeer, J. C.; Allen, H. E.; Drevnick, P. E.; Gorsuch, J. W.; Green, A. S.; Haldorsen, A.-K. L.; Mount, D. R. and 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.* Pensacola, F1, USA: SETAC Press, 59-112.

[6] Segner, H.; Wenger, M.; Möller, A.; Köllner, B. and Casanova-Nakayama, A. 2012. Immunotoxic effects of environmental toxicants in fish — how to assess them? *Environmental Science and Pollution Research,* 19**,** 2465-2476.

[7] SCENIHR 2010. Scientific Committee on Emerging and Newly Identified Health Risks), Scientific basis for the definition of the term "nanomaterial", Pre-consultation opinion.

[8] Handy, R.; Henry, T.; Scown, T.; Johnston, B. and Tyler, C. 2008a. Manufactured nanoparticles: their uptake and effects on fish - a mechanistic analysis. *Ecotoxicology,* 17**,** 396-409.

[9] Klaine, S. J.; Alvarez, P. J. J.; Batley, G. E.; Fernandes, T. F.; Handy, R. D.; Lyon, D. Y.; Mahendra, S.; Mclaughlin, M. J. and Lead, J. R. 2008. Nanomaterials in the environment behavior , fate, bioavailability, and effects. *Environmental Toxicology and Chemistry,* 27**,** 1825 -1851.

[10] Handy, R. D.; Al-Bairuty, G.; Al-Jubory, A.; Ramsden, C. S.; Boyle, D.; Shaw, B. J. and 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.

[11] Jovanović, B. and Palić, D. 2012. Immunotoxicology of non-functionalized engineered nanoparticles in aquatic organisms with special emphasis on fish—Review of current knowledge, gap identification, and call for further research. *Aquatic Toxicology,* 118–119**,** 141-151.

[12] Ding, L.; Stilwell, J.; Zhang, T.; Elboudwarej, O.; Jiang, H.; Selegue, J. P.; Cooke, P. A.; Gray, J. W. and Chen, F. F. 2005. Molecular characterization of the cytotoxic mechanism of multiwall carbon nanotubes and nano-onions on human skin fibroblast. *Nano Letters,* 5**,** 2448-2464.

[13] Tian, F.; Cui, D.; Schwarz, H.; Estrada, G. G. and Kobayashi, H. 2006. Cytotoxicity of single-wall carbon nanotubes on human fibroblasts. *Toxicology in Vitro,* 20**,** 1202-1212.

[14] Dobrovolskaia, M. A. and Mc Neil, S. E. 2007. Immunological properties of engineered nanomaterials. *Nat Nano,* 2**,** 469-478.

[15] Patlolla, A.; Knighten, B. and Tchounwou, P. 2010. Multi-walled carbon nanotubes induce cytotoxicity, genotoxicity and apoptosis in normal human dermal fibroblast cells. *Ethnicity & Disease* 20**,** S1-65-72

[16] Watts, M.; Munday, B. L. and Burke, C. M. 2001. Immune responses of teleost fish. *Australian Veterinary Journal,* 79**,** 570-574.

[17] Press, C. M. and Evensen, Ø. 1999. The morphology of the immune system in teleost fishes. *Fish and Shellfish Immunology,* 9**,** 309-318.

[18] Lange, M. A.; Govyadinova, A. A. and Khrushchev, N. G. 2000. Study on localization of hemopoietic tissue in sturgeon. *Russian Journal of Developmental Biology,* 31**,** 372-376.

[19] Handy, R. D.; Runnalls, T. and Russell, P. M. 2002c. Histopathologic biomarkers in three spined sticklebacks, *Gasterosteus aculeatus*, from several rivers in southern england that meet the freshwater fisheries directive. *Ecotoxicology,* 11**,** 467-479.

[20] Boyle, D.; Al-Bairuty, G. A.; Ramsden, C. S.; Sloman, K. A.; Henry, T. B. and 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.

[21] Handy, R. D.; Al-Bairuty, G.; Al-Jubory, A.; Ramsden, C. S.; Boyle, D.; Shaw, B. J. and 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.

[22] Peters, G. and Schwarzer 1985. Changes in hemopoietic tissue of rainbow trout under influence of stress. *Diseases of Aquatic Organisms,* 1**,** 1-10.

[23] Federici, G.; Shaw, B. J. and 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.

[24] Ramsden, C.; Smith, T.; Shaw, B. and Handy, R. 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.

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

Table 1Summary of the exposure conditions and nominal TiO_2 concentrations used in dietary or waterborne exposure studies with juvenile rainbow trout.

Nanoparticle	Types of exposure	Nominal concentrations	Actual measured Ti concentrations in the food or water	Exposure duration	Times when spleens were collected	References
TiO_2 NPs	dietary	10 and 100 mg kg^{-1}	5.4 and 53.6 mg kg^{-1} feed	10 weeks	8 weeks exposure followed by a 2 weeks recoveries	Ramsden *et al.* [24]
TiO_2 NPs	waterborne	0.1 and 1 mg l^{-1}	(mean ± S.E.M., *n* = 36/ treatments) 0.095 ± 0.006 mg l^{-1} 0.965 ± 0.021 mg l^{-1}	14 days	7 and 14 days	Federici *et al.* [23]

Table 2 The percentage of red and white blood cells in spleen prints from rainbow trout exposed to dietary TiO_2 nanoparticles for 8 weeks and followed by 2 weeks of recovery. (During recovery state fish feed on normal food)

% of haematopoietic cells	Time/ week	Treatments		
		Control	10 mg kg^{-1} TiO_2	100 mg kg^{-1} TiO_2
Haemocytoblast	8	5.4 ± 0.3 (5)	5.4 ± 0.2 (6)	5.7 ± 0.1 (6)
	2	6.6 ± 0.3(6) $^{+}$	6.8 ± 0.1 (6) $^{+}$	6.0 ± 0.6(5) $^{+}$
Erythroblast	8	13.6 ± 0.8 (5)	15.2 ± 0.3 (6) *	11.8 ± 0.2 (6) *$^{\#}$
	2	11.3 ± 0.4 (6) $^{+}$	11.4 ± 0.7 (6) $^{+}$	12.2 ± 0.4 (5)
Progranulocytes	8	0.3 ± 0.1 (5)	0.8 ± 0.1(6)	0.6 ± 0.2 (6)
	2	0.5 ± 0.3 (6)	Not observed (6) $^{+}$	0.3 ± 0.1(5)
Erythrocytes	8	77.2 ± 1.0 (5)	76.4 ± 0.2(6)	76.3 ± 0.5(6)

	2	77.8 ± 0.4 (6)	77.1 ± 0.6 (6)	76.1 ± 0.6(5)
Lymphocytes	8	0.2 ± 0.1 (5)	0.2 ± 0.1 (6)	2.9 ± 0.2 (6) *#
	2	0.4 ± 0.2 (6)	0.8 ± 0.2(6) +	Not observed (5) +#
Neutrophils	8	3.0 ± 0.2 (5)	2.6 ± 0.3(6)	4.1 ± 0.5 (5) #
	2	3.2 ± 0.4 (6)	2.8 ± 0.1(6)	3.0 ± 0.3 (6) +
Macrophages	8	Not observed (5)	0.2 ± 0.1(6)	0.3 ± 0.1 (6) *
	2	Not observed (6)	Not observed(6)	Not observed (5) +

Data are means of proportional cells ± S.E.M. (*n* = number of fish). (*) Statistically significant difference between control and treatment within row (ANOVA, *P*< 0.05). (#) Statistically significant difference between low and high concentration within row (ANOVA, *P*< 0.05). (+) Statistically significant difference between 8 weeks exposures and the subsequent 2 weeks recovery (time effects, ANOVA, *P*< 0.05). Not observed, the cell was absent from all the spleen prints examined from all the fish (a mean of zero).

Table 3 The percentage of erythrocytes with abnormalities in the spleen prints from rainbow trout exposed to dietary TiO_2 nanoparticles (NPs) for 8 weeks and followed by 2 weeks of recovery. (During recovery state fish feed on normal food)

Types of erythrocyte abnormality	Time/ week	Treatments		
		Control	10 mg kg^{-1}	100 mg kg^{-1}
Swollen cells	8	0.6 ± 0.1 (6)	1.4 ± 0.1 (6) *	2.5 ± 0.2 (5)*#
	2	0.4 ± 0.2 (5)	0.7 ± 0.1 (6) +	0.8 ± 0.2 (6) +
Cells with a dividing nuclei	8	12.5 ± 1.1 (6)	12.1 ± 0.4 (6)	12.4 ± 0.6 (5)
	2	6.2 ± 0.4 (5) +	7.6 ± 0.4 (6) +	6.8 ± 0.2 (6) +
Cells with membrane abnormality	8	1.3 ± 0.3 (6)	1.9 ± 0.2 (6) *	3.6 ± 0.2 (5) *#
	2	1.2 ± 0.1 (5)	0.8 ± 0.1 (6) +	1.4 ± 0.2 (6) +

Data are means of proportional cells ± S.E.M. (*n* = number of fish). (*) Statistically significant difference between control and treatment within row (ANOVA, *P*< 0.05). (#) Statistically significant difference between low and high concentration within row (ANOVA, *P*< 0.05). (+) Statistically significant difference between 8 weeks exposures and the subsequent 2 weeks recovery (time effects, ANOVA, *P*< 0.05).

Table 4 The percentage of red and white blood cells in spleen prints from rainbow trout exposed to waterborne TiO_2 nanoparticles for 7 and 14 days.

% of the haematopoietic cells	Time/ days	Treatments		
		Control	0.1 mg l^{-1} TiO_2	1 mg l^{-1} TiO_2
Haemocytoblast	7	Not observed(6)	0.8 ± 0.4 (6)	2.5 ± 0.4 (6) *#
	14	3.1 ± 0.3 (6)+	2.3 ± 0.2 (5) *+	2.3 ± 0.1(5)
Erythroblast	7	12.4 ± 1.0 (6)	7.7 ± 0.5 (6) *	8.1 ± 0.8 (6) *
	14	7.5 ± 0.7 (6)	7.0 ± 0.2 (5)	4.9 ± 0.1 (6) *+
Progranulocytes	7	0.3 ± 0.2 (6)	1.0 ± 0.1(6) *	0.6 ± 0.2 (6) #
	14	0.6 ± 0.1 (6)	0.8 ± 0.1(5)	0.4 ± 0.1(5)
Erythrocytes	7	86.0 ± 0.7 (6)	87.7 ± 1.3 (6)	86.1 ± 1.0(6)
	14	85.0 ± 0.4 (6)	86.5 ± 0.9(5)	86.7 ± 0.5(5)

Lymphocytes	7	1.0 ± 0.4 (6)	1.5 ± 0.2(6)	1.1 ± 0.1 (6)
	14	1.9 ± 0.2 (6)$^{+}$	0.9 ± 0.2 (5) *$^{+}$	3.1 ± 0.3 (5) *$^{\#+}$
Neutrophils	7	Not observed (6)	0.9 ± 0.4 (6) *	1.0 ± 0.2 (6) *
	14	1.5 ± 0.3 (6) $^{+}$	1.6 ± 0.2(5)	1.9 ± 0.1 (5) $^{+}$
Macrophages	7	Not observed (6)	0.3 ± 0.1(6)	0.6 ± 0.2 (6) *
	14	0.3 ± 0.1 (6)	0.7 ± 0.2 (5) *	0.6 ± 0.1(5)
Monocytes	7	Not observed (6)	0.3 ± 0.1 (6) *	Not observed(6)
	14	0.1 ± 0.1 (6)	0.3 ± 0.1 (5) *	Not observed (5)
Thrombocytes	7	0.9 ± 0.3 (6)	0.1 ± 0.1 (6) *	Not observed (6) *
	14	Not observed (6) $^{+}$	Not observed (5)	Not observed (5)

Data are means of proportional cells ± S.E.M. (*n* = number of fish). (*), statistically significant difference between control and treatment within row (ANOVA, *P*< 0.05). (#), statistically significant difference between low and high concentration within row (ANOVA, *P* < 0.05). (+), statistically significant difference between 7 and 14 days (time effects, ANOVA, *P*< 0.05). Not observed, the cell was absent from all the spleen prints examined from all the fish (a mean of zero).

Table 5 The percentage of erythrocytes with abnormalities in spleen prints from rainbow trout exposed to waterborne TiO_2 nanoparticles for 7 and 14 days.

% of erythrocytes abnormalities	Time/ days	Treatments		
		Control	0.1 mg l^{-1} TiO_2	1 mg $l^{-1}TiO_2$
Swollen cells	7	Not observed (6)	0.2 ± 0.1 (6)	0.9 ± 0.1 (6) *$^{\#}$
	14	0.2 ± 0.1 (6)	1.4 ± 0.2 (5) *$^{+}$	Not observed (5) $^{\#+}$
Cells with micronuclei	7	0.2 ± 0.1 (6)	2.1± 1.0 (6) *	Not observed (6) $^{\#}$
	14	Not observed (6)	0.2 ± 0.1 (5) $^{+}$	0.2 ± 0.1 (5)
Cells with a dividing nuclei	7	5.7 ± 0.8 (6)	8.2 ± 1.5 (6) *	5.5 ± 0.9 (6)
	14	18.4 ± 3.1 (6) $^{+}$	4.1 ± 0.3 (5) *	12.9 ± 1.7 (5) *$^{+\#}$
Cells with membrane abnormalities	7	1.8 ± 0.4 (6)	2.9 ± 0.6 (6) *	1.7 ± 0.4 (6) $^{\#}$
	14	0.2 ± 0.1 (6) $^{+}$	4.3 ± 0.4 (5) *	4.0 ± 0.3 (5) *$^{+}$

Data are means of proportional cells ± S.E.M. (*n* = number of fish). (*), statistically significant difference between control and treatment within row (ANOVA, *P*< 0.05). (#), statistically significant difference between low and high concentration within row (ANOVA, *P*< 0.05). (+), statistically significant difference between 7 and 14 days (time effects, ANOVA, *P* < 0.05). Not observed, the cell was absent from all the spleen prints examined from al/l the fish (a mean of zero).

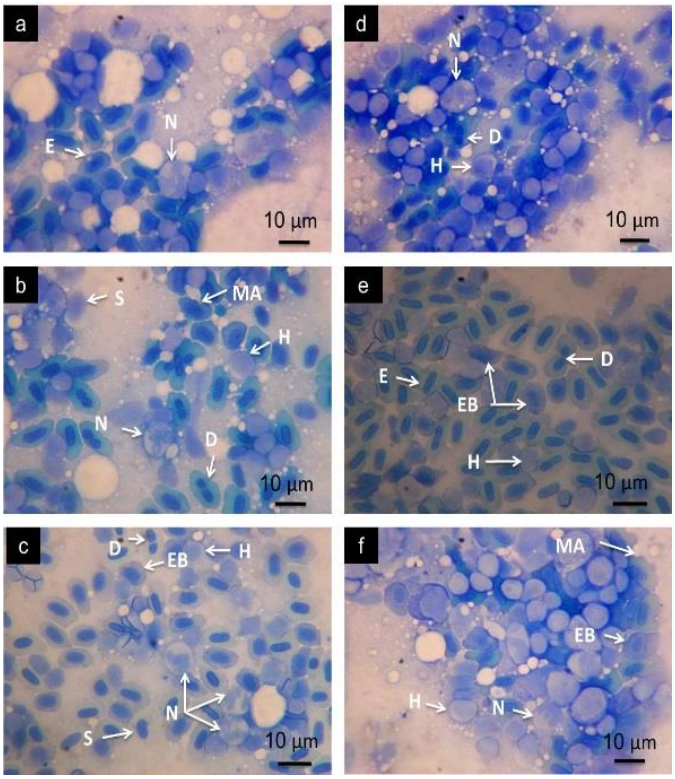

Figure 1 Spleen prints from rainbow trout following dietary exposure to TiO_2 NPs for 8 weeks (left column) and an additional 2 weeks of recovery which fish were feeding on normal food (right column). For 8 weeks, the panels include (a) control, (b) 10 mg kg^{-1} of TiO_2, (c) 100 mg kg^{-1} of TiO_2. After an additional 2 weeks recovery, the panels include (d) control, (e) 10 mg kg^{-1} of TiO_2, (f) 100 mg kg^{-1}. Erythrocytes (E), neutrophils (N), swollen red cells (S), cells with membrane abnormality (MA), haemcytoblast (H), red cells with dividing nuclei (D), and erythroblast (EB). Spleen prints were obtained from the fish used in Ramsden et al. [24]. Scale bar indicates magnification, smear stained with May-Grünwald Giemsa stain.

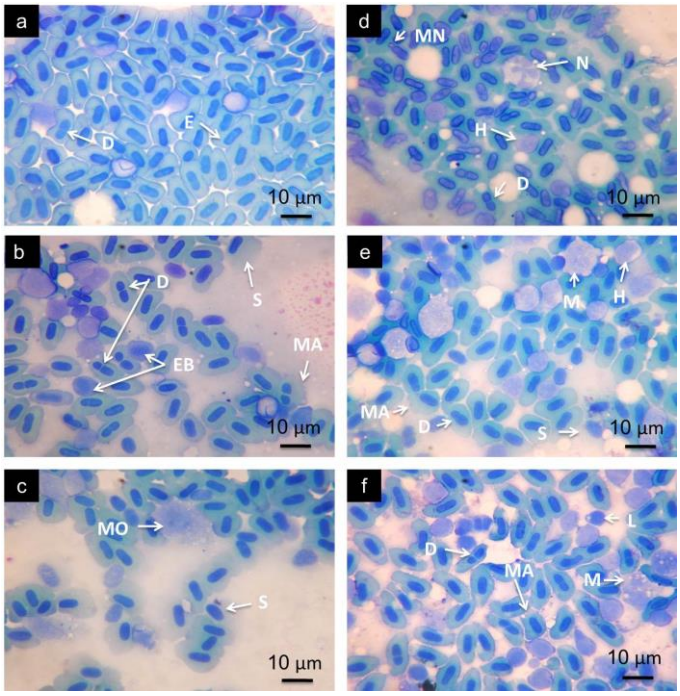

Figure 2Spleen prints from rainbow trout following exposure to waterborne TiO_2 NPs for 7 days (left column) and 14 days (right column). For 7 days, the panels include (a) control, (b) 0.1 mg l^{-1} of TiO_2, (c) 1 mg l^{-1} TiO_2. For 14 days, the panels include (d) control, (e) 0.1 mg l^{-1} of TiO_2 , (f) 1 mg l^{-1} of TiO_2. Swollen red cells (S), red blood cells with a dividing nucleus (D), erythroblast (EB), cells with membrane abnormality (MA), monocytes (MO), cells with micronuclei (MN), neutrophils (N), haemocytoblast (H), macrophages (M), lymphocytes (L). Spleen prints were obtained from the fish used in Federici et al. [23]. Scale bar indicates magnification, smear stained with May-Grünwald Giemsa stain