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Low hazard of silver nanoparticles and silver nitrate to the haematopoietic system of rainbow trout

Nathaniel J. Clark, Benjamin J. Shaw, Richard D. Handy*

School of Biological and Marine Sciences, University of Plymouth, Plymouth, United Kingdom

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ABSTRACT

Silver nanoparticles (Ag NPs) are known for their antibacterial properties and are used in a growing number of nano-enabled products, with inevitable concerns for releases to the environment. Nanoparticles may also be antigenic and toxic to the haematopoietic system, but the immunotoxic effect of Ag NPs on non-target species such as fishes is poorly understood. This study aimed to assess the effect of Ag NP exposure via the water on the haematopoietic system of rainbow trout, *Oncorhynchus mykiss*, and to determine whether or not the hazard from Ag NPs was different from that of $AgNO_3$. Fish were exposed for 7 days to a control (dechlorinated Plymouth freshwater), dispersant control, 1 $\mu g\,l^{-1}$ Ag as $AgNO_3$ or 100 $\mu g\,l^{-1}$ Ag NPs. Animals were sampled on days 0, 4 and 7 for haematology, tissue trace metal concentration, biochemistry for evidence of oxidative stress/inflammation in the spleen and histopathology of the blood cells and spleen. The Ag NP treatment significantly increased the haematocrit, but the haematological changes were within the normal physiological range of the animal. Thrombocytes in spleen prints at day 4, and melanomacrophage deposits at day 7 in the spleen, of Ag NP exposed-fish displayed significant increases compared to all the other treatments within the time point. A dialysis experiment confirmed that dissolution rates were very low and any pathology observed is likely from the NP form rather than dissolved metal released from it. Overall, the data showed subtle differences in the effects of Ag NPs compared to $AgNO_3$ on the haematopoietic system. The lack of pathology in the circulating blood cells and melanomacrophage deposits in the spleen suggests a compensatory physiological effort by the spleen to maintain normal circulating haematology during Ag NP exposure.

1. Introduction

In addition to naturally occurring deposits, silver is introduced into the aquatic environment through anthropogenic activities including those of the photographic and electrical industries (Purcell and Peters, 1998). The toxicology and ecophysiology of dissolved silver (Ag^+) has been relatively well studied in freshwater fish (review, Hogstrand and Wood, 1998). Dissolved silver exerts toxic effects via the fish gill and a Biotic Ligand Model has been developed to predict acute toxicity, depending on the water chemistry (Bury et al., 1999; Hogstrand et al., 2003; Bielmyer et al., 2007). The acute metal toxicity of dissolved silver is characterised by silver ion uptake at the gill epithelium with subsequent disturbances to sodium homeostasis and inhibition of the branchial Na^+/K^+-ATPase (Morgan et al., 1997). Defects in acid-base balance via interference with cytoplasmic carbonic anhydrase can also occur (Morgan et al., 1997). Ultimately, during acute toxicity, the depletion of plasma sodium and respiratory distress (Morgan et al., 1997; Bury et al., 1999) contribute to cardiovascular collapse (Wood et al., 1996).

Advances in material science have led to the development of colloidal forms of metals at the nanoscale, which are one type of engineered nanomaterials (ENMs). The classification of ENMs with respect to hazardous substances is still being debated (e.g., Stone et al., 2010), but they are generally defined as materials with at least one primary dimension between 1 and 100 nm, but other criteria such as the proportion of nanoscale material present in a chemical substance are also considered (European Commission, 2011). For metal oxide ENMs, there is a concern that their toxicity differs from the equivalent dissolved form of the same metal (Shaw and Handy, 2011). Nano forms of silver are a particular concern because some dissolution of Ag ions from the surface of Ag NPs is observed (e.g. Besinis et al., 2014), and the Ag^+ ion is arguably one of the most toxic elements to aquatic species with LC_{50} values typically in the low $\mu g\,l^{-1}$ for rainbow trout (Bury et al.,

* Corresponding author.
Email address: R.Handy@plymouth.ac.uk (R.D. Handy)

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1999). In contrast, the acute toxicity of Ag NPs to juvenile rainbow trout appear to be much less, with a 96 h LC_{50} of around 2.16 mg l^{-1} in freshwater (Johari et al., 2013). Models predict surface water concentrations of Ag NPs in the ng-μg l^{-1} range (Gottschalk et al., 2013) and while these predictions are likely below the acute toxicity thresholds, there are concerns for chronic and sub-lethal effects of Ag NPs. For Ag NP ecotoxicity to fish the focus has been on the gills (metal accumulation, alterations to gene expression, and oxidative damage; Griffitt et al., 2009; Scown et al., 2010) and the expected traditional mechanisms of dissolved metal toxicity (e.g., liver injury; Choi et al., 2010). Immune effects have been less well studied.

The particulate nature of ENMs has raised concerns that they may act as antigens, like viruses or proteins of a similar size. The concerns for modulation of the mammalian immune system by ENMs have been reviewed (Dobrovolskaia and McNeil, 2007), including the occurrence of immunosuppression as well as excessive stimulation of the immune system leading to inflammation and/or hypersensitivity reactions. In mammals, the research effort has focussed on respiratory exposure to ultrafine dusts (e.g., Duffin et al., 2007). These studies show that stiff high-aspect ratio nanofibers can cause asbestos-like inflammation in rodents that may lead to *meso*thelioma (Poland et al., 2008). The concerns are less for spherical metal particles that may pass through the lung or dissolve, but congestion of the lung as a result of Ag NP exposure has been observed (Schinwald et al., 2012). Less is known about the effects of ENMs on the haematopoietic system and immunity of aquatic species, including fishes (Handy et al., 2011; Jovanović and Palić, 2012). Teleost fishes, unlike mammals do not have discrete immune organs such as lymph nodes and thymus, although they have groups of cells (as yet not all identified) that may have analogous functions in different parts of the body. In trout, the haematopoietic system comprises the spleen, immune-like functions in cells clustered in the head kidney and elsewhere, as well as the blood cells. There is also evidence of ENM accumulation within the haematopoietic organs of fish. For example, Boyle et al. (2013) intravenously injected rainbow trout with 50 μg ml^{-1} TiO_2 over a 96 h period and reported significant accumulation of titanium in the spleen compared to bulk and control treatments. Similar to mammals, teleost fish have a range of different white blood cells and antibodies present in the cardiovascular system (reviews; Rice, 2001; Handy et al., 2011).

There are a few studies demonstrating potential effects of ENMs on the immune cells of trout. For example, Klaper et al. (2010) used rainbow trout macrophage cell cultures to show that additions of 0.1–10 μg ml^{-1} of functionalized carbon nanotubes cause dose-dependent up regulation of inflammatory cytokines in a similar way to lipopolysaccharide (LPS) positive controls. However, studies assessing Ag NP or dissolved silver (as Ag^+ or otherwise) on the immune functions of fish are sparse. Exposure of fresh suspensions of red blood cells from trout to high concentrations of Ag NPs (3.1–31 μg ml^{-1}) for 48 h causes lactate dehydrogenase (LDH) leakage and haemolysis (Massarsky et al., 2014). At least demonstrating that Ag NPs can be hazardous to the blood cells in vitro. A few in vivo studies are currently available that report some effects of Ag NPs on some parts of the haematopoietic system. Bruneau et al. (2016) exposed rainbow trout to a 10% (v/v) dilution of wastewater effluent with a Ag NP mass concentration of 40 μg l^{-1} for 96 h and found no decrease in lymphocyte or macrophage viability extracted from the pronephros. However, there was a significant decrease in the phagocytic activity of the isolated macrophages in the Ag NP treatment compared to unexposed controls and a $AgNO_3$ treatment. There was also increased cyclo-oxygenase activity in the livers of the fish (Bruneau et al., 2016), that could be interpreted as early evidence of inflammatory pathway activation. Katuli et al. (2014) also showed that acute exposure of zebrafish to 6.76 mg l^{-1} of Ag NPs for four days could cause biochemical disturbances to the blood including NaCl depletion, elevated cortisol and glucose, and inhibition of erythrocyte acetylcholinesterase activity. However, more complete in vivo measurements on the immune cells of fish blood are needed, and the effects of Ag NPs on the spleen of fishes is unclear. The aim of this study was to investigate the toxic effects of Ag NPs on the haematopoietic system of rainbow trout (*Oncorhynchus mykiss*) following a seven day waterborne exposure, and to determine whether the immune hazard for the nano compared to metal salt forms of silver are different. Animals were sampled on day 0, 4 and 7 for haematology (haematocrit, haemoglobin, total red and white cell counts) to overview the status of the blood. In addition, blood smears and spleen prints were collected to explore changes in the proportion of stored and circulating immune cells as well as cell morphology. Histological examination of the spleen enabled some considerations of functional integrity of this central organ of the haematopoietic system as well as anatomical evidence for inflammation. Oxidative stress in the spleen was also assessed biochemically by measuring total glutathione (GSH) and thiobarbituric acid reactive substances (TBARS).

2. Methodology

2.1. Experimental design

Juvenile rainbow trout (n = 138) were obtained from Exmoor Fisheries, then held in an aerated, re-circulating aquaria at Plymouth University. Stock fish were fed using a commercial trout feed (BioMar, 1–2 mm size pellets). A photoperiod of 12 h: 12 h light and dark was used throughout both the holding and experimental period. Fish weighing 12.3 ± 0.7 g (mean ± S.E.M., n = 120) were graded into twelve 20 l experimental aquaria (n = 10 fish per tank) and allowed to acclimate for 24 h prior to experimentation. Fish were not fed 24 h prior to, or during, the experiment. The entire experiment was subject to ethical approval according to the Home Office Scientific Procedures Act (1986) in the U.K. and the fish were inspected by an independent specialist on fish welfare and/or veterinary surgeon. *O. mykiss* were exposed, for 7 days, in triplicate tanks to one of four conditions: control (freshwater only), dispersant control (0.001% of 4% polyoxyethylene glycerol trioleate and polyoxyethylene [20] sorbitan momo-laurat [Tween 20]), 1 μg l^{-1} Ag as silver nitrate or 100 μg l^{-1} of Ag NPs. The Ag NP concentration was chosen after considering the 96 h LC_{50} of around 137 μg l^{-1} for zebrafish larvae in our laboratory (Shaw et al., 2016), and allowing for differences in water temperature and body size and the notion that the larger juvenile rainbow trout were likely to be less sensitive, a sub-lethal concentration of 100 μg l^{-1} was selected. The exposure duration of one week was chosen to enable time for blood parameters to change, but also to minimise the exposure (i.e., not several weeks) in compliance with ethical approval. The Ag NP was supplied pre-dispersed, and so to account for any toxic effects of the detergents present, a dispersant control of 0.001% was included to reflect the concentration of the dispersing agent in the Ag NP treatment. The study aimed to be sub-lethal for all treatments so that the biological effects were comparable. A sub-lethal value for the silver nitrate concentration was derived from published data. The LC_{50} of silver nitrate ranges between 2.9 and 12.7 μg l^{-1} for rainbow trout, depending on water chemistry (Bury et al., 1999). Therefore using a mass concentration of 100 μg l^{-1} of silver nitrate to match the same test concentration of the Ag NPs was not viable, and to ensure a sub-lethal response 1 μg l^{-1} of $AgNO_3$ was used.

Water changes of 80% of the total volume were carried out every 12 h to maintain water quality and to ensure Ag NP exposure. Water samples were collected after the morning water changes, and before the evening water changes, for pH (Hanna HI pH meter), dissolved and saturated oxygen (Hach HQ 40d multi), total ammonia (HI 95715, Hanna Instruments), total Ag, Ca^{2+}, Na^+ and K^+ concentrations (using inductively coupled plasma mass spectrometry, see below). Additional water samples were taken 6 h after each water change to check for early losses of silver from the water column. There were no tank effects

between general water quality parameters, so the data were pooled. Values were (means ± SEM, n = 27–144); total ammonia: 0.25 ± 0.03; pH, 6.98 ± 0.01; dissolved oxygen, 9.90 ± 0.07 mg l^{-1}; oxygen saturation, 96.6 ± 0.13%; temperature: 13.5 ± 0.1 °C. The electrolyte composition of dechlorinated, filtered, Plymouth freshwater was 10.07 ± 0.06, 1.47 ± 0.01 and 17.64 ± 0.17 mg l^{-1} for Na^+, K^+ and Ca^{2+}, respectively. Background concentrations of Ag were 0.09 µg l^{-1} ± 0.02 µg l^{-1}. Fish were randomly sampled on day 0, 4 and 7 for haematology, biochemistry and histology (see below).

2.2. Stock solutions and dosing

The Ag NPs (NM-300K) were supplied by the Joint Research Council as part of the European Commissions' MARINA Project, with the manufacturer's information stating a particle size of approximately 15 nm, with 99% of the particles being < 20 nm. Transmission electron microscopy (TEM, JEOL 1200EXII) at Plymouth determined a primary particle size of 15.5 ± 2.4 nm (mean ± SD, n = 156; Fig. 1A) and no other metal impurities were detected in the material as supplied. A stock of 400 mg l^{-1} of the Ag NPs was made by adding 197 µl of the liquid supplied by the manufacturer into a volumetric flask, which was then filled to 50 ml with ultrapure water (Millipore, 18.2 MΩ-cm resistivity, ion free and unbuffered) and sonicated (35-kHz frequency, Fisherbrand FB11010, Germany) for 2 h without the use of additional solvents. Nanoparticle tracking analysis (Nanosight LM10, Nanosight U.K.) revealed mean aggregate hydrodynamic diameters of 60 ± 41 nm with a mode of 34 nm (n = 3, Fig. 1B) in a 1 mg l^{-1} dispersion in ultrapure water. Nanoparticle Tracking Analysis could not be conducted reliably in the Plymouth freshwater at the selected test concentration of the Ag NPs due to the background of natural colloids in the water. For the silver nitrate control, a 4 mg l^{-1} stock of Ag as $AgNO_3$ (Sigma-Aldrich) was prepared in a two-step process. A 1 g l^{-1} solution was made by dissolving 1.575 g of $AgNO_3$ in 1 L of ultrapure water. Of this, 2 ml was taken and diluted to 500 ml using ultrapure water. The $AgNO_3$ stock was stored in the dark at room temperature when not in use. Dosing of tanks was carried out by adding the appropriate volume of the stock chemical within 10 min following the 9 a.m. and 9 p.m. water changes. The volume of stock added to each tank was reduced pro rata to reflect the 80% water changes.

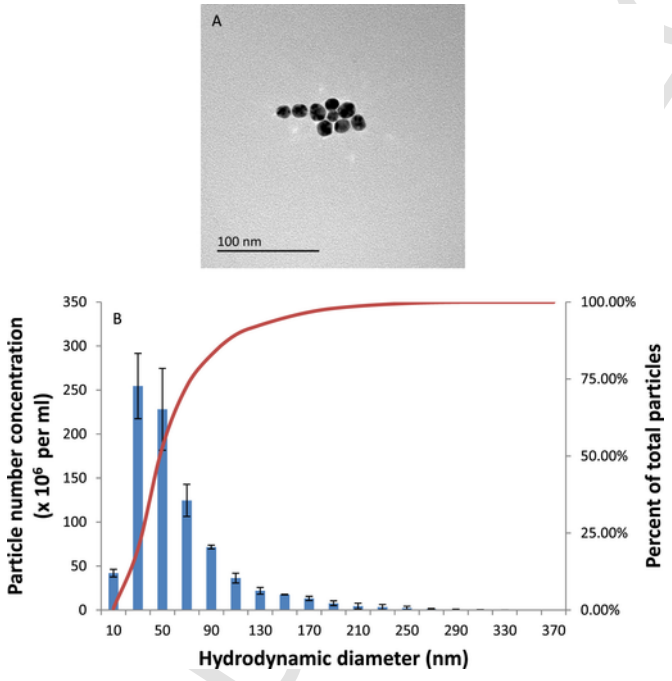

Fig. 1. Primary particle size and particle size distribution of a 1 mg l^{-1} Ag NP dispersion in ultrapure water. (A) Transmission electron micrograph showing Ag NPs with a mean primary particle size of 15.5 ± 2.4 nm (mean ± SD, n = 156 particles counted) and (B) Particle size distribution measured by Nanoparticle Tracking Analysis (NTA, Nanosight LM10) in a 1 mg l^{-1} dispersion showing the hydrodynamic diameters and particle number concentration at each bin size (bars ± standard error) and the cumulative percent of the total particles measured (solid line) in the dispersion in ultrapure water. The natural background of colloids in the Plymouth freshwater and the detection limits of NTA prevented reliable attempts to measure the dispersion during the experiment.

2.3. Haematology and plasma ion analysis

Haematological and plasma ion analysis were performed as described by Handy and Depledge (1999). Briefly, two fish were randomly selected from each tank (n = 6 for initial fish, or as otherwise stated at days 0, 4 and 7). The animals were carefully anaesthetised with buffered MS222 after which total fish length and weight was recorded. Blood was extracted via the caudal vein into heparinised syringes. Haematocrit (Hct) was calculated according to Handy and Depledge (1999) using microhaematocrit tubes (13,000 rpm, 2 min, Heraeus pico 17 microcentrifuge) and a Hawksley reader (Hawksley, Sussex, UK). The haemoglobin (Hb) concentration was determined from 20 µl of whole blood mixed immediately into 5 ml of Drabkin's reagent. Colour development was measured at 540 nm (Jenway 7315 spectrophotometer) and compared against cyanmethemoglobin standards. A further 20 µl of whole blood was fixed in Dacie's fluid (0.98 ml) for total red and white blood cell counts. Another drop of blood was used for blood smears prepared on glass microscope slides. After 24 h drying, the blood smears were fixed in 100% methanol and stained with Giemsa. Slides were then scored for normal and abnormal cells (e.g., cells with a dividing nucleus, shrunken cells and swollen cells). The rest of the blood was centrifuged for 2 min at 13,000 rpm (Micro Centaur MSE) and the plasma frozen at −80 °C until required. Plasma Na^+ and K^+ concentration was determined using a Corning 420 Flame Photometer as described by Burke et al. (2003).

Spleen prints were prepared as described by Peters and Schwarzer (1985), although these latter authors used the term 'spleen smear', the procedure is in fact to make a print of cells from the spleen. Briefly, spleens were removed from anesthetised fish, carefully cut in half and the prints prepared by pressing the exposed section of tissue onto a glass microscope slide (multiple presses per spleen). Samples were allowed to air dry for 24 h before fixation in 100% methanol. Once dry, slides were immersed in BDH buffer (tablets, pH 6.8), May-Grünwald stain (Sigma, 32856) and buffered Giemsa solution and allowed to dry. At least 200 normal cells were counted and the relative percentages calculated (as well as abnormal cells; Peters and Schwarzer, 1985).

2.4. Water and tissue ion analysis

Water sampling and tissue ion analysis were performed as described by Shaw et al. (2012). Briefly, water samples were taken to determine Na^+, K^+, Ca^{2+} and total silver concentrations within tanks from the treatments using inductively coupled plasma mass spectrometry (ICP-MS) against acidified matrix-matched standards and with blanks of 5% nitric acid in each sample run.

For the tissues, fish were anaesthetised (as above) and dissected to remove the spleen. Gills and liver from fish collected at day 7 from all treatments were also used to confirm aqueous exposure and internalisation of total Ag. The gill tissues were copiously rinsed with ultrapure water to remove externally bound Ag and clean instruments were used to collect organs from the body cavity to prevent cross-contamination during dissection. Tissue samples were digested in 2 ml of concentrated nitric acid for 2 h in a water bath at 70 °C following Shaw et al. (2012). Digests were then cooled and diluted to 8 ml with ultrapure deionised water. Procedural blanks of acid digestion with no tissue were included in the methodology. Samples were then analysed for total Mn, Fe and Ag using ICP-MS and total Zn, Ca, Mg, Na and K using

inductively coupled plasma optical emission spectrometry (ICP-OES). The standards were matrix matched for acidity and ionic strength as close as possible to the samples. Instrument blanks of nitric acid and also standards were included in the sample runs to correct for instrument drift. The detection limits of each analyte were determined from three times the standard deviation of the blanks. The detection limits for the cations measured by ICP-OES were 50 $\mu g\, l^{-1}$ or less depending on the analyte, and the detection limit for Ag by ICP MS was around 0.004 $\mu g\, l^{-1}$ depending on the sample matrix.

2.5. Biochemistry

Biochemical analysis was performed as described by Smith et al. (2007). Briefly, fish were anaesthetised on day 0, 4 and 7 using buffered MS222. Whole spleens were dissected and immediately snap frozen and stored at −80 °C until required for biochemistry. Spleens were homogenised into four volumes (2.0 ml) of ice cold isotonic buffer [in mmol l^{-1}; 300 sucrose, 0.1 ethylenediamine tetra acetic acid (EDTA), 20 (4-(2-hydroxyethyl)piperazine-1-ethane sulfonic acid (HEPES)), adjusted to pH 7.8 with a few drops of Tris (2-amino-2-hydroxylmethyl-1,3-propanediol)]. Crude homogenates were stored at −80 °C until required for analysis of thiobarbituric acid reactive substances (TBARS) and total glutathione (GSH). For TBARS 120 μl of homogenate was used and compared to standards of 0 (blank), 0.5–25.0 μmol l^{-1} of 1,1,3,3-tetra-ethoxypropane. For total GSH, 20 μl of homogenate was used for the kinetic determination of GSH. Both assays were conducted exactly as Smith et al. (2007) and absorbances (Versa Max micro plate reader) were measured in 96-well plates in triplicate.

2.6. Histopathology

This was performed as described by Smith et al. (2007). Briefly, at days 0 and 7 (n = 6 fish per treatment), fish were anaesthetised using buffered MS222, following which they were dissected for whole spleens. Organs were placed in individual vials containing buffered formal saline (Di sodium hydrogen orthophosphate monohydrate, sodium phosphate monobasic monohydrate, 36.6% formaldehyde). Tissues were processed and stained for routine wax histology. Spleen examinations were made at 5 μm and stained with Mallory's Trichrome. Photographs were taken with an Olympus Vanox T microscope with Olympus digital camera (C-2020 Z). Slides were processed in batches of treatments with controls to eliminate staining artefacts between treatments.

Quantitative histopathological measurements of the spleen were made by assessing fractional areas of the spleen in randomly selected areas using the point counting method described by Weibel et al. (1966). The fractional volume (area) $V_i = P_i/P_T$, where P_i is the number of points counted and P_T is the total number of points on the counting grid. This method was used to determine the relative proportions of red pulp, white pulp and sinusoidal space with counts of melanomacrophage also noted, similar to our previous studies on trout with ENMs (Al-Bariuty et al., 2013).

2.7. Dialysis experiment

Dialysis experiments were carried out to assess the dissolution rate of Ag NPs in dechlorinated Plymouth freshwater and ultrapure water. The method was based on Besinis et al. (2014). Glassware was acid washed using 5% nitric acid prior to use. The dialysis tubing (cellulose membrane with a molecular weight cut off at 12,000 Da, Sigma Aldrich) was soaked in ultrapure overnight, following which it was made into dialysis bags containing 8 ml of 100 mg l^{-1} Ag NPs using mediclips. After bags were filled, they were added to beakers containing 492 ml of the appropriate media (dechlorinated Plymouth freshwater or ultrapure water), giving a total volume of 500 ml. Each experiment was carried out in triplicate for 24 h at room temperature. A multipoint stirrer (Ro 15p power, Ika-Werke GmbH and Co. KG, Staufen, Germany) was used to gently agitate the media during the experimental period. Samples from the media of each beaker were collected at 0, 0.5, 1, 2, 4, 6 and 24 h as well as the dialysis tubing contents at the end of the experiment for determination of Ag concentration (ICP-MS). Glassware was monitored throughout the experiment for ion leakage by using dialysis blanks (dialysis bags also containing the external media).

2.8. Statistics

All data were analysed using Statgraphics Centurion XVI and dialysis curves fitted as appropriate using SigmaPlot 11.0. Data were checked for descriptive statistics and normality (skewedness and kurtosis), with deviations of more than ± 2 considered as non-normal distribution. No tank effects were observed in the data, and so data were pooled by treatment. Treatment effects or time-effects were analysed by two-way ANOVA after evaluating the data for unequal variance using Bartlett's test. For parametric data ANOVA was applied, followed by Tukey HSD post hoc test to confirm the location of significant differences ($P < 0.05$). For non-parametric data, the Kruskal-Wallis test was used with notched box and whisker plots to locate differences. All analysis used the default level for statistical significance in the software.

3. Results

3.1. Confirming aqueous exposure to silver and particle behaviour

Waterborne silver exposure was confirmed by measuring the total Ag concentrations in the tanks at 0, 6 and 12 h leading up to each water change and re-dosing of the test system. No significant differences were observed between tanks within time points and treatment for total Ag concentration, and so data were pooled by time point within treatment. The silver concentrations within the control and dispersant control treatments were not statistically different over the time period and remained low (< 0.09 $\mu g\, l^{-1}$ in control and < 0.04 $\mu g\, l^{-1}$ in dispersant control at 0, 6 and 12 h, respectively within water changes). The total silver present in experimental tanks (mean ± SEM, n = 14) were 0.96 ± 0.31, 0.81 ± 0.13 and 0.62 ± 0.06 $\mu g\, l^{-1}$ for $AgNO_3$ treatments at 0, 6, and 12 h before the next water change (no statistically significant difference, between time points, ANOVA, $P > 0.05$). The overall mean of measured total silver concentrations in the tanks were 0.76 ± 0.10 $\mu g\, l^{-1}$ during the whole experiment, demonstrating some loss of the nominal exposure concentration. Despite the water changes some settling of Ag NPs was apparent in the NP treatment. The average total silver concentration in Ag NP treated tanks (mean ± SEM, n =14) were 84.19 ± 2.14, 64.71 ± 5.99 and 56.67 ± 4.72 μg^{-1} in the Ag NP treatments (0, 6 and 12 h, respectively), which resulted in the samples at 6 and 12 h being statistically lower than that initially measured in the tank at the start (ANOVA, $P > 0.05$). Overall, the mean for the 7 days exposure was 65 ± 4.46 $\mu g\, l^{-1}$.

Tissue samples of the gill, liver and spleen were taken to confirm aqueous exposure and internalisation of Ag at day 7 of the experiment, which demonstrated treatment and some material effects. At day 7, the control and dispersant control gills had total Ag concentrations below the limit of detection (equating to between 2.8 and 53.0 ng g^{-1} dry weight of tissue). The gills of fish from the day 7 Ag NP treatment had significantly more total Ag (mean ± S.E.M, 3527.1 ± 392.1 ng g^{-1} dry weight) compared to the animals from the $AgNO_3$ treatment (746.1 ± 198.6 ng g^{-1} dry weight, ANOVA, $P < 0.05$). Some background levels of Ag were detected in the liver of control and dispersant control tissues. However, these values were significantly less (Kruskal-Wallis, P

< 0.05) than those of fish exposed to either $AgNO_3$ or Ag NPs (data are mean ± S.E.M., control, 0.31 ± 0.05; dispersant control, 0.32 ± 0.12; $AgNO_3$, 17.73 ± 4.87; and Ag NPs, 51.51 ± 12.61 μg g^{-1} dry weight tissue). No material-effect was observed. Spleens from the initial fish at time zero and those at day 7, regardless of treatment showed total Ag concentrations below the limit of detection (< 3 ng g^{-1}).

A dialysis experiment was conducted to assess dissolved Ag release from Ag NPs in ultrapure water and Plymouth freshwater. The dissolution was low and curves for the total cumulative Ag released into the medium (see Supplement data) followed a rectangular hyperbola achieving maximum apparent dissolved silver release of 84.7 and 52.4 of silver metal (absolute amount) in ultrapure water and Plymouth freshwater respectively; representing 10.6% and 6.5% of the initial total silver content in the dialysis bags. The fastest release of total dissolved silver was within the first 0.5 h, with maximum rates of dissolution of 0.62 and 0.61 μg min^{-1} in ultrapure water and Plymouth freshwater, respectively.

3.2. Haematology and plasma ions

Some statistically significant haematological differences were observed between $AgNO_3$, or Ag NPs, when compared to the control treatments; but no differences between silver materials were observed (Table 1). At day 4, total red blood cell counts of fish from the control and dispersant control were significantly higher compared to both $AgNO_3$ and Ag NP treatments (ANOVA, $P < 0.05$), but no differences were observed between the two silver materials. There were no statistical differences between treatment at any given time point, or time-effects within treatments in total white blood cell counts (ANOVA, $P > 0.05$). Haematocrit values were higher in fish exposed to Ag NPs than controls throughout, with transient differences to the dispersant control (ANOVA, $P < 0.05$). Haemoglobin concentration showed no treatment dependent affects at day 4 and day 7 in fish, although there were some time effects within treatments (Table 1).

Similar to the haematology, some statistical differences in plasma ion Na^+ and K^+ were observed between treatments after exposure to $AgNO_3$ and Ag NP, with some material-type effects (Table 1). Plasma Na^+ concentrations in the animals from the Ag NP treatment were significantly lower than controls, whereas those from the $AgNO_3$ treatment showed transient reductions compared to controls within the same day (ANOVA, $P < 0.05$). At day 7, Ag NP-exposed fish had a plasma Na^+ concentration that was significantly lower than that of $AgNO_3$-exposed fish at the same time point (Table 1). Plasma K^+ concentration showed treatment and time within treatment dependent changes. At day 7, plasma K^+ in Ag NP-exposed fish was significantly higher than those of the $AgNO_3$ treatment (ANOVA, $P < 0.05$; Table 1). There were no differences between treatment, or time within treatment, for the plasma osmolarity data (ANOVA, $P < 0.05$).

Some statistical differences were observed in trace metal concentration of the spleen between days within treatment, but no material-type affects were observed. Spleen Zn concentration of $AgNO_3$-exposed fish was significantly lower than the unexposed control (means ± SEM; $AgNO_3$ treatment, 0.42 ± 0.27 μmol g^{-1} dry weight tissue; control, 2.52 ± 0.52 μmol g^{-1} dry weight tissue; ANOVA, $P < 0.05$). Exposure to Ag NPs, but not $AgNO_3$, caused some depletion of K^+ and Mg^{2+} from the spleen (ANOVA or Kruskal-Wallis, $P < 0.05$). For example, spleen K^+ concentrations in Ag NP-exposed fish at the end of the experiment were 90.2 ± 28.6 μmol g^{-1} dry weight tissue compared to 621.3 ± 166.4 in the control. The spleen Mg^{2+} concentrations in Ag NP-exposed fish and controls at the end of the experiment were 8.1 ± 2.1 and 52.7 ± 14.3 μmol g^{-1} dry weight tissue respectively. The Mg^{2+} concentration in the spleens of Ag NP-exposed fish were also lower than dispersant controls (dispersant control, 63.0 ± 22.9 μmol g^{-1}; and Ag NP, 8.1 ± 2.1 μmol g^{-1}, Kruskal-Wallis, $P < 0.05$). The Ag NP treatment also depleted Mn in the spleen by day 7 (control, 75.93 ± 20.64; Ag NP treatment, 15.22 ± 6.91 μmol g^{-1} dry weight tissue; ANOVA, $P < 0.05$). However, no significant differences were observed between the type of Ag material on spleen Mn concentrations.

In the blood smears, statistical differences were observed between treatments, and some time-effects were found within treatments for erythrocyte, erythroblasts and lymphocyte counts, but no material-type effects were observed (Fig. 2). Erythrocyte counts at day 4 for fish from

Table 1
Haematology and plasma ions from rainbow trout exposed to 1 μg l^{-1} Ag as $AgNO_3$ or 100 μg l^{-1} Ag NPs for up to 7 days.

Parameter	Time	Treatment			
		Control	Dispersant control	1 μg l^{-1} $AgNO_3$	100 μg l^{-1} Ag NP
Haematocrit (%)	0	34.67 ± 0.80 (6)			
	4	28.50 ± 1.38 (6)	25.20 ± 1.59 (5)°	26.33 ±2.06 (6)°	33.50 ± 2.78 (6)*+#
	7	27.83 ± 1.52 (6)	30.6 ± 2.84 (5)	29.17 ± 0.83 (6)	36.40 ± 3.08 (5)*
Haemoglobin (g dl^{-1})	0	6.30 ± 0.24 (6)			
	4	1.82 ± 0.42 (5)°	1.74 ± 0.15 (5)°	2.00 ± 0.25 (6)°	2.47 ± 0.17 (5)°
	7	4.10 ± 0.55 (6)T	4.02 ± 0.82 (6)T	3.53 ± 0.16 (6)T	4.99 ± 1.10 (6)
Red blood cell count (cells × 10^6 mm^3)	0	0.10 ± 0.03 (6)			
	4	0.20 ± 0.01 (6)°	0.26 ± 0.03 (4)°	0.10 ± 0.02 (6)*+	0.09 ± 0.02 (6)*+
	7	0.13 ± 0.00 (6)	0.07 ± 0.02 (5)T	0.10 ± 0.02 (6)	0.09 ± 0.02 (5)
White blood cell count (cells × 10^3 mm^3)	0	0.83 ± 0.34 (6)			
	4	1.40 ± 0.33 (6)	1.85 ± 0.46 (4)	1.22 ± 0.44 (6)	1.22 ± 0.10 (6)
	7	1.33 ± 0.72 (6)	1.16 ± 0.23 (5)	2.80 ± 0.62 (6)	2.56 ± 0.53 (6)
Plasma Na^+ (mmol l^{-1})	0	144 ± 2.19 (4)			
	4	108.43 ± 14.43 (4)	123.78 ± 6.03 (4)	100.58 ± 16.85 (4)*	80.60 ± 18.23 (5)*+°
	7	111.3 ± 4.83 (5)°	114.83 ± 6.64 (4)°	111.70 ± 10.75 (4)°	83.50 ± 4.98 (3)*+#°
Plasma K^+ (mmol l^{-1})	0	3.15 ± 0.42 (4)			
	4	4.83 ± 0.53 (4)	4.49 ± 0.27 (4)	4.87 ± 0.10 (5)°	5.05 ± 0.03 (5)°
	7	3.74 ± 0.25 (5)	4.23 ± 0.40 (4)	3.94 ± 0.22 (4)T	4.86 ± 0.00 (3)*#°T
Osmolarity (mOsm kg^{-1})	0	322.50 ± 6.49 (4)			
	4	276.20 ± 33.06 (5)	284.25 ± 13.84 (4)	267.8 ± 16.69 (5)	276.00 ± 13.04 (5)
	7	293.17 ± 20.98 (6)	326.00 ± 31.28 (4)	303.40 ± 16.59 (5)	282.00 ± 31.09 (5)

Data are mean haematological parameter ± S.E.M. Brackets indicate number of samples per treatment. (*) significant difference from control within rows (ANOVA or Kruskal-Wallis, $P < 0.05$). (+) significant difference from dispersant control within rows (ANOVA or Kruskal-Wallis, $P < 0.05$). (#) significant difference between $AgNO_3$ and Ag NPs (ANOVA or Kruskal-Wallis, $P < 0.05$). (T) significant difference from day 4 data within treatment (ANOVA or Kruskal-Wallis, $P < 0.05$). (0) significant difference from initial fish (ANOVA or Kruskal-Wallis, $P < 0.05$).

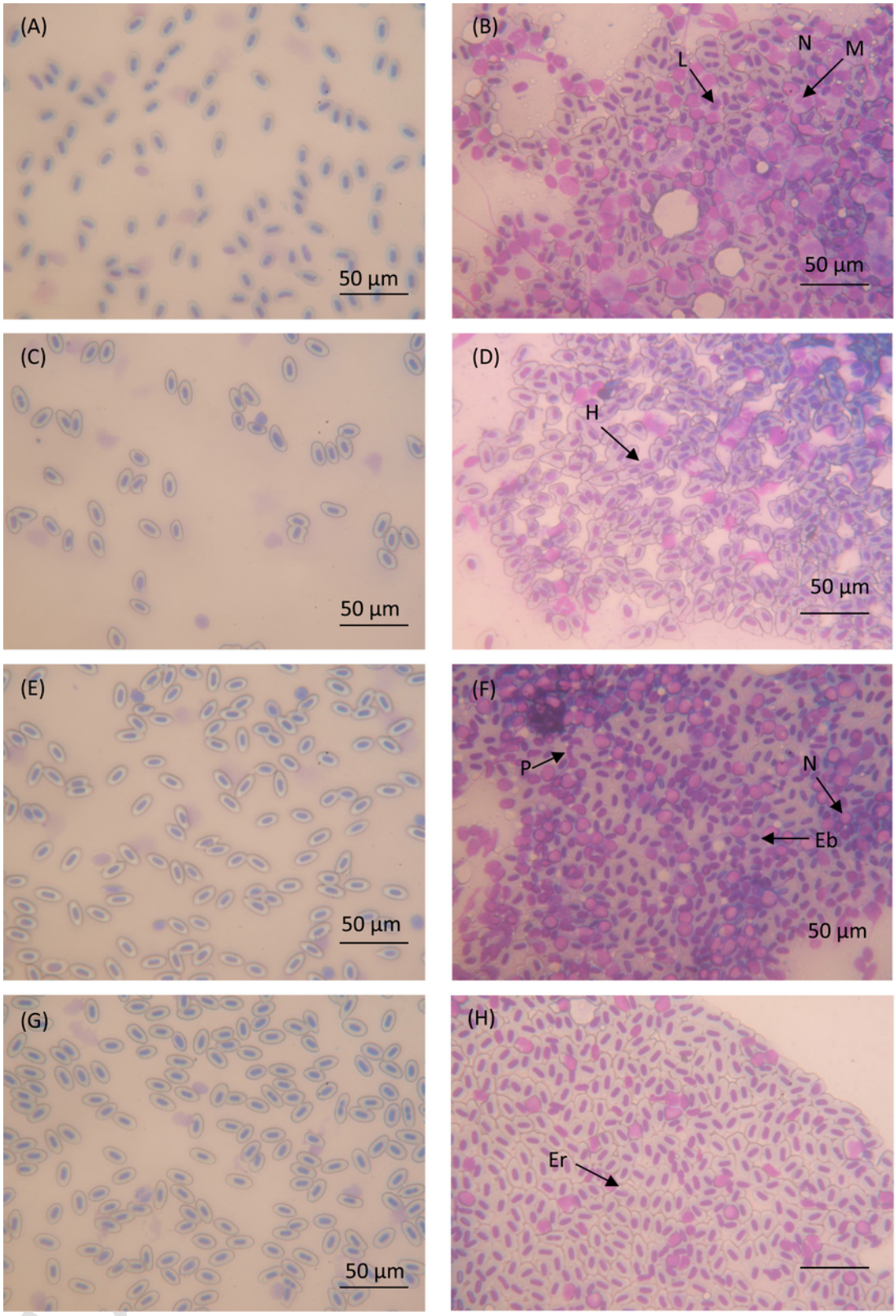

Fig. 2. Blood smears (left column) and spleen prints (right column) of rainbow trout following 7 days treatment. Panels are control (A and B), dispersant control (C and D), 1 µg l^{-1} Ag as $AgNO_3$ (E and F) and 100 µg l^{-1} of Ag NPs (G and H). Cells scored in the spleen prints (example cells indicated by arrows) included the hemoblasts (H), progranulocyte (P), erythrocytes (Er), erythroblasts (Eb), lymphocytes (L), macrophages (M), neutrophils (N). No overt pathologies of the blood cells were observed, although there were transient changes in some of the cell counts.

the dispersant control were significantly lower compared to Ag NP treatment (Kruskal-Wallis, $P < 0.05$). Also, day 4 erythroblast counts in both the control and Ag NP treatments were significantly lower compared to the dispersant control (Kruskal-Wallis, $P < 0.05$; Table 2). The total number of lymphocytes were counted on the blood smears and showed that Ag NP exposed fish at day 7 had significantly lower counts compared to that of the dispersant control and initial fish. There were some levels of background membrane abnormalities in control and dispersant control fish over time, but no significant difference between silver materials was observed.

Some statistical differences were observed in the total number of erythrocytes, lymphocytes and thrombocytes counted in spleen prints between treatments, and some time-effects within treatments. Notably, some differences between materials occurred (Fig. 2). The spleen print erythroblast counts decreased with exposure time in fish from the $AgNO_3$ treatment (ANOVA, $P < 0.05$), but not in the Ag NP treatment. The lymphocyte counts of fish from the control treatments showed a transient rise at day 4 compared to all the other treatments (ANOVA, $P < 0.05$). Some transient changes also occurred in the thrombocytes. At day 4, thrombocyte counts were significantly increased in the Ag NP treatment compared to those of the control, dispersant control and $AgNO_3$ treatments (material-type effect). No treatment or time effects were seen within hemoblast, progranulocytes or neutrophils counts. Background changes in abnormal cells were evident in control treatments, but no significant difference between silver materials was observed.

3.3. Histopathology and biochemistry

There were no treatment effects on the proportions of red pulp and white pulp measured in the spleen, but the values for individual fish did show some variation (Fig. 3). Furthermore, there was no constriction or expansion of the sinusoid space regardless of silver exposure or the form of silver (Kruskal-Wallis, $P < 0.05$). Melanomacrophage deposits were present in the spleens of control fish, as expected for the normal housekeeping of the spleen to remove damaged cells from the circulation. However, the dispersant and both forms of silver showed some time-dependent increase in melanomacrophage deposits compared to the control (ANOVA or Kruskal-Wallis, $P < 0.05$). There was also a material type-effect with the Ag NP-exposed fish showing higher melanomacrophage counts than that of the $AgNO_3$ treatment (ANOVA, $P < 0.05$).

Oxidative stress parameters were measured on initial, day 4 and day 7 fish to inform of potential inflammation injury to the spleen tissue. The TBARS concentrations showed some-time dependent changes within treatments, but there was no clear material-type effect. TBARS concentrations in spleen of exposed fish were significantly higher in $AgNO_3$ and Ag NP fish after 4 days compared to controls (controls, 0.34 ± 0.11; $AgNO_3$, 1.43 ± 0.44; and Ag NPs, 2.11 ± 0.63 nmol mg^{-1} protein), but this was transient and lost by the end of the experiment. There were no statistical differences in the total GSH concentration between silver treatments or the dispersion control, although by day 7 all these treatments show a decrease compared to the initial fish (initial fish, 1.11 ± 0.05; day 7 control, 0.71 ± 0.11; dispersant control, 0.56 ± 0.10; $AgNO_3$, 0.75 ± 0.06; and Ag NPs, 0.66 ± 0.16 μmol g^{-1} wet weight tissue, ANOVA, $P < 0.05$). Total GSH concentrations did not show any difference between silver materials.

4. Discussion

This study assessed the effects of Ag NPs compared to $AgNO_3$ on the haematopoietic system of rainbow trout. Exposure to Ag as $AgNO_3$ caused some disturbances in spleen lymphocyte counts. Exposure to Ag NPs caused significant changes in the Hct, plasma Na^+ and K^+ concentrations, as well as alterations in the spleen print thrombocyte and erythrocyte counts. For most parameters, the effects of Ag NPs were equal to or less than the effects of $AgNO_3$.

4.1. Confirming silver exposure

Waterborne exposure was confirmed by measuring total silver in the water and in the gills, liver and spleen tissues. The controls and solvent controls showed only trace levels of silver in the water as expected, and in the gills a background similar to that reported in trout by Bruneau et al. (2016). The $AgNO_3$ treatment achieved a measured concentration in the water that was very close to the nominal total concentration. However, the Ag NPs as expected from out previous semi-static exposure with metallic NPs (Cu NPs, Shaw et al., 2012) showed some decline in the measured total silver concentrations in the water column. The stock used for dosing the tanks with Ag NPs showed a good dispersion (Fig. 1), and the decrease in the fish tanks was almost

Table 2
Quantitative analysis of blood smears from rainbow trout exposed to 1 μg l^{-1} Ag as $AgNO_3$ or 100 μg l^{-1} Ag NPs for 4 or 7 days.

Percent of	Time	Treatment			
cells	(days)	Control	Dispersant	1 μg l^{-1} $AgNO_3$	100 μg l^{-1} Ag NP
Erythrocytes	0	92.62 ± 1.40 (6)			
	4	92.55 ± 1.69 (3)	79.96 ± 4.63 (5)	89.92 ± 3.12 (5)	92.10 ± 0.91 (5)$^+$
	7	93.41 ± 1.80 (6)	95.58 ± 0.65 (5)	95.84 ± 0.71 (6)	95.75 ± 0.86 (6)
Erythroblasts	0	2.05 ± 1.07 (6)			
	4	1.03 ± 0.81 (3)	4.47 ± 1.47 (5)*	1.58 ± 0.62 (5)	1.24 ± 0.35 (5)$^+$
	7	0.37 ± 0.29 (6)°	0.71 ± 0.29 (5)°	0.42 ± 0.02 (6)°	0.99 ± 0.37 (6)
Lymphocytes	0	1.24 ± 0.27 (6)			
	4	2.15 ± 0.44 (3)	3.88 ± 1.36 (5)	2.12 ± 0.71 (5)	1.72 ± 0.32 (5)
	7	1.75 ± 0.81 (6)	1.29 ± 0.42 (5)	0.66 ± 0.30 (6)	0.00 ± 0.00 (6)$^{+0}$
Macrophages	0	0.43 ± 0.36 (6)			
	4	1.84 ± 0.05 (3)	2.84 ± 0.87 (5)	0.75 ± 0.52 (5)	1.41 ± 0.48 (5)
	7	1.61 ± 0.69 (6)	1.01 ± 0.11 (5)	1.14 ± 0.49 (6)	0.97 ± 0.28 (6)
Neutrophils	0	0.39 ± 0.39 (6)			
	4	0.16 ± 0.16 (3)	0.28 ± 0.28 (5)	0.41 ± 0.41 (5)	0.56 ± 0.56 (5)
	7	0.45 ± 0.45 (6)	0.17 ± 0.17 (5)	0.07 ± 0.07 (6)	1.29 ± 1.29 (6)
Thrombocytes	0	3.26 ± 0.80 (6)			
	4	2.26 ± 0.84 (3)	8.58 ± 2.68 (5)	5.23 ± 2.14 (5)	2.98 ± 0.90 (5)
	7	2.41 ± 0.84 (6)	1.24 ± 0.51 (5)	1.87 ± 0.29 (6)	1.00 ± 0.42 (6)

Data are mean cell counts in blood smears ± S.E.M. Brackets indicate number of samples. (*) significant difference from control within rows (ANOVA, $P < 0.05$). (+) significant difference from Dispersant control within rows (ANOVA or Kruskal-Wallis, $P < 0.05$). (0) significant difference from initial fish (ANOVA, $P < 0.05$).

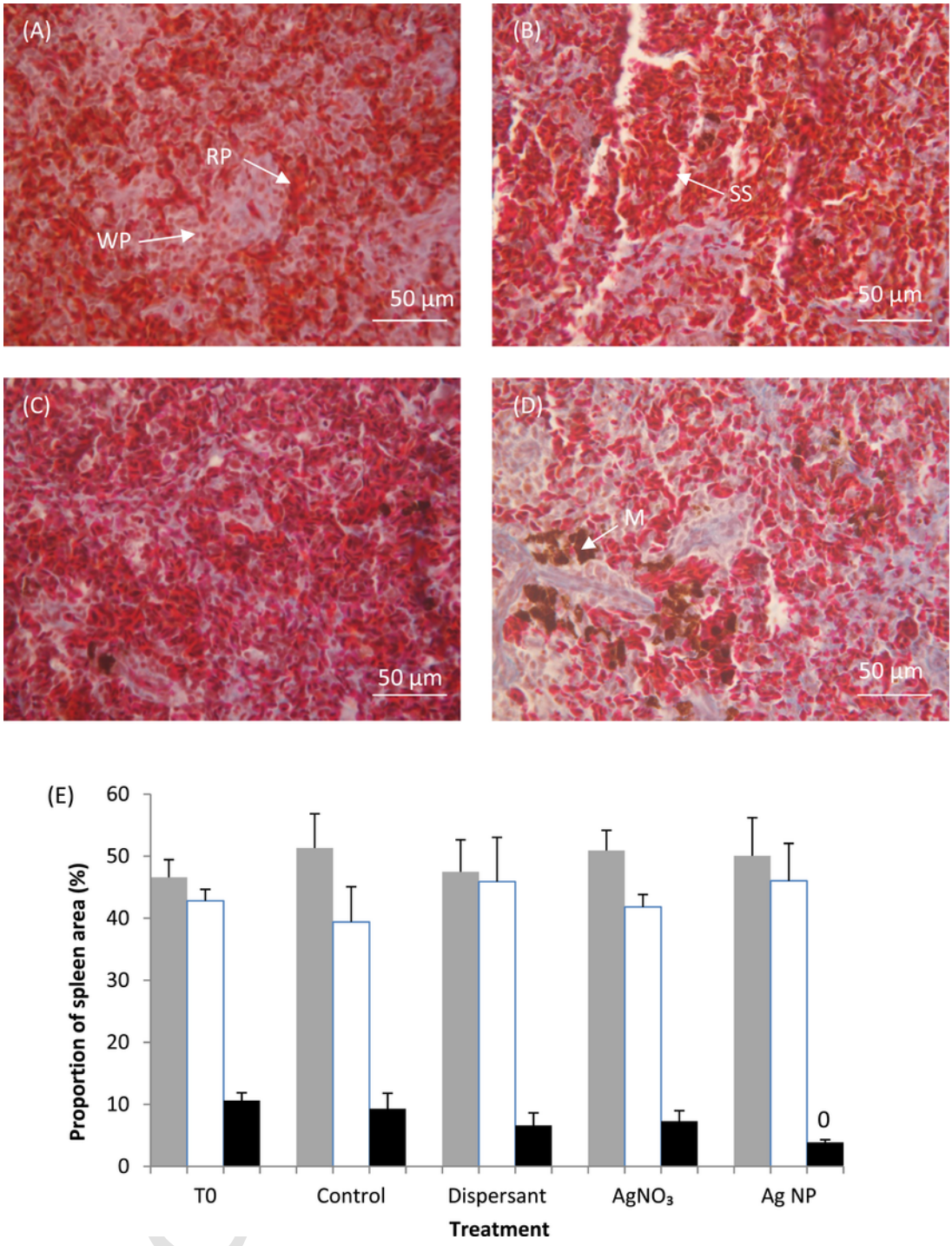

Fig. 3. Spleen morphology of rainbow trout following exposure to control (A), dispersant control (B), 1 µg l^{-1} of Ag as $AgNO_3$ (C), or 100 µg l^{-1} of Ag NPs (D). Scale bars indicate magnification; sections were 5 µm thick and stained with Mallory's trichome. The red pulp (RP), white pulp (WP) and sinusoidal space (SS) and melanomcarophage deposits (M) are indicated. Note the presence of melanomacrophage deposits in the Ag NP treatment. In panel (E) the quantitative measurements of the proportions of spleen area as red pulp (grey), white pulp (white) and sinusoidal space (black) are shown. Values are means ± S.E.M. Initial fish (T0). (0) indicates a significant difference from the initial fish (Kruskal-Wallis, $P < 0.05$). No other differences were observed.

certainly due to particle settling, resulting in overall silver concentrations of 65% of the nominal concentration during the exposure. While the exposure was confirmed by measurements of the water, from a perspective of regulatory testing, this would not be within the accepted 100 ± 20% nominal concentration criteria for a valid OECD (Organisation for Economic Cooperation and Development) test (Shaw et al., 2016). In terms of ENM testing, Ag NP exposures are especially challenging to maintain (see Shaw et al., 2016) and the dosing achieved here was relatively robust with the regular water changes. Both the $AgNO_3$ and Ag NP exposures were sub-lethal with only one mortality in the entire experiment, which occurred in the Ag NP treatment. This was attributed to bullying due to evidence of fin nipping.

The notion of a sub-lethal exposure for Ag NPs was also supported by the dialysis experiment which was conducted to assess total dissolved silver release from the Ag NPs in Plymouth freshwater. The percentage of overall dissolution of silver from the Ag NPs was 6.5% in

the dialysis experiments over 24 h. The maximum rate of dissolution was 0.61 μg min^{-1}, and even if this maximum dissolution rate were maintained for the duration of the 12-h period before the next water change, this would equate to a theoretical maximum release of 22 μg l^{-1} of Ag ions. This clearly did not occur, as there was no evidence of acute toxicity. This apparent lack of free ion toxicity can be attributed to any dissolution slowing in the fixed 20 L volume of the tank (i.e., reaching equilibrium), some particle settling, but more likely the presence of millimolar concentrations of Cl^- ions in the water that would rapidly chelate free silver ions to insoluble AgCl (see Besinis et al., 2014). Therefore, silver ion toxicity in the water column derived from the Ag NPs is not a likely explanation of the biological effects reported in the present study.

The total Ag in the gill tissues also confirmed that exposures had occurred compared to the controls, and notably the total Ag in the gills of fish from the Ag NP treatment were around 4× higher than those from the $AgNO_3$ treatment. This might be expected since the mass concentration of silver in the water for the latter was necessarily lower to maintain a sub-lethal exposure. The acid digestion method used for the tissues can only reveal total Ag, not the form of the silver (and was not intended to do so). Nonetheless both Ag treatments demonstrated internalisation of the metal (form unknown) with elevated total Ag levels in the livers of the fish at the end of the experiment. There was no silver accumulation in the spleen and this is consistent with the findings of Shaw et al. (2012) on Cu NPs, indicating the spleen is not an organ of direct toxicity.

4.2. Haematology and osmotic disturbances

The control animals showed normal ranges of haematology and plasma ions (Table 1) that were consistent with our previous reports for juvenile trout in Plymouth freshwater (e.g., Shaw et al., 2012). There were some transient changes in the haemoglobin concentration and red blood cell counts at day 4 (Table 1), but these recovered by day 7 and were within the normal physiological range. There was a gradual decline in plasma Na^+ in the control fish, which is associated with not feeding the fish during the experiment given that fish preferentially regulate plasma Na^+ from dietary salt intake (Smith et al., 1989). However, periods without food is normal for trout in the wild, and there was no impact on plasma osmolarity or any evidence of red cell swelling in the controls, showing that the control animals were not osmotically challenged.

Exposure to $AgNO_3$ or Ag NPs caused a fall in plasma Na^+ compared to the time-matched controls. This effect was much greater for the Ag NP treatment, and also with some evidence of cell swelling in the latter (increased Hct, Table 1). The sub-lethal effects of $AgNO_3$ on osmoregulation at the gill are well-known, and the small plasma Na^+ loss reported here is consistent with passive ion loss through the gills and/or a transient inhibition of the branchial Na^+ pump (Wood et al., 1996). The Ag NPs caused more disturbance with plasma Na^+ falling below 100 mmol l^{-1} (Table 1). This might be expected when the gill total Ag concentration was also higher than that in fish from the metal salt treatment. The loss of electrolytes from the blood and increased Hct are consistent with osmotic swelling of the red blood cells. Johari and Kalbassi (2016) also reported elevated Hct and sodium depletion in trout during a 21d day exposure to the same Ag NP concentration used here; although an Ag NP effect cannot be deduced from their study because there was no silver salt control in their study design or data presented on dissolution.

The percentage of erythrocytes in the blood smears also decreased (Table 2), suggesting that osmotically damaged red cells in the circulation were not being replaced by the spleen. There was no pathology in the spleen (see below) and the amount of red pulp within the spleen was normal and did not decrease with either form of silver exposure. This is consistent with biologically modest disturbances to the red blood cells that were not sufficient to initiate a splenic response, and was therefore within the physiological tolerance of the fish. The Hct values reported here, despite some statistical difference, are consistent with values for the normal range in trout (Handy et al., 1999). Furthermore, as the Hct values increased without a change in total Hb concentration, it is unlikely that the haematology is an artefact of a hypoxic response through gill damage.

A significant increase in plasma K^+ was observed in fish from the Ag NP treatment, but not in the $AgNO_3$-exposed fish by the end of the experiment. Rises in plasma K^+ concentrations are associated with vascular inflammation resulting from the loss of electrolytes (i.e., mainly K^+) from leaky muscle which makes up the bulk of the animal. This has been demonstrated in both metal and NP exposures (metals in shellfish: Cd, Sheir and Handy, 2010; Cu NP in trout, Al-Bariuty et al., 2013). Regardless, taken together, the greater loss of plasma Na^+, elevated plasma K^+ and increased Hct suggested that the effects of the Ag NPs were greater than that of the $AgNO_3$ exposure.

The morphology of the blood smears of fish were consistent with the haematology with the control and dispersant controls showing no evidence of swollen red blood cells, although there was some mild cell swelling in both silver treatments this was not statistically significant. There was also no effect of treatment on the number of shrunken cells or cells with a dividing nucleus. In addition, no particle aggregates were observed on the blood smears.

4.3. Splenic functions in the haematopoietic system

The spleen functions to ensure the composition and health of the circulating blood cells remain normal. It therefore has a role in the response to hypoxia with respect to maintain the number of circulating erythrocytes for the oxygen carrying capacity of the blood, but it also has an immune role in managing the circulating white cells (Handy et al., 2011). The total white cell counts in the blood were normal (Table 1), as were the proportions of white cells in the blood smears (Table 2), suggesting the spleen was maintaining the circulating white cells. There are few reports of white cell counts during in vivo Ag NP exposures in trout. Imani et al. (2015) reported elevation of white blood cell counts following exposure to 200 μg l^{-1} Ag NPs for eight days in trout. However, the changes, although statistically significant, were within the normal range for trout and unfortunately a metal salt control was not included in their study design or total silver measurements in the tissues to confirm the exposure.

Toxic metals are known to alter the response of the immune system in several ways, including excess immunostimulation and inflammation, or immunosuppression (e.g., Dobrovolskaia and McNeil, 2007). These concerns are also applied to metallic NPs since they are both metal and a foreign particle (potential antigen; Zolnik et al., 2010). In the present study, there was no evidence for excessive immunostimulation (e.g. congestion of blood or tissues with white cells, Sheir and Handy, 2010), or spleen pathology indicative of inflammation. Similarly, the absence of increases in TBARS in the spleen at day 7 and no treatment-dependent changes in total GSH also indicate that inflammation with the associated oxidative stress did not occur. Bruneau et al. (2016) also observed no elevation of TBARS in trout exposed to 40 μg l^{-1} Ag NPs for 96 h in gill or liver tissue, but did show an increase of cyclo-oxygenase activity in liver which could be a sign of emerging systemic oxidative stress.

The increase in melanomacrophage deposits of the spleen in Ag NP exposed fish (Fig. 3) suggests that the spleen had been coping with more housekeeping of debris from the turnover of blood cells. In addition, a transient elevation of thrombocytes was observed in the spleen prints only from the Ag NP-exposed fish. These cells secrete factors that are involved in the formation of the major histocompatibility complex (MHC) required for antigen recognition in fish (Köllner et al., 2004). Whether or not this indicates if the thrombocytes in the spleen were beginning to recognise Ag NP particles as antigen, or just responding to

the general debris in the tissue (Handy et al., 2011) cannot be deduced from the measurements here.

The spleen prints also showed a decrease in the number of lymphocytes in the Ag NPs and $AgNO_3$-exposed fish, as well as the dispersant control (Table 3). For the Ag NP treatment this effect could be entirely explained by the dispersing agent, but agents such as Tween tend to have non-specific effects on membrane fragility, and yet the dispersant control did not alter the other cell counts in the spleen. The $AgNO_3$ treatment showed a clear decline in lymphocytes in the spleen, but normal proportions in the blood smears from the circulation. This suggests the Ag was having an immune effect on lymphocytes, but it was readily managed by the spleen. The trout spleen contains several sub-populations of lymphocytes that are loosely analogous to mammalian T- and B-lymphocytes (Deluca et al., 1983). Further work would be needed on these sub-populations to determine the nature of the response.

4.4. Conclusions, water quality and environmental implications

Sub-lethal concentrations of Ag NPs did not cause adverse effects on the spleen or immune cells of trout in the short term with the experimental conditions used here. An acute hypersensitivity reaction with inflammation is also excluded. The early concerns that NPs, including Ag NPs, may be antigenic in mammals are not substantiated in this study for fishes. There are, however, some subtle differences between the effects of Ag as $AgNO_3$ and Ag NPs, with the latter altering more haematological and splenic endpoints. This might suggest the environmental hazard of immunotoxicty is a little greater with exposure to the NP form. However, the mass dose of total Ag must be considered. The fish were exposed to at least an order of magnitude more Ag in the NP than dissolved form in this study to elicit a sub-lethal response. Despite the substantial disparity in exposure dose, there were only modest difference between $AgNO_3$ and Ag NPs in terms of the biological importance of the endpoints; which were largely well within the physiological capability of the fish. Consequently, the current silver ion risk assessment is probably going to be protective for immune health during Ag NP exposures, at least in the short term in freshwater.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.01.030.

Table 3
Quantitative analysis of spleen prints from rainbow trout exposed to 1 μg l^{-1} Ag as $AgNO_3$ or 100 μg l^{-1} Ag NPs for 4 or 7 days.

Percent of	Time	Treatment			
cells	(days)	Control	Dispersant	1 μg l^{-1} $AgNO_3$	100 μg l^{-1} Ag NP
Hemoblast	0	15.19 ± 4.36 (6)			
	4	17.77 ± 2.65 (6)	19.13 ± 3.23 (6)	16.41 ± 1.00 (6)	16.47 ± 4.33 (6)
	7	19.79 ± 3.41 (6)	16.0 ± 5.23 (6)	15.85 ± 2.40 (6)	12.31 ± 2.64 (5)
Progranulocytes	0	3.77 ± 0.89 (6)			
	4	3.11 ± 0.88 (6)	5.88 ± 1.65 (6)	4.71 ± 0.83 (6)	3.48 ± 0.79 (6)
	7	4.34 ± 1.12 (6)	3.69 ± 0.84(6)	3.10 ± 0.56 (6)	3.67 ± 1.17 (5)
Erythroblasts	0	4.55 ± 1.04 (6)			
	4	2.29 ± 0.46 (6)	3.35 ± 0.77 (6)	3.30 ± 0.43 (6)	3.32 ± 0.94 (6)
	7	2.59 ± 0.71 (6)	2.06 ± 0.42 (6)	$0.87 \pm 0.21\ (6)^{T0}$	1.91 ± 0.90 (5)
Erythrocytes	0	61.03 ±2.72 (6)			
	4	66.67 ± 3.21 (6)	63.86 ± 4.41 (6)	66.18 ± 2.44 (6)	$55.69 \pm 4.54\ (6)^{*\#}$
	7	62.62 ± 5.34 (6)	72.62 ± 6.53 (6)	73.67 ± 2.93 (6)	$73.96 \pm 4.74\ (5)^{T}$
Lymphocytes	0	11.59 ± 2.96 (6)			
	4	6.60 ± 0.96 (6)	$1.73 \pm 0.64\ (6)^{*}$	$2.51 \pm 0.72\ (6)^{*}$	$2.44 \pm 0.89\ (6)^{*}$
	7	$1.0 \pm 0.39\ (6)^{T0}$	$0.88 \pm 0.38\ (6)^{0}$	$1.30 \pm 0.52\ (6)^{0}$	$0.69 \pm 0.22\ (5)^{0}$
Macrophages	0	0.90 ± 0.26 (6)			
	4	1.33 ± 0.54 (6)	1.21 ± 0.44 (6)	1.96 ± 0.38 (6)	2.59 ± 0.60 (6)
	7	0.85 ± 0.31 (6)	0.90 ± 0.53 (6)	$0.45 \pm 0.16\ (6)^{T}$	$1.01 \pm 0.69\ (5)^{T}$
Neutrophils	0	0.87 ± 0.23 (6)			
	4	1.03 ± 0.33 (6)	0.88 ± 0.34 (6)	2.05 ± 0.52 (6)	2.23 ± 0.98 (6)
	7	1.97 ± 0.47 (6)	1.45 ± 0.56 (6)	1.40 ± 0.38 (6)	1.18 ± 0.42 (5)
Thrombocytes	0	0.59 ± 0.24 (6)			
	4	0.08 ± 0.08 (6)	0.13 ± 0.08 (6)	1.22 ± 0.86 (6)	$7.59 \pm 1.28\ (6)^{*+\#0}$
	7	$2.54 \pm 0.53\ (6)^{T0}$	0.33 ± 0.07 (6)	$1.16 \pm 0.58\ (6)^{T}$	$0.76 \pm 0.47\ (5)^{*}$

Data are mean cell counts in spleen prints ± S.E.M. Brackets indicate number of samples. (*) significant difference from control within rows (ANOVA or Kruskal-Wallis, $P < 0.05$). (+) significant difference from dispersant control within rows (ANOVA or Kruskal-Wallis, $P < 0.05$). (#) significant difference between silver material type (ANOVA, $P < 0.05$). (T) significant difference from day 4 data within treatment (ANOVA or Kruskal-Wallis, $P < 0.05$).

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