Environmental Pollution 203 (2015) 153–164

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Titanium dioxide nanoparticles enhance mortality of fish exposed to bacterial pathogens

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ARTICLE INFO

Article history:
Received 10 January 2015
Received in revised form
2 April 2015
Accepted 7 April 2015
Available online 14 April 2015

Keywords:
Titanium dioxide
Nanoparticles
Immune response
Disease resistance
Fish
Bacteria
Histopathology

ABSTRACT

Nano-TiO_2 is immunotoxic to fish and reduces the bactericidal function of fish neutrophils. Here, fathead minnows (*Pimephales promelas*) were exposed to low and high environmentally relevant concentration of nano-TiO_2 (2 ng g^{-1} and 10 μg g^{-1} body weight, respectively), and were challenged with common fish bacterial pathogens, *Aeromonas hydrophila* or *Edwardsiella ictaluri*. Pre-exposure to nano-TiO_2 significantly increased fish mortality during bacterial challenge. Nano-TiO_2 concentrated in the kidney and spleen. Phagocytosis assay demonstrated that nano-TiO_2 has the ability to diminish neutrophil phagocytosis of *A. hydrophila*. Fish injected with TiO_2 nanoparticles displayed significant histopathology when compared to control fish. The interplay between nanoparticle exposure, immune system, histopathology, and infectious disease pathogenesis in any animal model has not been described before. By modulating fish immune responses and interfering with resistance to bacterial pathogens, manufactured nano-TiO_2 has the potential to affect fish survival in a disease outbreak.

1. Introduction

From 1916 to 2011, an estimated 165,050,000 metric tonnes of titanium dioxide (TiO_2) pigment (nano and bulk combined) were produced worldwide (Jovanović, 2015). Nano-TiO_2 is used as a constituent in personal, household, and food products. As an ingredient in food products nano-TiO_2 has an estimated human consumption of 1 mg kg^{-1} body weight per day (Weir et al., 2012). Nano-TiO_2 is also considered as an additive of drinking water in water treatment plants in a protocol for the removal of arsenic from water (EPA., 2010). The most frequent predicted concentration of nano-TiO_2 in surface water is 21 ng L^{-1} (Gottschalk et al., 2009), while the highest potential concentration is 16 μg L^{-1} (Mueller and Nowack, 2008). The nano-TiO_2 concentration of waste water effluent is documented in the μg L^{-1} range (Gottschalk et al., 2009; Kiser et al., 2009; Westerhoff et al., 2011). However, in urban runoff this concentration can be as high as 0.6 mg L^{-1} (Kaegi et al., 2008), and in raw sewage up to 3 mg L^{-1} of nano-TiO_2 has been detected (Kiser et al., 2009; Westerhoff et al., 2011). Nano-TiO_2 can be absorbed by the gills, skin, and intestine of fish, although the highest potential uptake is through diet (Handy et al., 2008). The experiments with perfused intestines of fish demonstrated TiO_2 uptake across the intestine both for the nano-TiO_2 and its bulk counterpart with average particle aggregates diameter of up to 1124 ± 331 nm (Al-Jubory and Handy, 2013). Although nano-TiO_2 is classified as a non-bioaccumulative substance in the fish embryos with the bioconcentration factor (BCF) < 100 (López-Serrano Oliver et al., in press), it is still present in the juvenile and adult fish body upon exposure (Ates et al., 2013; Fouqueray et al., 2013) with BCF of 181 (Zhu et al., 2010). Another study with adult fish determined BCF in the range 600–700, indicating possible increase of risk (Zhang et al., 2006). Since nano-TiO_2 can be transferred via the trophic food chain to fish (Fouqueray et al., 2013; Zhu et al., 2010), and although observed biomagnification factor is < 1, this suggests that

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http://dx.doi.org/10.1016/j.envpol.2015.04.003

the fish can internalize nano-TiO_2 on a daily basis through diet leading to chronic exposure (Zhu et al., 2010).

Nano-TiO_2 has a strong bactericidal effect and can kill fish pathogens *in vitro* (Cheng et al., 2008, 2009). Therefore, addition of nano-TiO_2 to the water of fish farms has been recommended in order to prevent or mitigate bacterial disease outbreaks (Cheng et al., 2008). However, methods that are successfully used for bacterial killing *in vitro* are frequently not efficient when applied to *in vivo* bacterial killing, due to the differences in the intracellular environment and the specific antibacterial function of phagocytic cells (Segal, 2005). It was recently demonstrated that nano-TiO_2 acts as a strong immunomodulator of fish neutrophil function (Jovanović et al., 2011). Cell-mediated immunity and the phagocytic cells are the primary targets of nano-TiO_2 immunotoxicity in aquatic animals. Immunotoxicity is manifested through lysosomal destabilization, frustrated phagocytosis, and change in function of the phagocytic cells (Jovanović and Palić, 2012).

Aeromonas hydrophila is a Gram-negative motile rod and one of the most important bacterial pathogens of aquatic animals in temperate waters (Angka, 1990; Esteve et al., 1993). *A. hydrophila* infection causes a systemic disease resulting in dermal ulceration, tail or fin rot, ocular ulceration, and erythrodermatitis, which leads to the descriptive disease appellations of "hemorrhagic septicemia", "red sore disease", "red rot disease", and "scale protrusion disease", among others (Cipriano, 2001). In the acute form of disease, rapid septicemia is the most common cause of mortality (Cipriano, 2001). Pathogenic mechanisms include the production of a cytotoxic enterotoxin, a type 3 secretion system, hemolysins, and an exotoxin (Grim et al., 2013), along with cytotoxic and haemolytic activities of the bacterial extracellular polysaccharides (Rodríguez et al., 2008), which collectively have lethal effects on renal tubular epithelium, precipitating acute renal failure. It is important to note that *A. hydrophila* is a member of the normal intestinal flora of healthy fish (Trust et al., 1979). The presence of the bacteria itself in fish does not indicate the disease *per se* and stress is often considered to be a contributing factor in disease outbreaks caused by *A. hydrophila* (Cipriano, 2001).

Edwardsiella ictaluri is a Gram-negative rod from Enterobacteriaceae family. It is the causative agent of Enteric Septicemia Disease that affects a variety of fish species (Baxa et al., 1990). Clinical signs, apart from signs of generalized systemic bacterial infection, include the presence of an open ulcer on the frontal bone of the skull between the eyes, and intradermal petechial hemorrhage of the jaws (Miyazaki and Plumb, 1985). The infection is initiated by transport of bacteria from the environment through the olfactory sac to the brain, with subsequent systemic dissemination of bacteria, causing generalized septicemic infection (Miyazaki and Plumb, 1985). During the infection, *E. ictaluri* may overcome phagocytic activities of neutrophils and other granulocytic cells, and multiplies intracellularly in foci of inflammation (Miyazaki and Kaige, 1985). Therefore, previously observed suppression of fish neutrophil function caused by nano-TiO_2 (Jovanović et al., 2011) has the potential to favor non-bactericidal phagocytosis of *E. ictaluri*.

Nano-TiO_2 is immunotoxic to fish and changes the function of fish neutrophils *in vivo*. After exposure of fathead minnows to 10 μg/g body weight of nano-TiO_2 for 48 h, respiratory burst, degranulation of primary granules, and neutrophil extracellular trap (NET) release were significantly reduced (Jovanović et al., 2011). The potential of nano-TiO_2 to interfere with resistance to infectious disease as a consequence of the ability to modulate immune responses has not been studied, and there are no available reports addressing possible outcomes of nanoparticle pre-exposure followed by bacterial challenge. The aim of this study was to determine if the outcome of bacterial challenge would be more severe in fish that are exposed to environmentally relevant concentrations of nano-TiO_2, as compared with bacterial-challenged fish without prior exposure to nano-TiO_2. Our hypothesis was that fish exposed to nano-TiO_2 would have higher morbidity and mortality than non-exposed fish after challenge with *A. hydrophila* and *E. ictaluri*.

2. Materials and methods

2.1. Animal care

Fathead minnows (*Pimephales promelas*) with average weight 2.5 ± 0.5 g were maintained in the Iowa State University, College of Veterinary Medicine, Ames, Iowa, USA. Fish were housed in a water recirculation system supplied with dechlorinated tap water at 20 °C in 120 L tanks, and fed twice daily with live brine shrimp larvae and dried flake food. Fish were cared for in accordance with approved Iowa State University animal care guidelines.

2.2. Bacterial culture

A. hydrophila (fish pathogen group, outbreak strain, USDA), and *E. ictaluri* (fluorescent transformed strain 93–146 pAKgfp1 (Karsi and Lawrence, 2007)) were plated on trypticase soy agar (TSA) with 5% of sheep blood plates and incubated at 37 °C overnight (*A. hydrophila*) or at 27 °C for two days (*E. ictaluri*). Morphologically distinct colonies were selected and placed in trypticase soy broth in a sterile tube. Cultures of *A. hydrophila* or *E. ictaluri* were incubated at 37 °C or 27 °C, respectively, to achieve logarithmic growth. The optical density of the broth culture was measured spectrophotometrically at 450 nm. Using a previously determined growth curve, colony forming unit (CFU) was determined based on optical density. After diluting the cultures with Hank's Balanced Salt Solution without Ca, Mg and Phenol Red (HBSS) to obtain the desired CFU, they were used immediately for intraperitoneal (i.p.) injections. To confirm the actual CFU used for bacterial challenge, the diluted cultures were plated on TSA sheep blood plates and enumerated.

2.3. Nanoparticle characterization

Nano-TiO_2 (anatase, nanopowder, < 25 nm, 99.7% purity; Sigma–Aldrich Corp, St. Louis, MO, USA) was used in all experiments. Nano-TiO_2 was suspended in sterile HBSS, pH = 7.3. The suspensions of nanoparticles were used as non-filtered or were filtered through a 220 nm general purpose filter. The non-filtered nano-TiO_2 suspension contained particles with average aggregate diameter of 585 nm, average zeta potential of −16.4 mV, and conductivity of 16 mS cm^{-1}. Polydispersity index (PDI) was 0.21. After filtration, the aggregate size had an average diameter of 86 nm, zeta potential of −8.87 mV, and conductivity of 15.4 mS cm^{-1} as determined by dynamic light scattering (DLS) technique with Malvern Zetasizer Nano ZS-90 instrument (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Since the fish were later injected with the 24 h aged suspension (concentration had to be analytically verified first) DLS measurements were also performed on a 24 h aged suspension. Prior to measurements suspension was sonicated for 10 min in a benchtop portable sonicator. The detailed characterization of the nano-TiO_2 is provided in the Supplementary Information.

2.4. Nano-TiO_2 accumulation in fish tissues

To determine the accumulation of nano-TiO_2 in fish organs, fish were injected i.p. with 10 μg g^{-1} body weight with non-filtered nano-TiO_2 suspension in HBSS. Negative control was injected

with HBSS and both groups fed *ad libitum* for 48 h. After 48 h fish were euthanized with an overdose of tricaine methane sulphonate (MS-222, Argent Laboratories, Redmond WA, USA) and kidney, spleen, and liver were each dissected from three individuals per treatment and control group. This time period was chosen to demonstrate that the administered nano-TiO_2 was present in the body at the beginning of the bacterial challenge study, as well as to provide information on biodistribution. Inductively coupled plasma mass spectrometry (ICP-MS) was performed according to our previously established methodology (Jovanović and Guzmán, 2014) with some minor modifications. Briefly, organs were weighed and digested in nitric acid. In addition, five whole individuals per group were digested with nitric acid. After digestion, the concentration of Ti isotopes 47 and 49 were measured with ICP-MS using a scandium (Sc) internal standard ($m/z = 45$). The preparation standard used for this analysis was created by spiking blank samples with Ti and Sc. Results were corrected for the natural abundance of Ti isotopes, averaged between the two Ti isotopes measured, and converted to TiO_2 concentration as $\mu g\ g^{-1}$ of sample weight.

2.5. Nano-TiO_2 treatment and challenge with *A. hydrophila*

To investigate the effects of *in vivo* application of nano-TiO_2 on the ability of the immune system to resolve bacterial infection, fish were anesthetized with 100 mg L^{-1} of aerated and buffered (sodium bicarbonate, pH 8.0) solution of the MS-222. Upon entering the third stage of anesthesia (Palić et al., 2006), fish were weighed and injected i.p. with sterile preparations of nanoparticles as described above or with HBSS. The fish were randomly divided into five groups (two nanoparticle treatment groups, and three control groups).

The first treatment group was injected with 10 $\mu g\ g^{-1}$ body weight of non-filtered nano-TiO_2 suspended in HBSS as a standard accepted approach for toxicological, disease challenge, and immunological studies in various species (Janeway et al., 2008). The second treatment group was injected with nano-TiO_2 suspended in HBSS and filtered through 220 nm general purpose filter to remove large aggregates. The concentration of nano-TiO_2 after the filtration was determined with ICP-MS and fish were administered nano-TiO_2 with a final concentration of 2 ng g^{-1} body weight. The high dose of 10 $\mu g\ g^{-1}$ is close to the concentration and particle size of nano-TiO_2 present in raw sewage (Kiser et al., 2009; Westerhoff et al., 2011). The low dose of 2 ng g^{-1} that was administered to the experimental fish is close to the estimated concentration of nano-TiO_2 in surface water (Mueller and Nowack, 2008). The low dose of 2 ng g^{-1} is, also, 10× less than the concentration of nano-TiO_2 present in treated effluent of waste water treatment facilities (Kiser et al., 2009; Westerhoff et al., 2011). Concomitantly, the low dose of 2 ng g^{-1} is 500× less than an estimated human consumption of 1 mg kg^{-1} body weight per day (Weir et al., 2012). It is however important to note that administered nano-TiO_2 concentration is the internal concentration in fish, not external concentration in the environment. In the aquatic ecosystem, fish internal and external (environmental) concentration of nano-TiO_2 will not necessarily be equal as this depends on many factors. Furthermore, although the selected internal concentrations correspond to externally environmentally relevant concentrations, the delivery method (i.p.) is not environmentally relevant.

Two control groups were included: one group of fish was injected with 10 $\mu L\ g^{-1}$ body weight of HBSS; and a no-injection control group was also included. All treatment and control groups had 30–32 individual fish per group. After the injections, fish were transferred to 38 L tanks, and fed to satiation for 48 h. We have previously determined that 48 h after the administration of nano-TiO_2 to fathead minnows, their neutrophil function is diminished (Jovanović et al., 2011). After the 48 h incubation period, fish from two treatment groups and one control group (bacteria control) were anesthetized again, as described above, and injected i.p. with 10 μL per gram body weight of 5.5×10^7 CFU mL^{-1} of live *A. hydrophila* culture suspension (previously determined to cause 10–15% mortality). Fish of the negative control group were injected with 10 μL of sterile soy broth g^{-1} body weight.

After the injections, fish were returned to their 38 L tanks and fed to satiation for 21 days. Mortality events were recorded twice per day (every 12 h) to the end of experiment. Dead fish were sampled for bacterial culture and observed by gross examination of the skin, peritoneal cavity, and kidney. In all instances, a culture of live *A. hydrophila* colonies was isolated on TSA sheep blood plates from fish that died before the end of the experiment.

In a subsequent experiment, *A. hydrophila* cultures were killed by exposure to 60 °C for 30 min. Inactivation was confirmed as no colonies grew on TSA sheep blood agar. Experimental design and injections of fish were performed as described above.

2.6. Nano-TiO_2 treatment and challenge with *E. ictaluri*

Experimental setup for *E. ictaluri* was the same as the one described for *A. hydrophila*. Two concentrations of live *E. ictaluri* (10 μL per gram body weight of 2.2×10^6 CFU mL^{-1} and 4.4×10^6 CFU mL^{-1}) were administered. The challenge lasted for 28 days. Mortality events and confirmation of an active infection were recorded as above. In all instances of fish deaths, live and fluorescent *E. ictaluri* were detected by fluorescent microscopy.

2.7. Phagocytosis assay

Phagocytosis of *A. hydrophila* was determined by flow cytometric detection of fluorescent bacteria in neutrophils. Fluorescein isothiocyanate (FITC) labeling of *A. hydrophila* was performed following the previously described method (White-Owen et al., 1992). Briefly, bacteria were grown in trypticase soy broth together with 50 $\mu g\ mL^{-1}$ FITC (Sigma F7250) at 37 °C overnight. Following two washes with PBS, bacteria were heat-killed at 60 °C for 30 min. Prior to killing, bacteria were plated on blood agar to confirm CFU. Heat-killing of bacteria was also confirmed by plating on blood agar. The bacterial pellet was resuspended with trypticase soy broth, divided into aliquots, and stored at 4 °C until used.

For the phagocytosis assay, a suspension of neutrophils was prepared as previously described (Palić et al., 2005) and adjusted to 1×10^7 cells mL^{-1}. Neutrophil suspensions were made from each experimental group, each containing pooled neutrophils from the anterior kidney of 10 randomly selected *P. promelas*. Cell suspensions (25 μL) were added to the wells of 96-well plate containing 50 μL of 3% fetal bovine serum (FBS), and 50 μL of non-filtered nano-TiO_2 in HBSS (final concentration in the wells of 333 $\mu g\ mL^{-1}$) or HBSS (negative control). After 1 h incubation at the room temperature, 25 μL of FITC-labeled and opsonized *A. hydrophila* at concentration of 1×10^9 CFU mL^{-1} in 5% carp serum (Common carp serum, courtesy of Wisconsin Department of Natural Resources, Yellowstone Lake, WI) were added to the wells. Additional wells without bacteria were prepared as controls. The whole experimental setup was performed in duplicate. Plates were centrifuged at 400 × g for 2 min and incubated at room temperature for 2 h. After incubation, plates were washed twice and centrifuged at 430 × g for 1 min, supernatant was discarded and cell pellets were resuspended with 1% paraformaldehyde. Flow cytometry data were acquired by a FACSCanto flow cytometer (BD

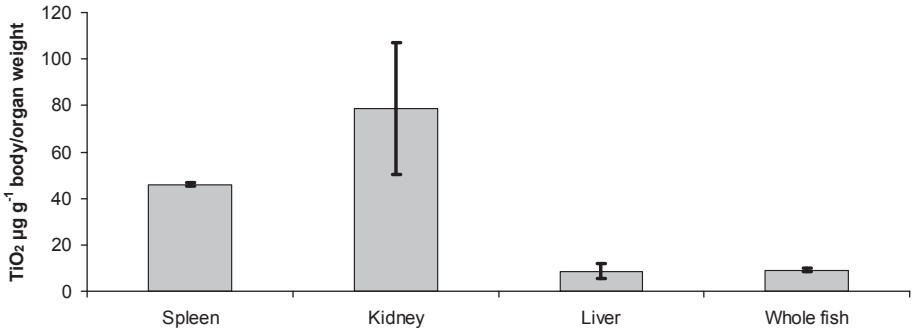


Fig. 1. Distribution of nano-TiO_2 in *P. promelas* organs 48 h after i.p. injection of non-filtered nano-TiO_2 10 μg g^{-1} body weight. Concentration was determined by ICP-MS. N = 5. Bars represent standard error of the mean.

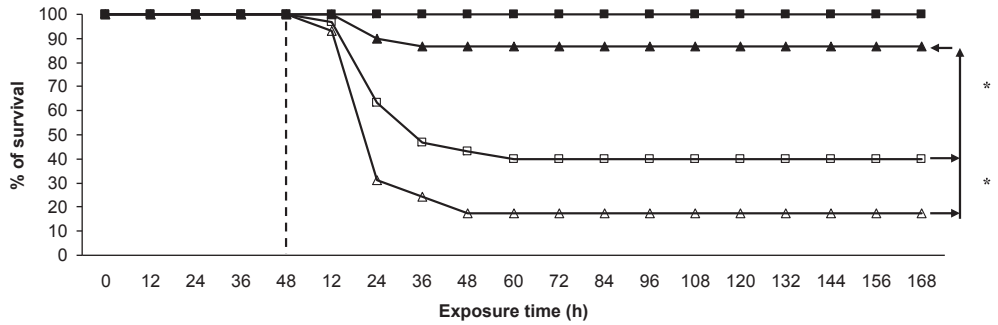


Fig. 2. Cumulative mortality rate of *P. promelas* exposed to nano-TiO_2 and challenged with *Aeromonas hydrophila*. Filled squares represent the negative control (HBSS without Ca, Mg and Phenol Red with soy broth); Filled triangles represent the bacteria control (HBSS without Ca, Mg and Phenol Red + 5.5 × 10^7 CFU mL^{-1} of live *A. hydrophila*); Empty squares represent a group treated with filtered nano-TiO_2 (2 ng g^{-1} body weight + 10 μL per gram body weight of 5.5 × 10^7 CFU mL^{-1} of live *A. hydrophila*); Empty triangles represent a group treated with non-filtered nano-TiO_2 (10 μg g^{-1} body weight + 10 μL per gram body weight of 5.5 × 10^7 CFU mL^{-1} of live *A. hydrophila*). At 0 h time point fish were anaesthetized and injected i.p. with either HBSS or nano-TiO_2 (treatment or control, respectively). The dashed line at 48 h time point indicates administration of *A. hydrophila* and reset of x axis to 0 h of exposure to *A. hydrophila*. The experiment was run for 21 days. Since no changes in survival rate occurred at a later time point, the last time point presented on x-axis is 168 h * indicates that the effect of nano-TiO_2 on survivability rate is statistically significant when compared with bacteria control (Chi − Square, $P < 0.05$).

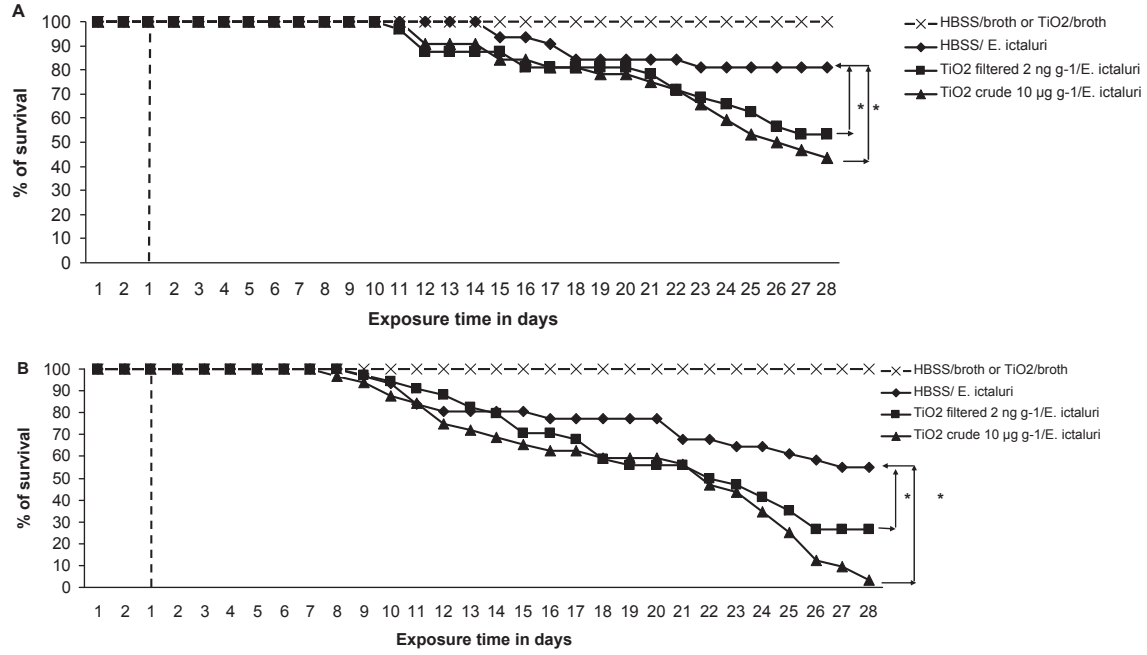


Fig. 3. Cumulative mortality rate of *P. promelas* exposed to nano-TiO_2 and challenged with 10 μL per gram body weight of 2.2 × 10^6 CFU mL^{-1} (A), and 4.4 × 10^6 CFU mL^{-1} (B) of live *E. ictaluri*. At 0 h time point, fish were anaesthetized and injected i.p. with either sterile preparations of HBSS or nano-TiO_2 (control or treatment, respectively). The dashed line at the day 2 time point indicates administration of *E. ictaluri* and reset of x axis to 0 h of exposure to *E. ictaluri*. * indicates that the effect of nano-TiO_2 on survivability rate is statistically significant when compared with bacteria control.

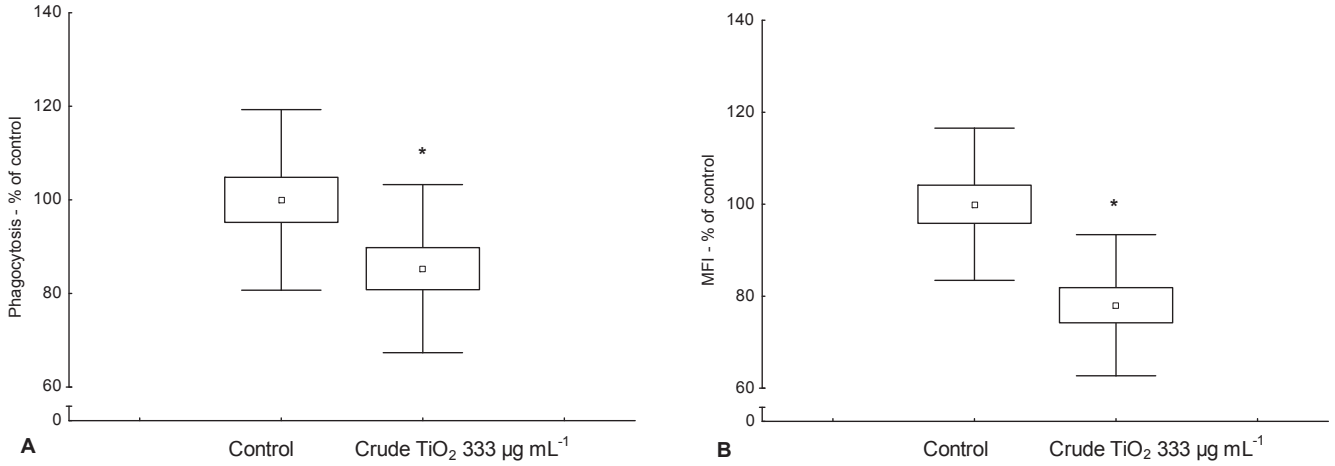


Fig. 4. Phagocytosis of *A. hydrophila* by *P. pomephales* neutrophils *in vitro* (A), and mean fluorescent intensity (MFI) of phagocytosed bacteria (B). Neutrophils were pretreated for 1 h with HBSS (control) or nano-TiO_2 prior to phagocytosis assay. * indicates that the effect of nano-TiO_2 is significant compared with control (t-test, $P < 0.05$).

Biosciences, San Jose, CA) and data were analyzed with FlowJo version 9.4.11 software. The phagocytic activity was reported as the percentage of neutrophils that had performed phagocytosis, and as mean fluorescence intensity (MFI) of phagocytosis-positive cells. In addition to *in vitro* evaluation, an *ex vivo* validation of phagocytosis was also performed. Fish were anaesthetized and injected i.p. with 10 µg g^{-1} body weight of non-filtered nano-TiO_2 and 2 ng g^{-1} body weight of filtered nano-TiO_2 suspended in HBSS. The control group was injected with HBSS. After 48 h, fish were euthanized and neutrophils were extracted as described above. For *ex vivo*

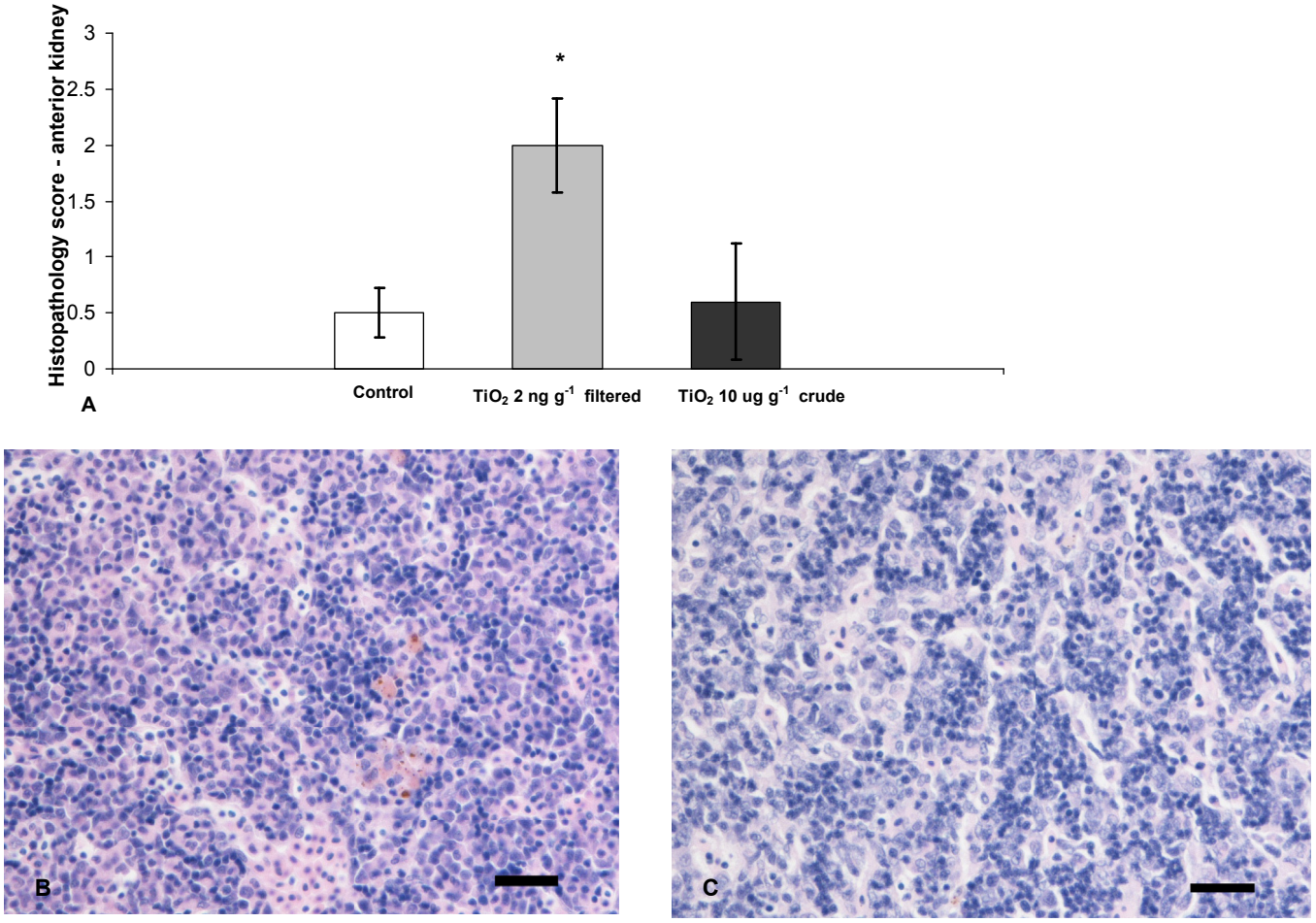
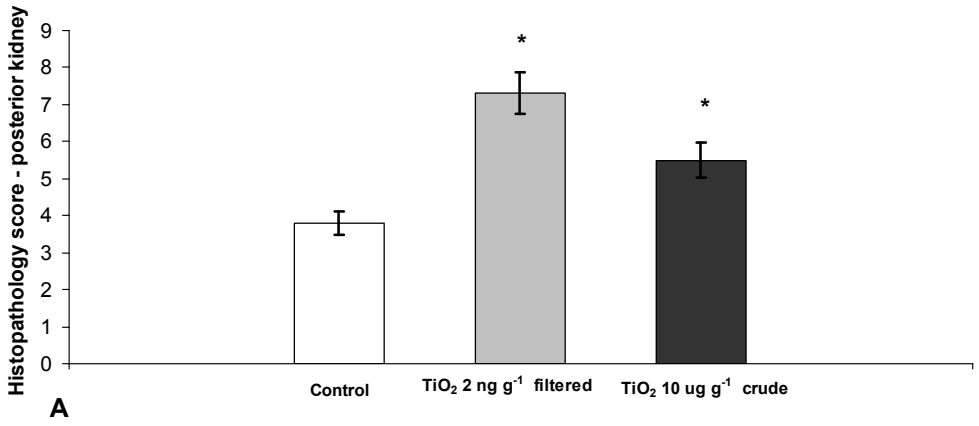
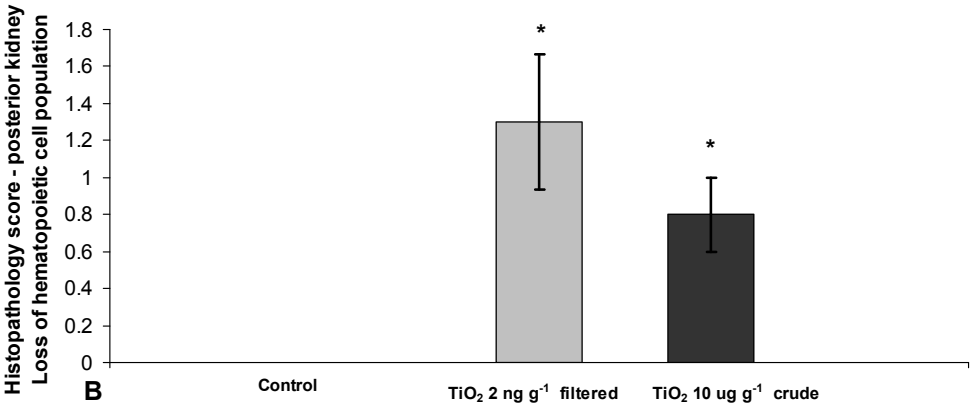
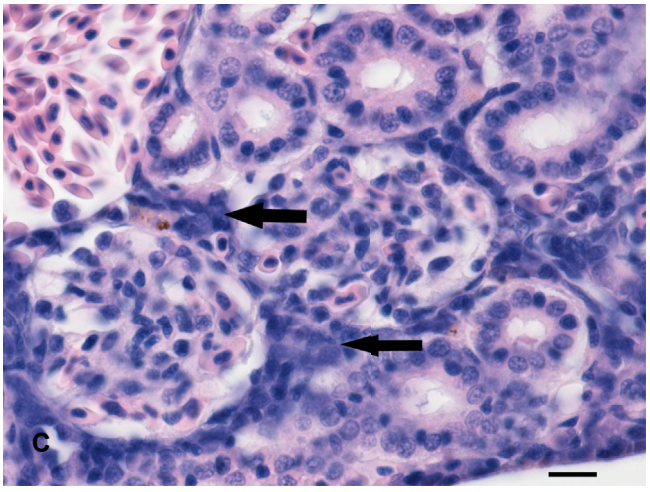
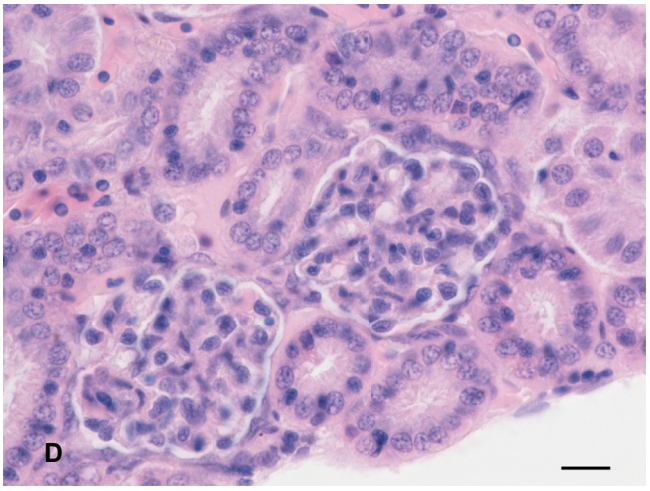


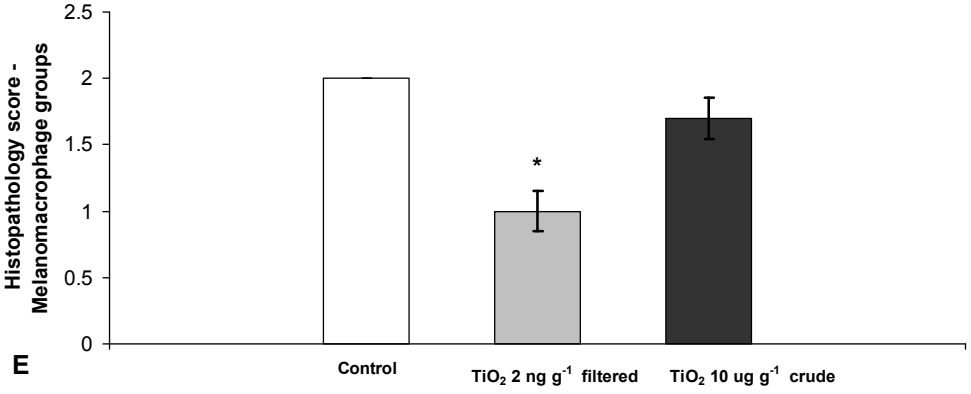
Fig. 5. Histologic changes in the anterior kidney associated with *in vivo* exposure of fathead minnows (*P. promelas*) to TiO_2 nanoparticles. Significant differences (ANOVA, $P < 0.05$) are present in the semi-quantitative histologic scores of the anterior kidney of fish injected with nano-TiO_2 (A). * indicates significant difference from the control group. Histopathology of filtered (2 ng/g) TiO_2 anterior kidney (B) and control (non-injected) (C). The scale bar is 5 microns in length.

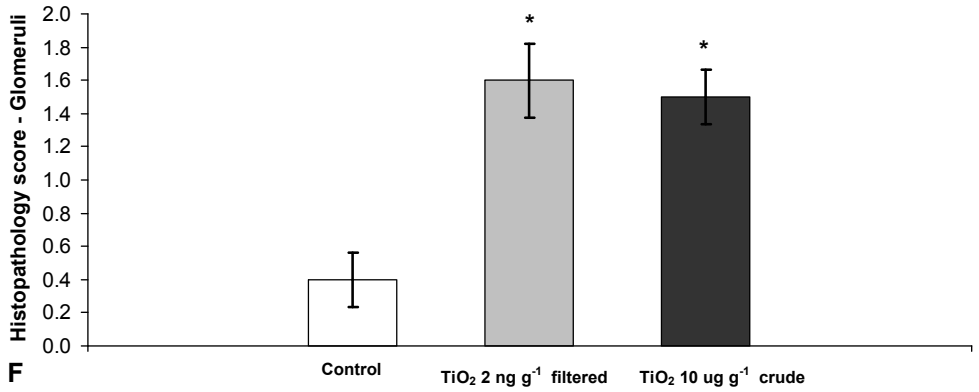

Histopathology score - posterior kidney
9
8
7
6
5
4
3
2
1
0
*
*
Control
TiO2 2 ng g-1 filtered
TiO2 10 ug g-1 crude
A

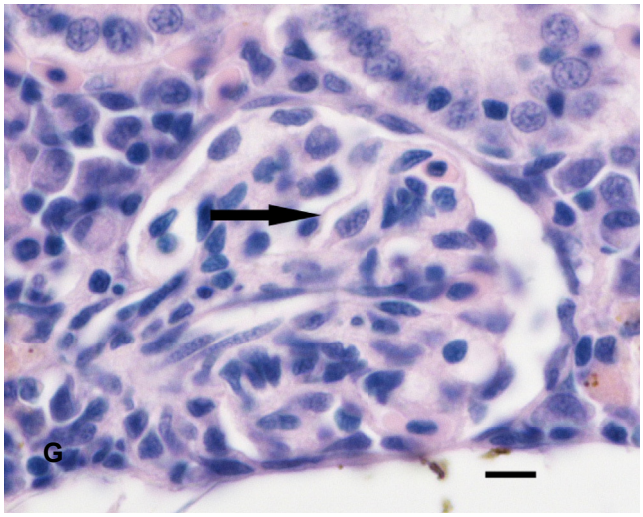

Histopathology score - posterior kidney
Loss of hematopoietic cell population
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
TiO2 2 ng g-1 filtered
TiO2 10 ug g-1 crude
B


C


D


Histopathology score -
Melanomacrophage groups
2.5
2
1.5
1
0.5
0
*
Control
TiO2 2 ng g-1 filtered
TiO2 10 ug g-1 crude
E





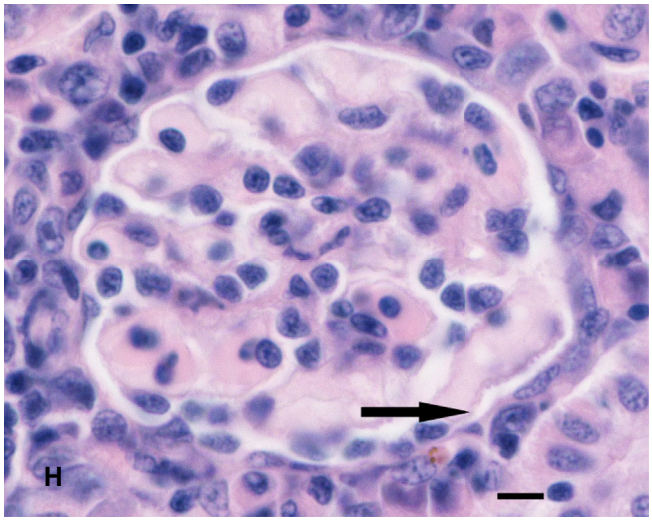


Fig. 6. (*continued*).

validation, kidneys of four fish from the same experimental group were pooled as a single sample and phagocytosis levels were determined for 4–7 samples per experimental/control group.

2.8. Histopathology

Under anesthesia, fish were administered i.p. filtered or non-filtered sterile preparations of nano-TiO_2, as described above. After 72 h, randomly selected fish (n = 10) from experimental and control groups were euthanized and immediately fixed in 10% neutral buffered formalin. Bodies were sectioned sagittally, placed in processing cassettes, demineralized for 18 h, and processed for routine paraffin embedding. Tissue sections were cut at 6 microns, with most blocks requiring at least two levels of sectioning to allow evaluation of major organs. Results were evaluated by a ACVP board-certified veterinary pathologist masked to treatments, using a modified scoring system as previously described (Jovanović et al., 2014), with additional criteria for morphologic changes in anterior and posterior kidney, including glomerular morphology, interstitial infiltration of neutrophils, and presence of pigment. In the glomerulus, thickening of capillary walls, capillary dilation and expansion of the mesangium were scored as no increase (0), mild (1), moderate (2), or severe (3). In the interstitium of the kidney, infiltration by neutrophils was scored as none (0), mild (scattered neutrophils) (1), moderate (2), or severe (broad sheets of neutrophils) (3). The presence of interstitial, intravascular, or intraepithelial rodlet cells was scored as none (0), 1–3 per 400× field (1), 4–8 per 400× field (2), and >9 per 400× field (3). Melanomacrophage groups in the posterior kidney were scored as number of groups in a 400× field, with no groups (0), one group (1), two groups (2), and three groups (3), with volume estimation when melanomacrophages were dispersed.

2.9. Ultrastructural pathology

Fish were injected i.p. with non-filtered or filtered, sterile suspensions of nano-TiO_2 or HBSS, and fed *ad libitum* for 72 h as above. Immediately after euthanasia, fish were necropsied and samples of

Fig. 6. Histologic changes in the posterior kidney associated with *in vivo* exposure of *P. promelas* to TiO_2 nanoparticles. Significant differences (ANOVA, $P < 0.0001$) are present in the semi-quantitative histologic scores of the posterior kidney of fish injected with filtered or non-filtered nano-TiO_2 (A). Nano-TiO_2 exposure resulted in reduced numbers of hematopoietic cells in the interstium (B). Note the significant differences in hematopoietic cell populations (black arrows in C, control fish) from the interstitium of the posterior kidney (filtered TiO_2, D). In each photomicrograph, bar size = 5 microns. Populations of melanomacrophages were reduced in the renal interstitium of fish receiving filtered nano-TiO_2, often with smaller or disseminated groups of cells (E). Significant differences in the glomerular morphology, after administration of nanoparticles, with mesangial edema and thickening of the glomerular capillary loop walls are presented in F, G (control) and H (filtered nano-TiO_2). * indicates significant difference from the control group.

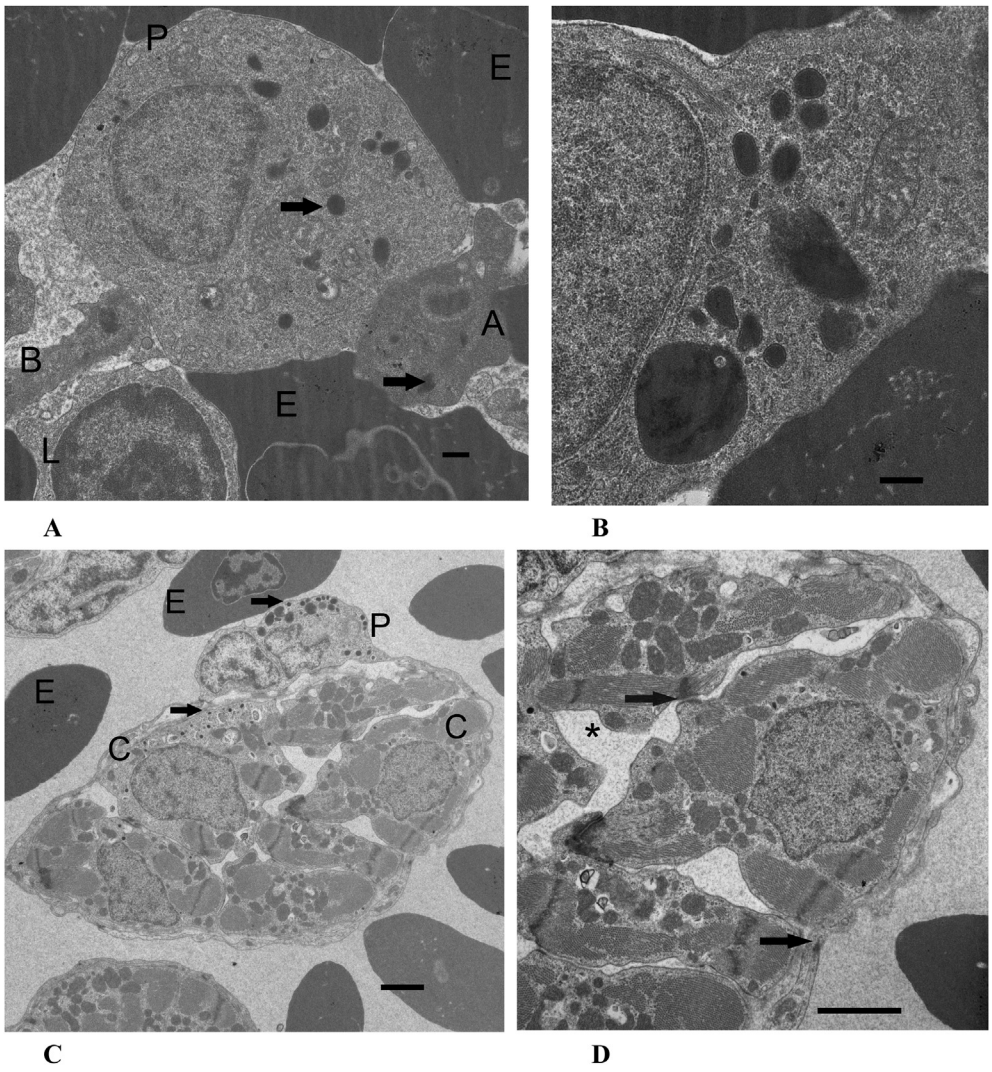


Fig. 7. Ultrastructural changes in the heart associated with *in vivo* exposure of *P. promelas* to TiO_2 nanoparticles. A phagocyte (P) in the atrium of a fish exposed to non-filtered TiO_2 contains electron-dense material (A and B) with two closely apposed, degenerating cells with electron-dense material (A and B), a lymphocyte (L), and many erythrocytes (E) in close proximity to the phagocyte in A. An atrial phagocyte (P) lining a fibromuscular trabecular and a cardiomyocyte (C) contain similar electron-dense material (C). In panels A and C, arrows point to electron dense material consistent with nano-TiO_2 morphology (Geiser and Kreyling, 2010; Mühlfeld et al., 2008). There is expanded space between cardiomyocytes (asterisk) and disruption of intercellular junctions (arrows) in D. Bars in A, B, D = 500 nm and C = 2 microns.

heart and anterior kidney were immersed in 2.5% glutaraldehyde in 0.1 M-cacodylate buffer at 4^{O}C. Fixed samples were rinsed in cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated in alcohols, cleared in propylene oxide, and embedded in epoxy resin. Areas of interest in the atrium and anterior kidney were selected by light microscopic examination of 1-micron thick, toluidine blue-stained, semi-thin sections, and ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined with a FEI Tecnai 12 Biotwin transmission electron microscope (FEI, Hillsboro, OR).

2.10. Statistics

The statistical difference in mortality rate between groups was calculated using the Chi–Square Test. The differences in nano-TiO_2 distribution among organs and in histopathology scores among treatments were evaluated with Analysis of Variance (ANOVA) or Kruskal–Wallis test, if the population was not in Gaussian distribution, followed by Tukey–Kramer or Dunn's multiple comparison tests, respectively. Phagocytosis assay data were subjected to the Dunnett's test procedure or Student t-test where applicable.

3. Results

3.1. Nano-TiO_2 accumulation in fish tissues

ICP-MS analysis confirmed the injected concentration of 10 μg g^{-1} body weight. After 48 h of exposure whole-body concentration was 9.0 ± 0.8 (mean ± standard error of the mean – SEM) μg g^{-1} body weight of TiO_2. TiO_2 had the highest bioconcentration in kidney (78.7 ± 28.3 μg g^{-1} kidney), followed by spleen (46 ± 0.9 μg g^{-1} spleen), and liver (8.6 ± 3.2 μg g^{-1} liver) (Fig. 1). The bioconcentration of nano-TiO_2 in kidney and spleen was significantly higher than in the whole body ($P < 0.01$ and $P < 0.05$, respectively). In contrast, the concentration measured in the liver was not statistically different from the average concentration of the whole body. In the control samples that did not receive any injections of nano-TiO_2, TiO_2 concentration was below the detection limit of the instrument (0.1 μg L^{-1}).

3.2. Challenge with A. hydrophila

The bacteria control (fish that received HBSS and then were

challenged with *A. hydrophila*) had cumulative mortality of 13.5% (Fig. 2). The fish in the group that was pre-treated with 2 ng g^{-1} body weight of filtered nano-TiO_2 and challenged with *A. hydrophila* had 60% cumulative mortality. The fish in the group pre-treated with 10 μg g^{-1} body weight of non-filtered nano-TiO_2 and challenged with *A. hydrophila* had 82.5% of cumulative mortality. Both filtered and non-filtered nano-TiO_2 treatments, followed by *A. hydrophila* challenge, caused statistically significant increase in mortality comparing to the bacteria control (not exposed to nano-TiO_2; challenged with *A. hydrophila*) (Chi–Square, $P < 0.05$). There were no mortalities observed in fish from nano-TiO_2 control (nano-TiO_2 treated; no bacterial challenge) or sham-injection control groups. Visual examination of fish that were pre-treated with nano-TiO_2 and challenged with *A. hydrophila* revealed development of larger and more severe hemorrhages than in the fish that were subjected to *A. hydrophila* challenge only. In fish injected with heat-killed *A. hydrophila,* there were no mortalities observed in any of the treatment or control groups.

3.3. Challenge with E. ictaluri

Bacteria controls treated with 10 μL per gram body weight of 2.2×10^6 CFU mL^{-1}, and 4.4×10^6 CFU mL^{-1} of live *E. ictaluri* had cumulative mortality of 19% and 45% respectively. The fish in the groups that were pre-treated with 2 ng g^{-1} body weight of filtered nano-TiO_2 and challenged with 2.2×10^6 CFU mL^{-1}, and 4.4×10^6 CFU mL^{-1} *E. ictaluri* had significantly (Chi–Square, $P < 0.05$ and $P < 0.01$ respectively) higher cumulative mortality rate of 47% and 74% respectively, as compared with bacteria control (Fig. 3A). The fish in the groups pre-treated with 10 μg g^{-1} body weight of non-filtered nano-TiO_2 and challenged with 2.2×10^6 CFU mL^{-1} or 4.4×10^6 CFU mL^{-1} *E. ictaluri* had significantly higher cumulative mortality rate of 56% and 97% respectively, as compared with bacteria control (Chi–Square, $P < 0.01$ and $P < 0.001$ respectively) (Fig. 3B). There were no mortalities in nano-TiO_2 (nano-TiO_2 treated; no bacterial challenge) and no injection control groups.

3.4. Phagocytosis assay

Exposure of neutrophils for one hour to nano-TiO_2 results in significantly reduced phagocytosis *in vitro*. The percentage of neutrophils that have performed phagocytosis of *A. hydrophila* was 15% lower (Fig. 4A) in nano-TiO_2 group as compared with the control (t-test, $P < 0.05$). MFI was lower by 22% (t-test, $P < 0.05$), indicating that the neutrophils that have performed phagocytosis in nano-TiO_2 group have phagocytized on average 22% fewer bacteria per neutrophil (Fig. 4B). *Ex vivo* validation revealed the same pattern of reduction in phagocytosis rate 48 h after exposure to nano-TiO_2. In the control group 44.3 ± 3.5% (mean ± SEM) of neutrophils were phagocytic while only 36.1 ± 2.9% and 24.4 ± 0.5% of neutrophils from fish exposed to 10 μg g^{-1} body weight of non-filtered nano-TiO_2 and 2 ng g^{-1} body weight of filtered nano-TiO_2, respectively, were capable of performing phagocytosis.

3.5. Histopathology

Semi-quantitative morphologic scoring of tissue changes in fish administered filtered or non-filtered nano-TiO_2, as compared with sham-injected (negative) controls, revealed significant differences in the anterior kidney and posterior kidney ($P < 0.01$ and $P < 0.0001$, respectively). In the anterior kidney, fish injected with filtered nano-TiO_2 often had increased numbers of neutrophils with abundant, homogeneous eosinophilic cytoplasm and mild to moderate loss of mononuclear cells with the morphology of macrophages and lymphocytes (Fig. 5). There was a mild increase of neutrophils or loss of mononuclear cells in the anterior kidney of fish exposed to non-filtered nano-TiO_2. In the posterior kidney, histologic changes, including mild to moderate interstitial congestion, reduction in melanomacrophage groups, increased numbers of neutrophils with abundant cytoplasm, and irregular thickening of capillary walls, dilated capillaries and/or expansion of the mesangium, contributed to statistically significant differences between fish exposed to filtered nano-TiO_2 compared to control (Fig. 6A). Among these histologic features in the posterior kidney, there were significantly reduced numbers of hematopoietic cells after exposure to filtered nano-TiO_2 (Fig. 6B, C, D). Also, there were significant differences in the glomerular morphology with administration of filtered or non-filtered nanoparticles, with mesangial edema thickening of the glomerular capillary loop walls (Fig. 6F). Populations of melanomacrophages were reduced in the renal interstitium of fish receiving filtered nano-TiO_2, often with smaller or disseminated groups of cells (Fig. 6E). Statistically significant differences were not observed in the brain, coelomic cavity, or liver.

3.6. Ultrastructural pathology

In fish exposed to non-filtered or filtered nano-TiO_2, many phagocytes lining the fibromuscular trabeculae of the atrium or admixed with atrial blood contain variably sized, intracytoplasmic aggregates of electron-dense material when compared to the control group (Fig. 7A). Some intracellular aggregates are membrane-bound, while a membrane was not observed to surround other aggregates of this material (Fig. 7B). This electron-dense material did not have the fine tubular ultrastructure of Weibel-Palade bodies, secretory organelles typical of endothelia (Valentijn et al., 2011), although Weibel-Palade bodies were present in some endothelial cells. The atrial trabeculae of fish exposed to a filtered preparation of nano-TiO_2 were markedly expanded by clear space between cardiomyocytes, consistent with edema (Fig. 7C), with disruption of many intercellular junctions between endothelial cells and between cardiomyocytes (Fig. 7D) and regional loss of myofibrillar architecture. Rarely, endocardial phagocytes with intracytoplasmic aggregates of electron-dense material were observed in a control fish.

4. Discussion

In vivo exposure of *P. promelas*, a common freshwater teleost, to environmentally relevant concentrations of nano-TiO_2 has important biological effects. TiO_2 bioaccumulated in the kidney and spleen, both critical organs involved in hematopoiesis and immune protection. Groups of fish pre-treated with nano-TiO_2 and subsequently exposed to common bacterial pathogens of fish, *A. hydrophila* or *E. ictaluri*, resulted in markedly increased mortality. Based on results, mechanisms of toxicity appear to target neutrophil function, hematopoiesis, and renal glomerular architecture. The results of this study are in accordance with previous research demonstrating the potential for nano-TiO_2-mediated immunotoxicity in fish (Jovanović and Palić, 2012). The reduced function of *P. promelas* neutrophils after exposure to nano-TiO_2 has been demonstrated previously (Jovanović et al., 2011).

Present study also demonstrated that the kidney is one of the major organs for nano-TiO_2 bioaccumulation in fish as soon as 48 h after parenteral administration, which is in accordance with previous investigations. Rainbow trout exposed to nano-TiO_2 by i.p. injection demonstrated renal bioaccumulation; with almost no clearance even 90 days post-exposure (Scown et al., 2009). Another recent study demonstrated that in rainbow trout up to 94% of TiO_2 concentrated in the kidney after intravenous injection (Boyle et al.,

2013). In fish, the kidney has a major role in hematopoiesis and serves as a neutrophil depot (Zapata, 1979). Therefore, the neutrophil population that is continuously produced and resides in the fish kidney can be chronically exposed to nano-TiO_2. Histopathologic examination of tissues from TiO_2-exposed fish support the storage of nanoparticles in renal neutrophils, as sheets of large cells with nuclear morphology consistent with neutrophils with a distended cytoplasmic compartment were observed in some fish exposed to filtered nano-TiO_2. The loss of mature and progenitor hematopoietic and immune system cells in the anterior and posterior kidneys in fish exposed to nano-TiO_2 represents another mechanism through which nanoparticles may cause an ineffective antibacterial response. Loss of hematopoietic and immune system cells in the fish kidney after exposure to nanoparticles appear to be a common histopatological feature after nanoparticle exposure, irrespective to the type of nanoparticles (Jovanović et al., 2014). Significant differences in the glomerular morphology with administration of filtered or non-filtered nanoparticles, with thickening of the glomerular capillary loop walls and mesangial edema were also observed by light microscopy in this study. The irregularly thickened exterior surfaces of glomerular capillary loops suggest pathology of the glomerular filtration barrier, which is composed of fenestrated capillaries, basal lamina of endothelial cells, and podocytes, with a charge- and size-selective sieve between podocytes foot processes (Tryggavason, 1999).

Phagocytes lining the atrial trabeculae have critical functions in removal of particulate matter in teleosts, similar to macrophage populations in mammalian lymph nodes (Nakamura and Shimozawa, 1994). The cardiac endothelium also functions as a semi-selective barrier between the plasma compartment and underlying contractile cardiomyocytes with a critical role in maintaining cardiac performance (Brutsaert, 2003). Many endocardial phagocytes in fish exposed to nano-TiO_2 in these experiments had ingested electron-dense particulate material (which in its appearance resembled nano-TiO_2) and had a rounded morphology with abundant cytoplasm, consistent with an activated phenotype. Since followup elemental analyses of those cells were not performed by electron spectroscopic imaging and parallel electron energy loss spectroscopy there is no definite proof that the observed electron-dense material is indeed TiO_2. However, the electron microscopy presentation of previously imaged nano-TiO_2 anatase in tissues (Geiser and Kreyling, 2010; Mühlfeld et al., 2008) is consistent with material observed in samples from nano-TiO_2 exposed fish (Fig. 7). In fish exposed to filtered nano-TiO_2, we observed separation of endocardial cells from the underlying trabeculae, with disruption of endothelial tight junctions as well as multifocal dissolution of intercellular junctions of the intercalated disk between cardiomyocytes. Disruption of the endothelial lining, accumulation of interstitial edema, and loss of cardiomyocyte communication in muscular trabeculae of the atrium is expected to potentiate intravascular coagulation and to reduce cardiac function. Scant electron-dense particulate matter in rare endothelial cells of control fish was much less than in TiO_2-treated fish and probably represents background environmental exposure.

Heat-inactivated *A. hydrophila* did not cause mortality in any of the experimental groups. This is consistent with previous findings (Rodríguez et al., 2008). The mechanism of *A. hydrophila* pathogenicity include the use of a type 3 secretion system and secreted toxins (Rosenzweig and Chopra, 2013), with both mechanisms requiring viable bacteria. Samples of heat-killed *A. hydrophila*, as used in this experiment, are expected to contain abundant lipopolysaccharide (LPS), because LPS is a component of the outer membrane of Gram-negative bacteria. While nano-TiO_2 can act as a carrier of LPS through protein corona formation (Ashwood et al., 2007), the absence of mortality in the experimental group exposed both to nano-TiO_2 and subsequently heat-killed *A. hydrophila* suggests that delivery of LPS by nano-TiO_2 to the kidney cells is not an important pathogenic mechanism. The observed increase in mortality with live *A. hydrophila* pretreated with nano-TiO_2 suggests direct immunotoxic effects of nano-TiO_2 on renal granulocytic population (Jovanović et al., 2011), as these cells are critical for successful anti-bacterial defenses.

Present study further demonstrated that the neutrophil phagocytosis rate of *A. hydrophila in vitro* was decreased by 15% after exposure to nano-TiO_2. On average, neutrophils that have performed phagocytosis had phagocytized 22% fewer individual bacteria per neutrophil after exposure to nano-TiO_2. We theorize that this reduction in phagocytosis is due to nanoparticle competition. Nano-TiO_2 is heavily internalized by immune cells through macropinocytosis (Bartneck et al., 2010); thus, there is a partitioning of available phagosomal and cytoplasmic space between nano-TiO_2 and bacteria, as reflected by the significant decrease in MFI we observed in association with nano-TiO_2 exposure. Treatment of freshwater invertebrates with various nanoparticles, including TiO_2, is known to reduce phagocytosis by immune cells (Couleau et al., 2012; Gagné et al., 2008). Cumulatively, results support the concept that nano-TiO_2 increases the pathogenenicity of *A. hydrophila* infection through loss of immune cell populations and reduced phagocytic function, resulting in immunosuppression and failure to mount an effective antibacterial response.

Infection by *E. ictaluri* yielded results similar to *A. hydrophila* challenge. Pretreatment with nano-TiO_2 significantly increased mortality. Although *E. ictaluri* is susceptible to phagocytosis by fish neutrophils, intracellular killing is not an effective means to control this bacterium, and the main bactericidal effects are expressed through neutrophilic extracellular killing mechanisms (Waterstrat et al., 1991). There are numerous references documenting that *E. ictaluri* can survive and replicate within fish neutrophils and macrophages (Ainsworth and Chen, 1990; Baldwin and Newton, 1993; Booth et al., 2006; Morrison and Plumb, 1994). Thus, decreased neutrophil extracellular function, predominately NET release *in vivo* after exposure to nano-TiO_2 (Jovanović et al., 2011) can contribute to bacterial survival in, and in the vicinity of, phagocytes and result in progression of infection to death (Arazna et al., 2013).

The concentrations of nano-TiO_2 to which the fish were exposed in present study are environmentally relevant, and represent external concentration that the fish may encounter in the environment. A dose of 2 ng g^{-1} body weight of nano-TiO_2 falls close to or within the estimated environmental concentration in the surface water that ranges from 21 ng L^{-1} (Gottschalk et al., 2009) to 16 μg L^{-1} (Mueller and Nowack, 2008). The concentration of 2 ng g^{-1} is also 10 times less than the concentration of nano-TiO_2 present in effluent of the waste water treatment facilities (Kiser et al., 2009; Westerhoff et al., 2011). It is important to note that since the fish were injected they were exposed internally to this concentration. In the aquatic ecosystem, fish internal and external (concentration in water) concentration of nano-TiO_2 will not necessarily be equal as this depends on many factors. However, this internal concentration of 2 ng g^{-1} is 500 times smaller than the internal concentration to which humans are exposed orally on a daily base − 1 μg g^{-1} body weight per day of nano-TiO_2/E171 (Weir et al., 2012). Therefore, this level of exposure raises potential health concerns regarding the current practice of supplementing drinking water, food, and pharmaceuticals with TiO_2 intended for human consumption.

In conclusion, the interaction of nano-TiO_2 with innate immune cells and their progenitors impaired host defenses of fish sufficiently to result in significantly increased mortality and morbidity during subsequent challenge by bacterial pathogens. Morphologic

changes in glomerular and endocardial architecture suggest that there may also be altered renal filtering function and cardiac function, respectively, with nano-TiO_2 exposure. These findings indicate that environmental contamination by nano-TiO_2 could negatively affect fish survival by interfering with immune responses and internal organ function during disease outbreaks.

Acknowledgments

We are thankful to Travis Witte for his help with ICP-MS analysis; Diane Gerjets for histopathology processing; and Judith Stasko for electron microscopy. This research was partially supported by a Marie Curie FP7 Career Integration Grant (B. Jovanović) within the 7th European Union Framework Programme - Project No PCIG13-GA-2013-618006.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.04.003.

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