www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 18: 739-746 (2018) DOI: 10.4194/1303-2712-v18_5_09

PROOF RESEARCH PAPER

Effects of Waterborne ZnO Nanoparticles and Zn^{2+} Ions on the Gills of Rainbow Trout (*Oncorhynchus mykiss*): Bioaccumulation, Histopathological and Ultrastructural Changes

Borhan Mansouri[1], Seyed Ali Johari[2,*], Nammam Ali Azadi[3], Mehrdad Sarkheil[4]

[1] Birjand University of Medical Sciences, Medical Toxicology and Drug Abuse Research Center (MTDRC), Birjand, Iran.
[2] University of Kurdistan, Fisheries Department, Natural Resources Faculty, P.O. Box 416, Sanandaj, Kurdistan, Iran.
[3] Iran University of Medical Sciences, Biostatistics Department, School of Public Health, Tehran, Iran.
[4] University of Hormozgan, Faculty of Marine Science and Technology, Bandar Abbas, Iran.

* Corresponding Author: Tel.: +98.56 32395000;
E-mail: a.johari@uok.ac.ir

Received 29 May 2017
Accepted 19 September 2017

Abstract

The aim of this study was comparing the toxic effects of zinc oxide nanoparticles (ZnO NPs) versus zinc ions (Zn^{2+}) at a high non-lethal (500µg/L) and a low environmental relevant (0.05µg/L) concentrations on gills of rainbow trout (*Oncorhynchus mykiss*) following 14 days of waterborne exposure. Structural alterations, histopathological anomalies, and zinc bioaccumulation were investigated in the gills using field emission scanning electron microscopy (FESEM), hematoxylin and eosin staining (H&E), and graphite furnace atomic absorption spectrophotometry (GFAAS) respectively. Some damages such as shortening and fusion of secondary lamellae, surface epithelium hypertrophy, and hyperplasia of the primary lamellae were observed in the gill tissue. Histopathological alterations of gills were minimum in both none exposed (control) fish and fish exposed to 0.05µg/L Zn^{2+}. The severity of gill damages were higher in fish exposed to 500µg/L ZnO NPs compared to 500µg/L Zn^{2+} and 0.05µg/L ZnO NPs. The Zn accumulation in the gills was concentration-dependent such that bioaccumulation order was as 500µg/L Zn^{2+}> 500µg/L ZnO NPs ≈ 0.05µg/L Zn^{2+}> 0.05µg/L ZnO NPs> control. In summary, the results of present study showed that although the accumulation capability of Zn^{2+} was higher than ZnO NPs, but NPs cause more structural damages to gills compare to ions.

Keywords: Zinc, nanoparticles, aquatic nanotoxicology, gill, trout.

Introduction

The life standards of human societies have been rigorously changed after introduction of modern technologies. One aspect of these new technologies is daily increase of emerging nanoproducts. Recent report shows 1,358 companies are currently involved in fabricating 6,928 nanoproducts (Nanotechnology Products Database, 2017). The production of nano-materials worldwide is expected to excess US $ 75.8 Billion by 2020 (RNCOS, 2015). However, broad applications of nanotechnology have raised concerns about their safety for human use and their environmental impacts, mainly due to use of silver, carbon, titanium, silicon/silica, zinc, gold, and other consuming nanoproducts (Woodrow Wilson Database, 2013).

Zinc oxide nanoparticles (ZnO NPs) have been used extensively due to their optoelectronic, catalytic, electrical, photochemical, and antimicrobial properties (Sirelkhatim *et al*., 2015). ZnO NPs are used in pharmaceutical, medical, and personal care products as well as other industrial applications such as paints and coatings (Woodrow Wilson Database, 2013). Releasing nano ZnO to aquatic systems was estimated 3,700 tons worldwide in 2013 (Keller, McFerran, Lazareva, & Suh, 2013). Moreover, according to the predictions, environmental concentrations of ZnO NPs in aquatic ecosystems was estimated to be from 76 µg/L to ≤ 2 mg/L (Yao, Chen, Zhao, Yang, & Zhang, 2012; Luo *et al*., 2015).

In the review of literature, many studies have evaluated the toxicity of ZnO NPs after waterborne exposure to different aquatic organisms (Hao & Chen, 2012; Adam, Vergauwen, Blust, & Knapen, 2015; Falfushynska *et al*., 2015). Zhao, Ren, Zhu, Luo, and Ren (2016) showed that the acute exposure to ZnO NPs induces developmental toxicity, oxidative stress, and DNA damage in zebrafish. In study of Kaya *et al*. (2015), oxidative stress arised from ZnO NPs was observed in tilapia and the magnitude of oxidative stress was influenced by the size and concentration of ZnO NPs. The rainbow trout is a well-established laboratory model organism with an important place in the world of aquaculture breeding. Moreover, rainbow trout recommended as model organism in aquatic ecotoxicology by the organisation for economic co-operation and development (OECD, 1984). However,

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in cooperation with Japan International Cooperation Agency (JICA), Japan

research on the effect of waterborne exposure to ZnO NPs on rainbow trout is much rarer. To our knowledge, we found only one study on dietborne toxicity of ZnO nanoparticles to rainbow trout with a claim that high levels of Zn from ZnO NPs can accumulate in the gills, liver and intestine of fish and cause oxidative stress (Connolly *et al*. 2016).

The fish gills are the ideal sentinels for toxic chemical exposure due to their constant and direct contact with the aquatic environment and susceptible to any alteration in the physicochemical characteristics of the habitat, and moreover, the results of Hao, Chen, Hao, & Zhang (2013) and Connolly *et al*. (2016) illustrated that the gill epithelia of carp and rainbow trout is potential routes of uptake for ZnO NPs. The aim of present study was to investigate the Zn bioaccumulation and toxicity effect of ZnO NPs and Zn^{2+} on gill ultrastructure and histopathology of rainbow trout (*Oncorhynchus mykiss*).

Materials and Methods

The ZnO NPs (10-30 nm) used in this study were made by US Research Nanomaterials, Inc (3302 Twig Leaflane, Houston, TX77084) and purchased from Nanosany Co. (Mashhad, Iran). SEM and EDX analyses of the powder form ZnO NPs were performed using a MIRA3 TESCAN field emission scanning electron microscope (FESEM) equipped with energy dispersive X-ray spectroscopy (EDS). Also, TEM analyses of ZnO NPs were performed using a Philips EM-208 transmission electron microscope with an acceleration voltage of 100kV. The diameters of randomly selected 60 particles were measured at a magnification of 50,000 using Axio Vision digital image processing software (Release 4.8.2.0, Carl Zeiss Micro Imaging GmbH, Germany). A stock suspension of 1,000 mg/L was prepared by vigorous stirring of 0.1g ZnO NPs powder in 100 ml double distilled water, followed by 60 min sonication in a bath-type sonicator (SOLTEC 2200 MH-SD). In addition, by dissolving appropriate amount of Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$, Merck) in double distilled water a stock solution of 1,000 mg/L Zn^{2+} was prepared.

A population of 100 juvenile rainbow trout with a mean total body weight of 10.15 ± 1.08 g (mean ± SD) was obtained from a local trout farm in Sanandaj city, Iran. Prior to the experiments, the fish were kept in a 500L tank with a daily water exchange and 16-h light/8-h dark cycle. They fed with pellet trout feed (Biomar, France) at 1% of their body weight. Aerated, dechlorinated tap water was used at all experimentations (dechlorinating was reached using vigorous aeration of at least 4 days). After ten days of acclimation, fish moved to 10 glass aquariums (10 fish/tank) of 30L volume, in a duplicated design. They allowed an additional 24 hours adaptation period before establishment of experimentation. Each tank was continuously aerated using a 2cm spherical air stone. The chronic (14 days) toxicity tests were conducted in accordance with standard OECD guideline number 204 (Fish, prolonged toxicity test; OECD, 1984).

Low environmentally relevant concentration of ZnO simulated using 0.05µg/L ZnO NPs (Wu *et al*, 2013). Also, concentration of 500µg/L ZnO NPs was chosen as high non-lethal concentration based on a series of preliminary experiments (data not shown). For comparison purposes, the same concentrations (0.05 and 500µg/L) of Zn^{2+} were also used. Groups of fish without any exposure were also considered as control. Using duplicated tests, the fish in experimental groups were exposed to low and high concentrations of either ZnO NPs or Zn^{2+}, according to a semi-static exposure regime (100 % water change and re-dosing on days 4, 8, and 12). At each concentration, 10 healthy fish were examined (hence 20 fish per treatment).

To avoid overestimating the toxicity, minimizing the risk of chemical absorption in the fecal material or food, and minimizing the dissolved organic materials (DOMs) in the exposure tanks (Welsh, Lipton, Mebane, & Marr, 2008), the feeding stopped 48h before beginning and over the toxicity examinations. For ethical considerations and to prevent fish mortality due to starvation, fish received a pellet feed (approx. ten pellets per fish) on days 8 and 12, one hour before the water exchange. All fish were treated humanely to alleviate of suffering. The entire laboratory procedures involving fish were reviewed and approved by an Animal Care and Use Committee in accordance with the Animal Welfare Act and Interagency Research Animal Committee guidelines. Ethical considerations and animal rights were approved by Ethics Committee of the University of Kurdistan.

After 14 days of exposure, fish anesthetized with 100 mg/L of clove powder. The right gill filaments were used for ultrastructure and histological studies and left gills were used for determination of Zn bioaccumulation. For ultrastructure study, a part of gill filaments separated and rinsed in ice-cold 0.1M phosphate buffer (pH 7.4) to remove excess mucus and blood quickly. Samples fixed using 2.5% glutaraldehyde in 0.1M phosphate buffer at 4°C for 24h. Then, they rinsed three times in phosphate buffer, and post-fixed in unbuffered 1% osmium tetroxide at room temperature under the laboratory hood for one hour. The fixed samples were then dehydrated in ethanol series (70, 80, and 90%, each 3 times × 5 min), absolute methanol (3 times × 5 min), and dried acetone (2 times × 5 min). Finally, the gill filaments were air dried and mounted on stubs, coated with gold and viewed and imaged with a MIRA3 TESCAN field emission scanning electron microscope (FESEM).

Histopathological alterations in gill tissues were assessed using a procedure described by Johari,

Kalbassi, Yu, and Lee (2015). Briefly after fixing the samples in Bouin's solution, tissues dehydrated and placed in paraffin wax. Samples sliced in 5µm using a microtome (MicroTec, Rotary microtome, CUT 4050), stained with Hematoxylin–Eosin (H&E), and surveyed microscopically. At the end, gill dimensions including the length of secondary lamellae, diameter of the secondary lamellae, and diameter of the primary lamellae were measured by AxioVision Microscopy Software (Release 4.8.2).

Zinc concentrations were obtained by dissecting left gill filaments, oven dry at 70 °C, and grounding into powder by a mortar and pestle. Then, 0.1g of the dried tissue powder weighed using PA214 analytical balance (OHAUS). In order to breakdown the tissue and dissolve the zinc content, the dried tissue was then digested using 3ml of concentrated nitric acid. To promote digestion, sample containers were put on the bain-marie for 120 minutes at 100°C. The digested tissues were then cooled down and diluted with 10 to 100ml (as required) deionized water. Finally, Zn concentration was quantified using a graphite furnace atomic absorption spectrophotometer (GFAAS, Biotech, Model Phoenix 961). The tissue Zn concentrations were expressed as micrograms per gram of dried weight.

For description purpose, the mean and standard deviation of data were calculated. The statistical analysis performed using open access R statistical software. Normality assumption of data evaluated using the Kolmogorov–Smirnov test. The statistical evaluation was performed using an analysis of variance (ANOVA) following multiple comparison tests using Tukey's test approach. The level of statistical significance was set at P<0.05.

Results

The ZnO NPs observed by TEM were spherical in shape, with an average diameter of 17.32 ± 5.80nm (Figure 1). The SEM images displayed nanoscale sizes (Figure 1). EDX analysis showed Zn and O as the main component of nanoparticles. Gills exposed to low concentration of Zn^{2+} exhibited minor histopathological alterations; while, exposure to high concentration (500µg/L) of Zn^{2+} led to hyperplasia-like thickening of the primary lamellae and the shortening of secondary lamellae. Moreover, common observed alterations in fish gills treated with high ZnO NPs (500µg/L) were aneurism of the primary lamellae, fusion of lamellae, epithelium shortening, and oedema (Figure 2 and 3). Hypertrophy of the surface epithelium (both in lamellae and filaments) was abundant leading to the fusion of secondary lamellae. Moreover, irregular mode of gill filaments was observed and increased secretion of mucous was covered the epithelial surface. The mean and standard deviation of each gill's parameter, including length of the secondary lamellae, diameter of the secondary lamellae, and diameter of the primary lamellae under different experimental conditions are presented in Table 1. Regardless of the type of Zn used, thicker primary diameter and shorter filament length in higher concentration are notable.

In order to test whether the mean differences were statistically significant, a two-way ANOVA with main effects of Zn type (ZnO NPs and Zn^{+2}) and group (concentration of Zn: 0, 0.05, and 500µg/L), was performed for each parameter (LSL, DSL, and DPL). For LSL, there was a significant main effect of Zn type ($F_{1,30}$ = 14.38, P<0.001), group ($F_{2,30}$ = 9.84, P<0.001), and Zn type × group interaction effect ($F_{2,30}$ = 10.69, P<0.001). For DSL both main effects and their interaction weren't significant. For DPL there was a significant main effect of Zn type ($F_{1,30}$ = 21.58, P<0.001), group ($F_{2,30}$ = 92.56, P<0.001), and Zn type × group interaction effect ($F_{2,30}$ = 5.48, P<0.009). Since the interaction effect was significant for both LSL and DPL, the results of main effects should be interpreted with care. This is because, the

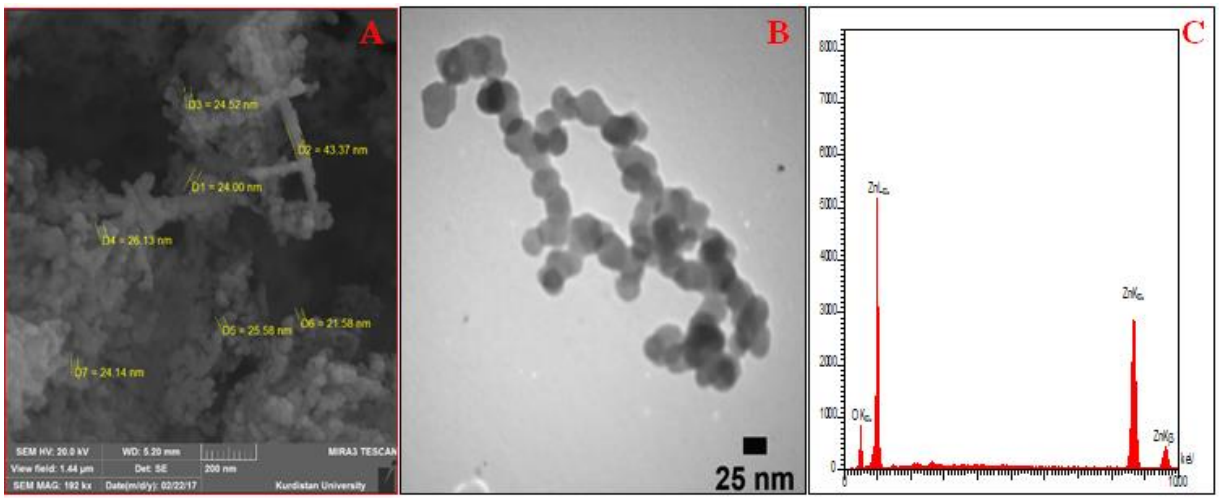

Figure 1. SEM (A), TEM (B), and EDX spectra (C) of ZnO NPs used in this study.

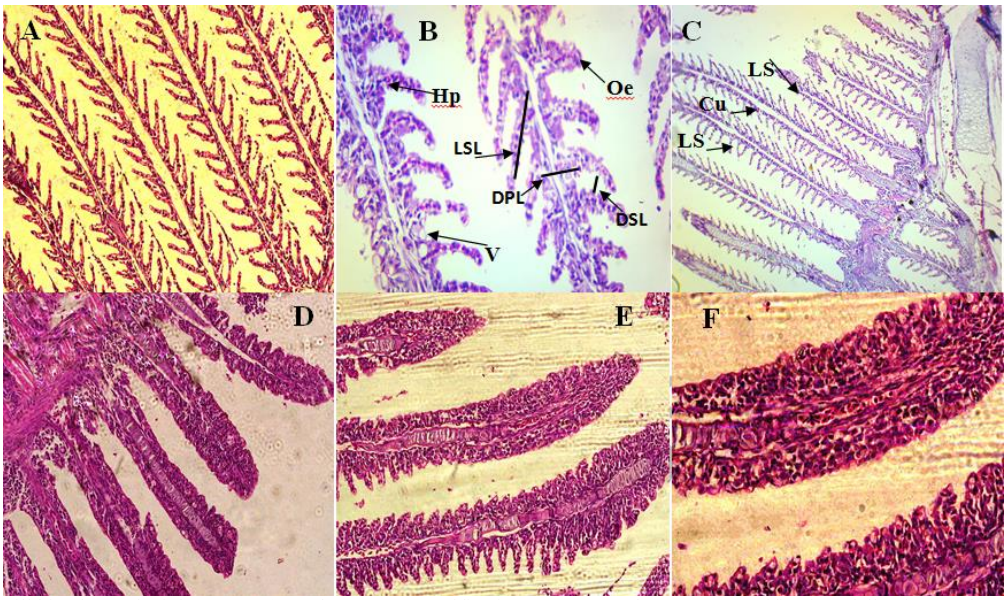

Figure 2. Gill histology of rainbow trout (A and C: 200× magnification; B, D, E, and F: 400× magnification) following 14 days of exposure to ZnO NPs or Zn^{2+}; Control (A) and treatment groups (B and C: Zn^{2+}; D to F: ZnO NPs); Aneurism (An), hyperplasia (Hp), oedema (Oe), curvature (Cu), fusion of lamellae (F), hypertrophy and proliferation in the erythrocytes of cartilaginous core (HPC), epithelium shortening (ES), lamellar synechiae (LS), vacuoles (V), necrosis (N). Gill dimensions such as the length of secondary lamellae (LSL) and diameter of secondary lamellae (DSL) as well as diameter of primary lamellae (DPL) are shown in image B.

direction of the mean difference between groups depends on the type of Zn (Figure 4). The mean difference of DPL between two 0.05 and 500µg/L of ZnO NPs was positive but it was negative (direction changed) for Zn^{+2}. The results of Figure 4 also showed that the length of secondary lamella got shorter as the concentration of ZnO NPs increased, but totally different behavior was seen for the filament length after exposure to various concentration of Zn^{+2}; they got shorten at 0.05µg/L but it increased as the level of concentration raised to 500µg/l.

After 14 days of exposure to ZnO NPs and Zn^{2+}, the Zn contents in the gill tissues of rainbow trout increased significantly in both ZnO NPs and Zn^{2+} treatments compared to the control groups (Figure 5; $P<0.05$). Meanwhile, at equal exposure concentrations, the accumulated amount of zinc in the gills was higher in the Zn^{2+} compare to the ZnO NPs ($P<0.05$). Zinc accumulation following exposure to 0.05µg/L of Zn^{2+} did not alter significantly compare to 500µg/L ZnO NPs ($P>0.05$). The highest zinc accumulation was observed following exposure to 500µg/L Zn^{2+} which was significantly higher than the amount accumulated after exposure to 500µg/L of ZnO NPs ($P<0.05$).

Discussion

Gill is an invaluable organ for studying the toxic effects of chemicals including nanomaterials on fish and other aquatic organisms. Gill is the main entry of nanoparticles into the fish's body (Handy, Henry, Scown, Johnston, & Tyler, 2008). We found that exposure to sublethal concentrations of zinc oxide nanoparticles (ZnO NPs) and zinc ions (Zn^{2+}) can increase accumulation of zinc in rainbow trout gill. The degree of bioaccumulation was closely related to both concentration and type of zinc. Increasing the concentration led to higher Zn accumulation in both types, though the bioaccumulation ability of Zn^{2+} was higher than ZnO NPs.

Johnston *et al*. (2010) studied the Zn bioaccumulation in zebrafish gills (*Danio rerio*) and found no significant bioaccumulation of this element after 14 days of exposure to 500 and 5,000µg/L ZnO NPs. Hao *et al*. (2013) reported that 50mg/L of nano-ZnO can cause more severe histopathological alterations than what the same concentration of bulk-ZnO may do. This was mainly due to the induction of higher levels of intracellular oxidative stress. Because of their tiny size, NPs can cross the small intestines and distribute further into other tissues (De Jong *et al*., 2008). Kaya *et al*. (2015) exposed tilapia (*Oreochromis niloticus*) to small (10-30nm) and large (100nm) ZnO NPs and found higher accumulation of small ZnO NPs in various tissues (including gills). Fan, Li, Yang, and Zhang (2013) showed that Zn bioaccumulation in different tissues of goldfish (*Carassius auratus*) depends on types of Zn (ZnO NPs, bulk ZnO, and Zn^{2+}). However, more researches are still required to understand the exact mechanisms of toxicity caused by each types of Zn in different organs of aquatic organisms.

One reason behind the Zn accumulation in the fish's gill can be the secretion of mucoproteins on the epithelium. Khan and McGeer (2013) claimed that the hyper-enriched zinc diet can increase significantly

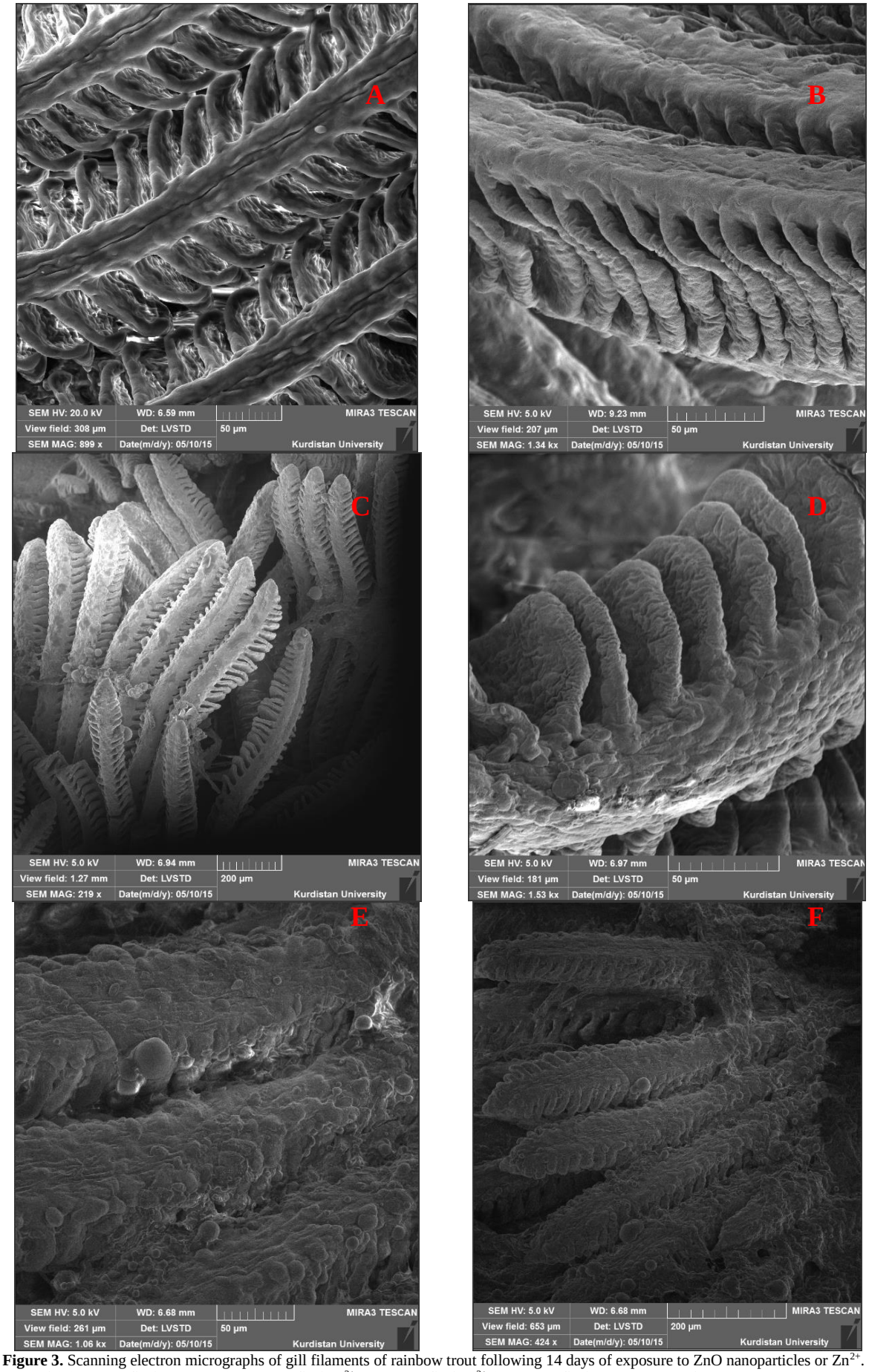

Figure 3. Scanning electron micrographs of gill filaments of rainbow trout following 14 days of exposure to ZnO nanoparticles or Zn^{2+}. Control (A), 0.05µg/l of ZnO NPs (B), 0.05µg/l of Zn^{2+} (C), 500µg/l of Zn^{2+} (D), 500µg/l of ZnO NPs (E and F).

Table 1: Mean (± SD) of the length and diameter of the secondary lamellae as well as diameter of the primary lamellae (µm) of rainbow trout (n=6 per treatment) following 14 days of exposure to ZnO NPs or Zn^{2+}

		Experimental group		
Parameter*	Types of Zn	0.05µg/L	500µg/L	Control
LSL	Zn^{2+}	37.1 ± 3.2	45.9 ± 2.1	45.9 ± 8.6
	ZnO NPs	34.5 ±6.6	20.8 ± 10.5	45.9 ± 8.6
DSL	Zn^{2+}	6.9 ± 1.1	7.7 ± 1.4	7.9 ± 0.84
	ZnO NPs	6.9 ± 0.8	9.57 ± 3.6	7.9 ± 0.84
DPL	Zn^{2+}	26.4 ± 2.0	34.7 ± 1.9	19.7 ± 4.1
	ZnO NPs	33.9 ± 3.1	43.4 ± 4.7	19.7 ± 4.1

*LSL: Length of secondary lamellae; DSL: Diameter of secondary lamellae; DPL: Diameter of primary lamellae.

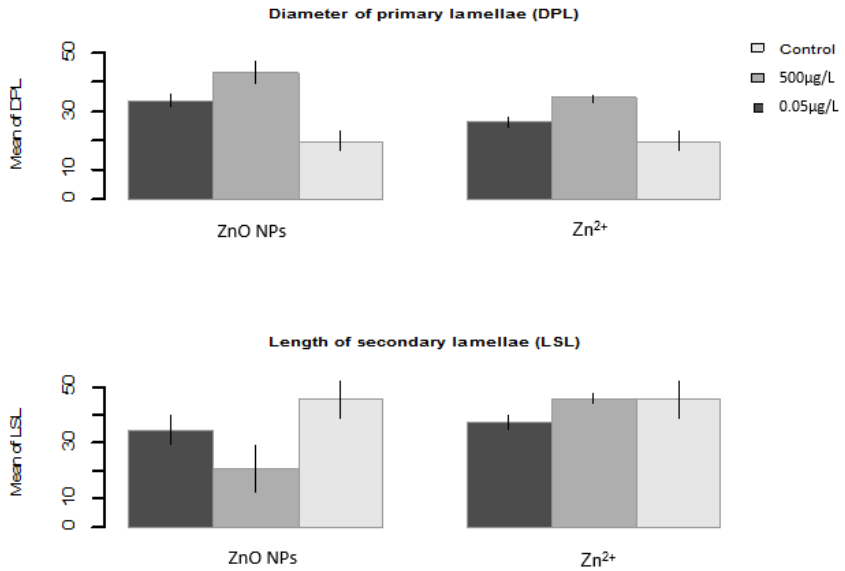

Figure 4. Mean (±SD) of length of secondary lamellae (LSL) and diameter of primary lamellae (DPL) of gills in juvenile rainbow trout following 14 days exposure to low (0.05µg/L) or high (500µg/L) concentrations of ZnO nanoparticles or Zn^{2+}.

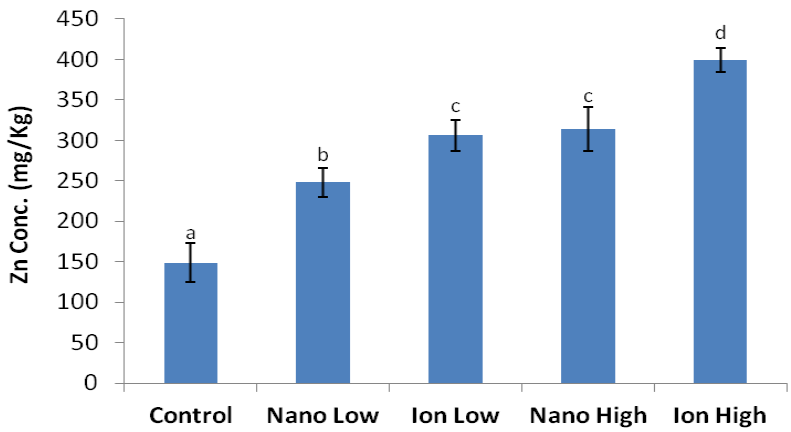

Figure 5. Zn concentration in the gills of rainbow trout (n=6 per treatment) after 14 days of exposure to low (0.05µg/L) or high (500µg/L) concentrations of ZnO nanoparticles or Zn^{2+}. Significant difference from control group (Duncan's, P<0.05) is denoted by different letters (a, b. . .).

intestinal mucus secretion which prevents cadmium accumulation in the rainbow trout intestine. Although elevated mucus secretion may act as a defense mechanism to prevent direct contact between gill surface and polluted water, it can cause impairment of the gill functions such as respiration and ion regulation.

Although gill anomalies and structural changes caused by environmental pollutants such as NPs exposure have been observed for various fish species and different nanoparticles, in the case of nanoparticulate zinc we found only one study by Subashkumar and Selvanayagam (2014) reporting several histopathological alterations in gill tissue of

common carp (*Cyprinus carpio*) after 21 day of exposure to sublethal concentrations of ZnO nanoparticles. In our study, the severity of ultrastructural changes in the gill of rainbow trout was both materials and concentration dependent, so that fish treated with higher concentration of ZnO NPs showed more intense damages. Most important observed damages were fusion of secondary lamellae, hyperplasia, and aneurism. These histopathological anomalies were defense mechanisms against toxic substances (Zn^{2+} or ZnO NPs) to guard for further damages (Mansouri, Maleki, Johari, & Reshahmanish, 2015). In other words, the aneurism in the gill which is represented through blood-filled and swelling blood vessel may lead to disturbances in blood flow in the gills, increase risk of rupture, and result in severe hemorrhage and bleeding or death (Rajkumar, Kanipandian, & Thirumurugan, 2012).

We observed structural alterations such as hyperplasia, hypertrophy, and fusion of secondary lamellae. They are the most common symptoms in gills exposed to pollutants such as metals and nanomaterials (Griffitt, Hyndman, Denslow, & Barber, 2009; Johari *et al*. 2015). It has been shown that hypertrophy can accelerate the absorption of zinc ions into the body of rainbow trout (Spry & Wood, 1998). This is likely the reason why we observed high zinc accumulation in gill after exposure to high concentration of ZnO NPs and Zn^{2+}.

We also found higher Zn bioaccumulation in gill after treating fish with Zn^{2+} whereas gills exhibited sever structural changes and histopathological damages for fish exposed to ZnO NPs. Thus, it appears that ionic and nanoparticulate zinc have different toxic mechanisms. A series of studies on bacteria (*Vibrio fischeri*) and crustacean (*Daphnia magna* and *Thamnocephalus platyurus*) have shown that ZnO NPs are the only cause of their toxicity (Adam *et al*, 2015). In contrast, however, some authors highlight the role of solubilized ions from NPs surface as the main reason of toxicity. Franklin *et al*. (2007) claimed that the most likely toxic effect of ZnO (nanoparticulate or bulk) on microalga (*Pseudokirchneriella subcapitata*) was dissolution, presumably, as Zn^{2+} or small inorganic complexes. Some studies on water flea (*Daphnia magna*) and fish (*Danio rerio*, *Oryzias melastigma*, and *Cyprinus carpio*) suggest the toxicity of ZnO NPs origin from both solubilized ions and NPs themselves (Hao *et al*. 2013; Cong, Jin, Wang, & Mu, 2017).

The purpose of this study was to determine whether exposure of rainbow trout to one environmental relevant concentration (ERC) and one sublethal concentration of ZnO NPs and equivalent amounts of Zn^{2+} can cause accumulation of Zn and structural damages to the gill tissue. Taken together, the results of present study showed that although the accumulation capability of Zn^{2+} was higher than ZnO NPs, but ZnO NPs cause more structural damages to gills compare to Zn^{2+}. In conclusion, our data emphasize the importance of concentration and form of any compound that aquatic organisms such as fish will be exposed to and ecotoxicological assessment of any chemicals for identification of their environmental hazards.

Acknowledgement

This research was funded by University of Kurdistan under the research grant No. 4.14423.

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