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Effects of ZnO nanoparticles in the Caspian roach (*Rutilus rutilus caspicus*)

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Abstract

Most studies investigating the toxicity of zinc oxide nanoparticles (ZnO NPs) focused on the effect of size, whereas exposure concentration and duration remained poorly understood. In this study, the effect of acute and sub-acute exposures of ZnO NPs on Zn compartmentalization and biomarkers' expression were investigated in *Rutilus rutilus caspicus* (Caspian roach) considering various exposure scenarios: i) the assessment of the concentration-response curves and median lethal concentration (LC_{50}); ii) the assessment of the effects of organisms exposed at LC_{50} value and one tenth of LC_{50} value of ZnO NPs suspensions for 4 d and 28 d, respectively; iii) the assessment of 14 d depuration period. Same concentrations of $ZnSO_4$ were investigated. The highest Zn accumulation was detected in gill after sub-acute exposure (4.8 mg/L; 28 d) followed by liver, kidney and muscle. In gill, liver and muscle, Zn from Zn NPs accumulated higher concentrations. Depuration (14 d) decreased Zn content in each organ, but no complete removal occurred except for muscle. Biomarkers' activity was significantly over expressed after treatments, but depuration brought back their values to background levels and most effects were related to acute concentrations (48 mg/L; 4 d) and in presence of $ZnSO_4$. Histopathological analyses showed that the exposure to ZnO NPs increased lesions in gill, liver and kidney, with a direct proportionality between alterations and Zn accumulated in the target organs. After depuration, lesions regressed for both ZnO NPs and $ZnSO_4$, but not in a complete way. These data could contribute to increase the knowledge about ZnO NPs risk assessment in aquatic vertebrates, suggesting that the size of ZnO NPs can influence biomarker and histopathological effects.

Keywords

Toxicokinetic; acute and sub-acute concentrations; fish; ZnO NPs; $ZnSO_4$

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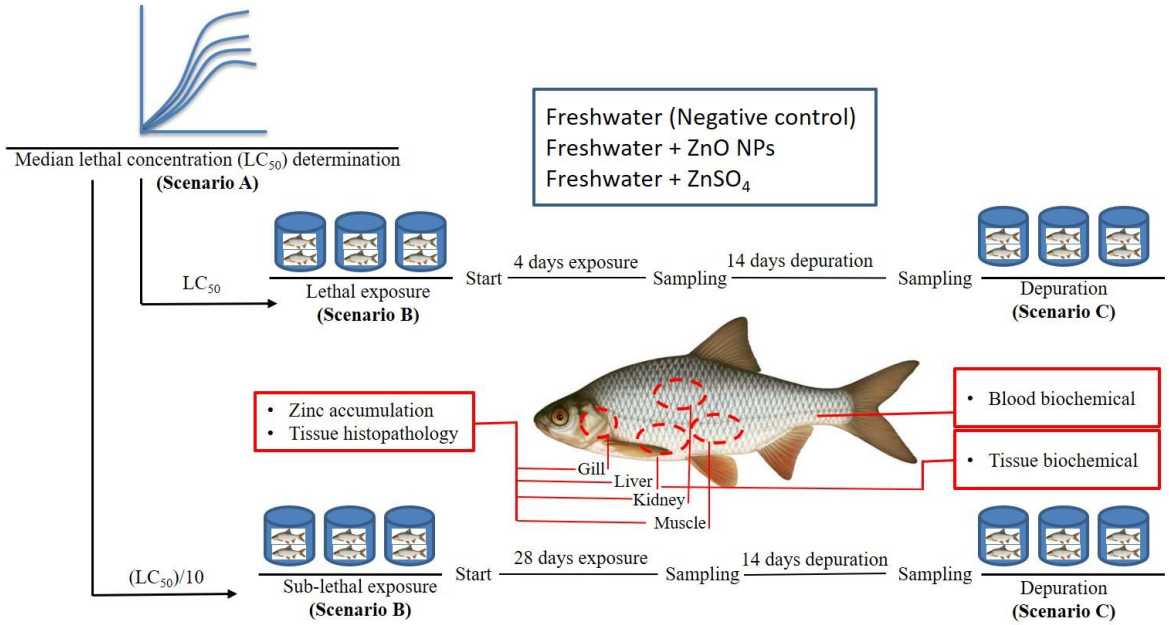
Graphical abstract



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1. Introduction

Nanotechnology is one of the most innovative field of the century exploiting physico-chemical properties of the matter related to its size (~ 10^{-9} m) (Minetto et al., 2016; Minetto et al., 2014). Nanoparticles (NPs) are used in a range of household and industry products (Lofrano et al., 2016; Maurer-Jones et al., 2013). Large-scale production and use are likely to result in the release of NPs-based products into the environment making them one of the emerging class of contaminants (Castiglioni et al., 2015; Libralato et al., 2016; Vale et al., 2016). Metal oxide NPs are commonly used in different fields like sunscreen creams, toothpaste, cosmetics, paint, paper, plastic, textile, ceramics, medicine, electronics, and wastewater treatment. Zinc oxide NPs (ZnO NPs) are produced for several industrial and household applications as reported in Table 1s (Supplementary Information).

The toxicity of ZnO NPs has been shown to vary according to the exposure dose/concentration, test duration, reference protocol, and considered biological model (Christen and Fent, 2012). ZnO NPs effects towards bacteria (mortality) and marine phytoplankton (growth rate, Miller et al. (2011)), *Caenorhabditis elegans* (survival, Ma et al., (2009)), zebra fish (mortality and hatching rate, Bai et al. (2010)), and common carp (oxidative stress and organ-specific accumulation of Zn, Hao and Chen (2012)) were all dependent on NPs size. Thus, most studies focused on the effect of NPs size, while exposure concentration and duration remained poorly investigated, also, despite importance of the released Zn ion, just few studies compared the toxicity of Zn-based NPs with ionic Zn in vertebrates (Hao et al., 2013).

Zhao et al. (2013b) evidenced that NPs could be uptaken and compartmentalized in various tissues generating oxidative stress and histopathological damage (Zhao et al., 2013b), but there is a gap into the knowledge about ZnO NPs toxicokinetic including bioavailability, uptake dynamic, tissue distribution, accumulation, and depuration in organisms. Few studies have examined the bio-distribution of ZnO NPs in vertebrates. Ates et al. (2015) compared the effects of dietary and waterborne exposure to ZnO NPs stating that diet can play a major role in Zn bioaccumulation

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primarily via intestine, gills and liver in *Carassius auratus*. Zn bioaccumulation in liver and gill of *Cyprinus carpio* after 21 d exposure to ZnO NPs and ionic form (Zn^{2+}) was reported by Hao et al. (2013) showing severe histopathological changes by increasing cellular oxidative stress response; Zn bioaccumulation was reported in mice after intravenous injection accumulating preferentially in kidney, thigh bone and the gastrointestinal tract (Yeh et al., 2012). Zn from ZnO NPs accumulated also in plants like *Zea mays* with significant reduction of root and shoot biomass production (Zhao et al., 2013a). Antioxidant defence responses are commonly used as biomarkers to detect state, susceptibility and exposure to environmental pollutants (Rudneva, 2013). There are limited studies on the toxic effects of ZnO NPs on the antioxidant system of vertebrate species (Kaptaner et al., 2016). Plasma glucose and cortisol have been measured as general stress markers in response to different pollutants (Katuli et al., 2014a; Katuli et al., 2014b) and their measurement can help to determine the effects of contaminants on living organisms.

Aquatic environments can be considered as the ultimate sink for many environmental pollutants such as NPs (Degger et al., 2015; Lenartova et al., 1997), therefore, aquatic wildlife species are increasingly at risk. Gottschalk et al. (2009) reported that ZnO NPs forecast concentrations can range from 10 and to 430 ng/L in natural surface water and in treated wastewater at European level, respectively. Given their widespread application, it is expected that their environmental levels could further increase in the near future (Osmond and Mccall, 2010). Generally, fish is considered an interesting biological model, but data on ZnO NPs are still scarce (Degger et al., 2015; Katuli et al., 2014b). Bai et al. (2010) investigated the toxicity of ZnO NPs reporting on the effects on embryos of *Danio rerio*. No data about Zn compartmentalization in organs are available, but it showed to be able to kill embryos (50 and 100 mg/L), retard their hatching (1-25 mg/L) and reducing their body length and causing tail malformations (after 96 h).

Up to now, many studies have examined the toxicity properties of different NPs on different aspects of aquatic organisms' life (Jang et al., 2014; Krysanov et al., 2009; Rajkumar et al., 2016), nevertheless their toxic potential are still not completely understood, and sometimes different

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results have been obtained mainly due to the differences in exposure methods. While some studies considered short-term exposure of acute NPs concentrations (Krysanov et al., 2009), Lee et al. (2015) examined the effects of long-term chronic exposure to NPs. Few studies simultaneously investigated acute and sub-acute effects of NPs (Katuli et al., 2014b). Despite the current use of ZnO NPs and the increasing possibility of exposure to aquatic organisms to them, there are many uncertainties regarding the potential toxicity of ZnO NPs on different aspects of aquatic organisms especially towards vertebrates.

This research was carried out to evaluate the effect of acute (4 d) and sub-acute (28 d) exposure to ZnO NPs considering uptake, Zn accumulation in target tissues and Zn depuration like as the potential disruption in the expression of some biomarkers (superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), lactate dehydrogenase (LDH), glutathione (GSH), malondialdehyde (MDA), protein concentrations, blood cortisol and glucose, and tissue histopathology) in the Caspian roach (*Rutilus rutilus caspicus*). Effects of ZnO NPs were compared to ionic Zn (Zn^{2+} from $ZnSO_4$). To the best of our knowledge, this is the first study investigating the effect of acute and sub-acute concentrations of ZnO NPs on these parameters after depuration period contributing to elucidate the pathway of ZnO NPs toxicity.

2. Materials and methods

2.1. Experimental design

Experiments were carried out considering: i) the assessment of the concentration-response curves and median lethal concentration (LC_{50}) data after 1, 2, 3 and 4 d of Caspian roach exposure to ZnO NPs suspensions – static renewal acute test (Scenario A); ii) the assessment of the effects of ZnO NPs and $ZnSO_4$ in organisms exposed at equivalent zinc concentrations (LC_{50} value and one tenth of the LC_{50} value of ZnO NPs suspensions) for 4 d and 28 d, respectively, looking for Zn

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compartmentalization and biomarkers' expression (Scenario B); iii) the assessment of 14 d depuration period, looking for Zn compartmentalization and biomarkers' expression (Scenario C). All scenarios were carried out in triplicate according to the Organization for Economic Cooperation and Development protocol (OECD, 1992). Selected exposure concentrations were in accordance to previous results from Hao et al. (2013) on *C. carpio* exposed to ZnO NPs.

2.2. Chemicals

Commercial ZnO particles were purchased from Pishgaman Cop. (Mashhad, Iran). According to the manufacturer, NPs were spherical with an average size of 30 nm, specific surface area of 60 m^2/g (Brauer Emmett Teller) and purity of 99.9**%**. The particle shape and size was characterised starting from the dry powder and stock suspension by transmission electron microscopy (TEM) (Hitachi, Japan) (Figure 1A and 1B) and assessed via ImageJ 1.51j8 (Schneider et al., 2012). $ZnSO_4$ $7H_2O$ with a purity 99.9% were provided by Sigma- Aldrich (Steinheim, Germany). The stock suspensions were prepared by adding ZnO NPs and $ZnSO_4$ dry powder into aerated ultra-pure water followed by 30 min of sonication (40 kHz, 100W; USH650, Sonicator, USA) in an ice water bath. Testing suspensions were prepared diluting the stock suspension with aerated tap water (7 mg/L of dissolved oxygen; pH =7.8 ± 0.1 at 23 ± 1 °C; salinity of 0.25 ± 0.01 mg/L; hardness of 165 ± 8 mg/L of $CaCO_3$; SO_4^{2-} of 37 ± 1 mg/L; N-NH_4^+ of < 0.01 mg/L; N-NO_2^- of < 0.01 mg/L; N-NO_3^- of 17 ± 0.01 mg/L; F^- of 0.01 ± 0.01 mg/L; total As, Cd, Cu, Pb, and Zn < 1 μg/L). Before use, tap water was filtered on activated carbon after ultra-filtration (0.2 μm) The same water was used for $ZnSO_4$ solutions.

The particle size distribution (hydrodynamic diameter) and zeta (ζ)-potential in the exposure suspension were measured by Dynamic Light Scattering (DLS) (Zetasizer, Malvern Instruments). Total Zn content and dissolution rates were measured after 24 h (for each exposure concentration of Scenario B). Hydraulic radius distribution of ZnO NPs and zeta (ζ)- potential were analysed by collecting samples from fish water tanks every 24 h (i.e. starting from the zero time) for five days

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(i.e. four half-tank water renewals) considering the 48 mg/L of ZnO NPs exposure concentration. TEM analysis of particle size distribution in water samples collected from fish tanks was carried out immediately after dosing.

Air bubbling facilities present in fish tanks was used to prevent ZnO NPs from settling keeping the suspension constantly mixed. The dissolution rate of ZnO NPs in exposure media was assessed using the Amicon ultra-centrifugal filters (3 kDa, Millipore, Germany). A volume of 15 mL of ZnO NPs suspensions (at 48 and 4.8 mg/L collected after 24 h from dosing from fish tanks) were transferred to the filters and centrifuged at 7168g for 40 min (27 ± 1 °C). The amount of Zn was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo Finnigan) (Reed et al., 2012) both in the filtrate and in the whole exposure media, and the amount of ion released was expressed as percentage of the total Zn content. The ICP-MS was calibrated with standard Zn solution (1000 mg/L Zn in nitric acid; Sigma, USA).

2.3. Ecotoxicity

2.3.1. Organisms

Caspian roach specimen with a mean body weight of 16.7 ± 0.76 g and length of 17.3 ± 1.32 cm were provided from the Sijowal Fish Reproduction Center (Golestan province, Iran). Acclimatization to test conditions prior to exposure consisted in keeping organisms in three tanks of 100 L for 2 weeks under natural photoperiod regime (14: 10 h light: dark) and fed *ad libitum*. All procedures were carried out in accordance with the Animal Care and Use Committee guidelines at the Faculty of Sciences of the University of Tehran (357, 8 November 2000).

Experiments were conducted in 100 L fiberglass tanks that were continuously aerated. Main water parameters were kept constant during all the experimental activity: 25 ± 1 ºC, 7.0 ± 0.1 mg/L of DO, pH 7.8 ± 0.8 165 ± 8 mg/L of total hardness). Organisms were fed at a rate of 1% body weight per day. The chemical composition of feed for both acclimatization and experiment is shown in

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Table S2 (Supplementary Information). Every 24 h, half of the exposure medium was renewed with freshly spiked water to remove the metabolic waste of fish within the Scenario A. The experimental design included the analysis of the effects at 0, 10, 20, 40, 50, 80 mg/L of ZnO NPs (nominal concentrations). Fishes were deprived of feed for 24 h prior to and during the toxicity test. A batch of ten organisms was used during exposure and fish mortality recorded every day for the whole test duration; dead organisms were systematically removed. Effects were reported as LC_{50} and calculated using the Probit method for each exposure period (Katuli et al., 2014b). According to Scenario B, fishes were exposed to 48 mg/L of ZnO NPs and $ZnSO_4$ for 4 d (acute exposure) and 4.8 mg/L of ZnO NPs and $ZnSO_4$ for 28 d (sub-acute exposure). Within Scenario C, depuration was investigated in triplicate following the same experimental design of Scenario B, except for the fact that after 4 and 28 d of exposure, living organisms were transferred to clean water (free of ZnO NPs and Zn^{2+}) to allow depuration to occur for 14 d.

Living specimens, collected at the end of the exposure scenario, were euthanized with an overdose of 100 mg/L of clove oil for 30 s. After euthanasia, fish gill, liver, kidney and muscle were collected. Tissues were stored at -80 ºC for the quantification of total Zn and the analysis of antioxidant enzyme activities, or fixed in 10% buffered formalin solution (Roberts, 2012) and stored at 4 ± 1 ºC until for histopathology. Blood was drawn from caudal vessels and centrifuged (15 min at 16000*g*) to extract the serum that was stored at -80 ºC until further analysis.

Within each exposure scenario, data were tested for normality (Kolmogorov-Smirnov test) and descriptive statistics. Student's t-test ($\alpha = 0.05$) and one-way analysis of variance (ANOVA) with post-hoc Tukey's test ($\alpha = 0.05$) described the potential significant difference between the considered exposure conditions. The SPSS software version 19.0 (SPSS Inc., Chicago, IL, US) was used.

2.3.2. Zn compartmentalization

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Gill, liver, kidney and muscle samples were removed from liquid nitrogen and lyophilized. Lyophilized tissues were separately homogenized with porcelain mortar, then an amount of ~ 0.1 g of tissue was transferred to 50 mL digestion vessel containing 5 mL of nitric acid (Merck, Germany) and 2 mL of hydrogen peroxide (Merck, Germany). Digestion occurred in an oven using a three-stage protocol: i) 10 min at 150 ºC; ii) 15 min at 180 ºC; iii) >10 min of cooling phase. Digested samples were diluted to a final volume of 50 mL using ultra-pure water. ICP-MS analysis was carried out to determine the total Zn concentration as well as Zn content in dry-weight target organs expressed as µg of Zn per g of dry weight (d.w.) biomass (µg/g d.w.).

2.3.3. Biomarkers and histopathology

Several biomarkers (SOD, CAT, GST, LDH, GSH, MDA, protein content) were assessed in liver tissues. Liver samples were weighed and homogenized at a ratio of 1:10 in sodium phosphate-buffered saline (pH 7.3, 300 mmol sucrose, 1 mmol EDTA, and 1.4 mmol dithioerythritol). Specimens were centrifuged at 16128g for 15 min at 4 ºC. Supernatants were assessed for activity of SOD, CAT, GST, LDH, GSH and MDA. SOD, CAT, GST, LDH, GSH, MDA, and proteins were determined according to Winterbourn et al. (1975), Aebi et al. (1984), Habig et al. (1974), Paramon Com. (Tehran, Iran), Tietze (1969), Satoh (1978), and Bradford (1976), respectively. Blood cortisol and glucose were determined according to Katuli et al. (2014b). All specifications about SOD, CAT, GST, LDH, GSH, MDA and protein determination are available in Supplementary Information (S1).

To detect histopathological markers, gill, kidney, liver and muscle samples were removed from formalin solution and embedded in paraffin blocks and sectioned (4 µm) using microtome (Microdis, 4055) (Roberts, 2012). Sections were stained with haematoxylin and eosin as described by Selmurugan et al. (2007). Morphological examination was carried out under a light microscope and pictures were captured using Nikon EC 600 Eclipse microscope.

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3. Results

3.1. Particle characterization, ZnO NPs suspension stability, and Zn dissolution rate

ZnO NPs characterization is shown in Figure 1. According to TEM analysis, primary particle size of dry powder ZnO NPs ranged between 20-70 nm (mean = 45 ± 15 nm, median = 55, n = 100) (Figure 1A), while particle size distribution in water samples collected from fish tanks (with fishes) immediately after dosing (48 mg/L) ranged between 40-130 nm (mean = 90 ± 16 nm, median = 85, n = 100) (Figure 1B).

Hydraulic radius of ZnO NPs present in water samples collected from fish tanks (with fishes) immediately (0 h) after dosing (48 mg/L) were presented in Figure 1C ranging between 37-141 nm (mean = 79.3 ± 15.8 nm, median =73 nm, at 25 °C). Hydraulic radius of ZnO NPs present in water samples collected from fish tanks (with fishes) after 24 h from dosing (48 mg/L) were presented in Figure 1D ranging between 58-342 nm (mean = 163 ± 37 nm, median =141 nm, at 25 °C). The increase of the average values of hydraulic radius indicated aggregation/agglomeration with an increase of 48% after 24 h. After fish tank water renewal and 1 h equilibration post-renewal each time for five days, the hydraulic radiuses still indicated aggregation/agglomeration. Differences ranged up to 33%, but they were not significantly different ($p < 0.05$) than hydraulic radius distribution after 24 h. The zeta-potential of ZnO NPs in water samples collected from fish tanks (with fishes) was -17.545 ± 7.27 mV and -21.46 ± 4.81 mV at 0 h and after 24 h from dosing (pH = 7.7 ± 0.1), in that order. The ZnO NPs suspension was relatively stable potentially supporting slight aggregation/agglomeration according to Patel and Agrawal (2011) as also suggested by DLS analysis. The zeta-potential of not spiked tap water was 2.55 ± 1.07 mV (pH = 7.6 ± 0.1). After fish tank water renewal and 1 h equilibration post-renewal each time for five days, zeta-potential did not significantly differ than after 24 h with values suggesting a trend towards slight/medium aggregation/agglomeration (up to 28% more than after 24 h). These data suggested that testing suspensions were relatively stable during the exposure time.

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The ZnO NPs dissolution rates were assessed in water samples collected from fish tanks (with fishes) at 48 h (acute exposure) and 4.8 (sub-acute exposure) mg/L of ZnO NPs. The amount of Zn^{2+} released as percentage of the total Zn content was 4 ± 2% and 7 ± 5% for the sub-acute and acute exposure, respectively. They showed to be statistically independent from the starting exposure concentration ($p > 0.05$), and relatively constant during the exposure period (i.e. first five exposure days and last exposure day) (up to a maximum of 28% variation).

3.2. Scenario A: acute toxicity of ZnO NPs suspensions and $ZnSO_4$ solutions

The mortality of Caspian roach in acute exposure assays (Scenario A) showed a concentration-time dependent relationship (Figure 2). In negative controls, no mortality effects were highlighted during the exposure period (up to 4 d). Toxicity as LC_{50} (and relative 95% confidence limit values) for ZnO NPs suspension increased over time: 24 h (78 ± 7 mg/L), 48 h (61 ± 5 mg/L), 72 h (53 ± 6), and 96 h (48 ± 3 mg/L). This information was used for the sub-sequent exposure scenarios (Scenario B and C) also to test $ZnSO_4$. For acute and sub-acute exposures, real concentrations of Zn in ZnO NPs suspension were 34.1 ± 6.1 and 2.9 ± 0.8 mg/L, and in $ZnSO_4$ solutions were 26.78 ± 7.22 and 3.14 ± 1.82 mg/L, respectively.

3.3. Scenario B and C

3.3.1. Partitioning of Zn in target organs

The concentration of Zn in target organs was summarised in Figure 3 as total Zn content (μg/g d.w.) in gill, liver, kidney and muscle after 4 d (acute exposure, 48 mg/L of ZnO NPs and $ZnSO_4$) and 28 d (sub-acute exposure, 4.8 mg/L of ZnO NPs and $ZnSO_4$) of contact time (Scenario B), and 14 d depuration (Scenario C). Results showed that all tissues significantly ($p < 0.05$) increased their total Zn concentration compared to negative controls in both exposure conditions and in both Scenario B and C. The only exception was for muscle after depuration. Also, the comparison between Scenario

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B and C in each Zn forms and concentrations (identified by asterisk) showed that Zn had a significant reduction in Scenario C except for the acute concentration of $ZnSO_4$ in gill and muscle. For both ZnO NPs and $ZnSO_4$, bioaccumulation trends of Zn were similar in gill, kidney ($p < 0.05$) and liver with higher Zn levels after sub-acute exposure ($p < 0.05$). In muscle, Zn content did not significantly change ($p < 0.05$) between acute and sub-acute exposure, but decreased to background levels (not significantly different from negative controls, $p < 0.05$) after depuration. In kidney, Zn from $ZnSO_4$ suspension presented the highest concentration, while in other tissues Zn from ZnO NPs was prevalent.

The partitioning of Zn into the target organs of non-exposed organisms (negative controls) was: i) acute (4 d): muscle (14 ± 1 μg/g d.w.), kidney (36 ± 7 μg/g d.w.), liver (67 ± 18 μg/g d.w.), and gill (57 ± 3 μg/g d.w.); ii) sub-acute exposure (28 d): muscle (14 ± 1 μg/g d.w.), kidney (49 ± 21 μg/g d.w.), liver (49 ± 32 μg/g d.w.), and gill (46 ± 8 μg/g d.w.). Data from control groups were averaged within the target organ (being not statistically different, $p < 0.05$) and used to define the Zn background level used in Figure 3. In summary, Zn partitioned similarly after the two exposure periods (not considering the 14 d depuration) within Scenario B: i) acute exposure (4 d at 48 mg/L): muscle (34 ± 1.5 μg/g d.w. for Zn from ZnO NPs; 19 ± 2 μg/g d.w. for Zn from $ZnSO_4$), kidney (1429 ± 157 d.w. for Zn from ZnO NPs; 1817 ± 123 μg/g d.w. for Zn from $ZnSO_4$), liver (2123 ± 243 d.w. for Zn from ZnO NPs; 1749 ± 176 μg/g d.w. for Zn from $ZnSO_4$) and gill (2541 ± 127 d.w. for Zn from ZnO NPs; 1226 ± 174 μg/g d.w. for Zn from $ZnSO_4$); ii) sub-acute exposure (28 d at 4.8 mg/L): muscle (38 ± 3 d.w. for Zn from ZnO NPs; 18 ± 2 μg/g d.w. for Zn from $ZnSO_4$), kidney (1922 ± 237 d.w. for Zn from ZnO NPs; 2450 ± μg/g d.w. for Zn from $ZnSO_4$), liver (2314 ± 154 d.w. for Zn from ZnO NPs; 2453 ± 372 μg/g d.w. for Zn from $ZnSO_4$) and, gill (3036 ± 221 d.w. for Zn from ZnO NPs; 2012 ± 176 μg/g d.w. for Zn from $ZnSO_4$). The highest Zn content was detected in gill after 28 d after sub-acute exposure to ZnO NPs and the lowest was shown in muscle (with slight differences between the two exposure scenarios) (data are presented in Supplementary Data Figure 1s A).

After the 14 d depuration (Scenario C), the partitioning of Zn into the target organs of not exposed organisms (negative controls) was: i) acute (4 d): muscle (15 ± 1.5 μg/g d.w.), kidney (15 ± 7 μg/g d.w.), liver (74 ± 29 μg/g d.w.) and gill (68 ± 9 μg/g d.w.); ii) sub-acute exposure (28 d): muscle (9 ± 0.5 μg/g d.w.), kidney (61 ± 10 μg/g d.w.), liver (53 ± 17 μg/g d.w.) and gill (68 ± 3 μg/g d.w.). Data from control groups were averaged within the target organ (being not statistically different, $p < 0.05$) and used to define the Zn background level used in Figure 3. After the 14 d depuration (Scenario C), the partitioning of Zn into the target organs of exposed organisms was: i) acute exposure (4 d at 48 mg/L): muscle (14 ± 3 for Zn from ZnO NPs; 13 ± 1.5 μg/g d.w. for Zn from $ZnSO_4$), kidney (479 ± 47 for Zn from ZnO NPs; 521 ± 84 μg/g d.w. for Zn from $ZnSO_4$), liver (1518 ± 58 for Zn from ZnO NPs; 978 ± 121 μg/g d.w. for Zn from $ZnSO_4$) and gill (708 ± 152 for Zn from ZnO NPs; 727 ± 186 μg/g d.w. for Zn from $ZnSO_4$); ii) sub-acute exposure (28 d at 4.8 mg/L): muscle (15 ± 2.5 for Zn from ZnO NPs; 11 ± 1 μg/g d.w. for Zn from $ZnSO_4$), kidney (856 ± 82 for Zn from ZnO NPs; 684 ± 176 μg/g d.w. for Zn from $ZnSO_4$), liver (1721 ± 54 for Zn from ZnO NPs; 1384 ± 89 (μg/g d.w. for Zn from $ZnSO_4$) and gill (1716 ± 160 for Zn from ZnO NPs; 7503 ± 236 μg/g d.w. for Zn from $ZnSO_4$). After 14 d depuration period, highest Zn content was detected in liver in sub-acute concentration of ZnO NPs and the lowest was still shown in muscle (with slight differences between exposure scenarios) (data are presented in Supplementary Data Figure 1s B).

3.3.2. Liver biomarkers

Results from the analysis of biomarkers (SOD, CAT, GST, LDH, GSH and MDA) were presented in Figure 4. Results from negative controls did not significantly differ between exposure conditions (acute and sub-acute) for both ZnO NPs and $ZnSO_4$, so mean activity values were used for negative controls (i.e. a mean value for acute and sub-acute exposure, and a mean value after depuration including results after 4 d and 28 d exposure).

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All biomarkers were significantly overexpressed compared to negative controls ($p < 0.05$) for both both ZnO NPs and $ZnSO_4$, and acute and sub-acute exposures with the except for GSH activity, and LDH activity exposed to the sub-acute concentration of Zn, and GST activity exposed to the sub-acute concentration of $ZnSO_4$. CAT, GST, LDH and GSH activity were higher for the acute exposure than the sub-acute one (only changes in the GST activity was significant in ZnO NPs suspension); while the activities for SOD and MDA in sub-acute concentration was higher (only changes in the SOD activity was significant in $ZnSO_4$ suspension) (Scenario B). Within Scenario B, ZnO NPs affected more GST ($p < 0.05$) and LDH, while $ZnSO_4$ affected more SOD, CAT ($p < 0.05$), and MDA.

After 14 d depuration (Scenario C), there was no significant differences between negative controls and all biomarkers (except for MDA at acute concentration of ZnSO4). Also the activity of SOD, CAT, GST (except for 4.8 mg/L for ZnSO4), LDH (except for sub-acute concentration), and MDA (except for 48 mg/L of $ZnSO_4$) significantly decreased compared to the levels in Scenario B (identified by asterisk).

3.3.3. Cortisol and glucose in blood

Cortisol and glucose results were presented in Figure 5. Results from negative controls did not significantly differ between exposure conditions and mean activity values were used for negative controls. After exposure to both ZnO NPs and $ZnSO_4$, cortisol and glucose levels significantly increased compared to control groups ($p < 0.05$). The comparison exposures (Scenario B) showed that after 4 d at 48 mg/L of ZnO NPs and $ZnSO_4$ (acute) cortisol significantly augmented compared to the sub-acute one ($p < 0.05$). For glucose, the highest level was showed after 28 d at 4.8 mg/L of $ZnSO_4$. After the 14 d depuration (Scenario C), both cortisol and glucose concentrations were back to the pre-exposure levels (i.e. not significantly different from negative controls, $p < 0.05$), demonstrating that their alteration was reversible. Also, the comparison between Scenario B and C

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for both ZnO NPs and $ZnSO_4$ (identified by asterisk) showed that cortisol and glucose had a significant reduction in Scenario C.

3.3. Histopathological effects

Histopathological changes in gill, liver and kidney of Caspian roach within acute and sub-acute exposure scenarios were summarized in Figure 6 and Figure 2s (in Supplementary Materials) and also in Table 1, 2 and 3. Microscopy observations (Figures 6 and 2s) revealed alterations in the target organs after the exposure of organisms to ZnO NPs and $ZnSO_4$ as from Scenario B and C. Main histopathological changes in gill included: shortening of secondary lamellae, collapse of secondary lamellae, curling of secondary lamellae, epithelial lifting, epithelial hyperplasia and lamellar fusion (Figure 6A, B, C, Figure 2s A and B and Table 1). Nuclear conjunction, hypertrophy of hepatocytes, hepatic lipolysis and focal necrosis were the most frequent lesions observed in Caspian roach liver after exposure to different concentrations of ZnO NPs and $ZnSO_4$ (Figure 6D, E, F, Figure 2s C and D and Table 2). Acute and sub-acute concentrations of ZnO NPs and $ZnSO_4$ produced lower impacts on kidney, compared to other tissues, but evidencing the degeneration of Bowman's capsule, glomerulus and renal tubule (Figure 6 G, H, I, Figure 2s E and F and Table 3). No changes of muscle structural tissues were found after exposure to different concentration of ZnO NPs (un-published data), probably because it was the less target organ about Zn bioaccumulation. After 14 d depuration (Scenario C), most histopathological changes were substantially recovered (Figures 6 and 2s, and also Table 1, 2 and 3). Depuration was less effective in recovering gill tissues especially in fish after sub-acute exposure (4.8 mg/L of ZnO NPs for 28 d) (Table 1).

4. Discussion

4.1. Zn partitioning and effects

Zn accumulation in tissues of aquatic animals showed to affect their structure and function (Krysanov et al., 2009). Few studies assessed the partitioning and effects of ZnO NPs in fish (Kasprzak et al., 2003; Shukla et al., 2007), thus some comparisons with other biological models and NPs were introduced into the discussion.

Gills are the most important organs involved in breathing (Cengiz, 2006) having also other physiological roles such as metabolites excretion, ion exchange and regulation of acid-base balance (Bonga and Lock, 1991). Results from this study showed that the highest Zn accumulation was observed in gill similarly to other biological models. Trevisan et al. (2014) reported that in *Crassostrea gigas* after 4 d exposure to ZnO NPs, Zn preferentially accumulated in gill and then in the digestive gland with a time-dependent progression of mitochondrial impairment within these organs. Similar results, but with other engineered nanomaterials were presented by Krysanov et al. (2009) with *Poecilia reticulata* that evidenced after 5 d exposure to single walled carbon nanotubes the preferential accumulation in gill (i.e. gill > spleen > liver > gonad). Due to their vulnerable external position and primary contact with suspended contaminants and their large surface area, gills can be considered at high risk. In previous studies, alterations in gill such as mucus secretion and hyperplasia can increase the likelihood of NPs and other chemicals of sticking onto the same target orgna (Spry et al., 1988; Smith et al., 2007; Bilberg et al., 2010).

Liver is another critical organ due to its involvement in detoxification processes (Haschek and Rousseaux, 2013). From this study, the accumulation trend of Zn in both acute and sub-acute exposures is like muscle with no significant difference between acute and sub-acute concentrations. (Figure 3). Moreover, there was little difference between ZnO NPs and $ZnSO_4$ exposure about Zn accumulation in liver. In Krishnaraj et al. (2016), after 14 d exposure to sub-acute concentrations of Ag NPs, Ag accumulated in liver of adult zebrafish. In study of Krysanov et al. (2009), guppy (*P. reticulata*) were exposed to sub-acute concentration of SnO_2 NPs with Sn accumulating preferentially in liver. Zinc can reach the liver passing through the gills and via gut absorption then

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using blood circulation (Wang et al., 2011).Thus, this could be the potential reason of high Zn content in Caspian roach liver.

Kidney is an organ involved in the metabolism of excreta such as ammonia and creatinine (Katuli et al., 2014a) and acts as a filter in fish for particles present in the blood stream. Results showed for both ZnO NPs and $ZnSO_4$ that total Zn accumulated more easily in sub-acute concentrations with the highest Zn concentrations of accumulated Zn in relation to $ZnSO_4$. Karakoc et al. (2003) showed that after 30 d exposure, Zn accumulated in kidney> gill > liver of *O. niloticus* with levels directly proportional to exposure time. Al-Bairut et al. (2013) compared the toxicological effects of Cu NPs and $CuSO_4$ concluding that in some tissues, $CuSO_4$was more bioavailable than the nano-form, similarly ZnO NPs could act in the present case study potentially explaining the high levels of Zn from $ZnSO_4$ accumulated in kydney.

According to the present paper results, there was no relation between Zn accumulation in kidney Zn accumulation in muscle was lower than all other investigated tissues suggesting that it cannot store Zn probably due to the lack of metal binding proteins. No significant differences were found between acute and sub-acute exposures of Zn considering the Zn levels accumulated in muscle. Rajkumar et al. (2016) observed the accumulation of Ag in muscle after 7 d exposure to *Labeo rohita* (10, 25, 50 and 100 mg/L) of Ag NPs. Similarly, Jang et al. (2014) showed that after 7 d exposure to 0.62 mg/L Ag NPs, lowest concentrations of Ag were observed in muscle and brain of common carp.

Elimination of Zn from tissues of Caspian roach was observed after 14 d depuration. Zn accumulated in gill, liver, kidney and muscle in both acute and sub-acute exposures decreased compared to the relative treatments (Figure 3). In summary, the following Zn level reduction percentages were observed for gill, liver, kidney and muscle, in that order: i) acute exposure to ZnO NPs and $ZnSO_4$: 72% and 41%, 29% and 44%, 66% and 71%, 59% and 31%; ii) sub-acute exposure to ZnO NPs and $ZnSO_4$: 43% and 63%, 26% and 44%, 55% and 72%, 61% and 39%. Zn reduction trend in gill, liver and kidney was proportional to the relative treatment concentration,

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being higher after the sub-acute exposure. Zn after acute exposure seemed more "labile" and more efficiently eliminated during the depuration phase. Zn from $ZnSO_4$ presented the highest accumulation in kidney, but a significant reduction after depuration as well even if background levels (i.e. negative controls) were not reached after 14 days. In muscle, Zn concentration was back to background levels after depuration.

4.2.3. Effect of ZnO NPs on oxidative stress

The antioxidant defence system prevents the occurrence of oxidative damage caused by reactive oxygen species (ROS), and could be remarkably increased under different stress conditions (Lindley, 1998). Results from Caspian roach exposure to acute and sub-acute concentrations of ZnO NPs and $ZnSO_4$ were reported in Figure 4.

SOD is the first antioxidant enzyme against oxidative toxicity catalysing dismutation of highly superoxide radical O^{2-} to O_2 and H_2O_2 (Panda, 2012). SOD significantly increased than the control groups ($p < 0.05$) but no significant differences were present between results after acute and sub-acute exposures. This result may be due to the high concentrations of NPs and self-scavenging capacity of SOD. And also in groups that exposed with $ZnSO_4$, SOD activity significantly increased in sub-acute concentration. Muralisankar et al. (2014) observed that the freshwater prawn *Macrobrachium rosenbergii* exposed to ZnO NPs, increased its SOD activity in a dose-dependent manner. Similarly, Cozzari et al. (2015) observed that the estuarine ragworm *Hediste diversicolor* exposed to Ag NPs augmented in a significant way SOD activity. Conversely, Hao and Chen (2012) reported that *C. carpio* exposed to ZnO NPs (0, 0.5, 5, and 50 mg/L) after an initial SOD increase (0.5 mg/L) it significantly decreased (more than 80%) in a time-concentration manner.

CAT, located in peroxisomes, facilitates the removal of H_2O_2 (namely the product of SOD activity) that is metabolized to molecular oxygen and water (Lindley, 1998). CAT activity significantly increased in exposed animals thus it prevented greater oxidative damage in the exposed fish. Induction of CAT as a response to NPs exposure was previously reported in Cyprinidae (Gül et al.,

2008), *Scrobicularia plana* (Buffet et al., 2011), *H. diversicolor* (Buffet et al., 2011; Cozzari et al., 2012), and *M. rosenbergii* (Muralisankar et al., 2014).

GST by using GSH increases dismutation of hydrogen peroxide (Habig et al., 1974; Völker et al., 2015) and can bind Zn to thiol group of GST leading to detoxification, and thus increasing exposed animals tolerance and survival in critical situations (Gül et al., 2004; Yuan et al., 2016). GST activity increased after exposure to acute and sub-acute concentrations of ZnO NPs, with the highest levels reached after the acute scenario (Figure 5). Also, comparison between exposed groups showed that the lowest activity was related to sub-acute exposure to $ZnSO_4$. According to Brown et al. (2000), Zn can bind to thiol groups (Brown et al., 2000) supporting detoxification, and thus GST reduction after 28 d. In previous studies, GST increased in clams exposed to Ag NPs (Völker et al., 2015), similarly to *Mytilus galloprovincialis* exposed to CuO NPs after 15 d (Gomes et al., 2013).

LDH, catalysing the conversion of lactate to pyruvic acid and back, followed the GST trend with higher activity after acute exposure than sub-acute one (Figure 4) suggesting the presence of non-specific injuries like cell death (Agrahari et al., 2007). It can be said that Zn caused cell death in exposed fish. However to better understand toxicity mechanism of NPs on LDH activity, further investigation are needed. Ates et al. (2016) and Lee et al. (2014) found that rainbow trout and common carp exposed to Fe NPs and ZnO NPs, respectively, presented levels of LDH activity significantly increased compared to the control groups.

GSH is a non-enzymatic antioxidant acting as a protective agent against numerous toxic substances and catalysing hydrogen peroxide (Hao and Chen, 2012). GSH activity in exposed groups did not show any significant difference compared to negative controls (Figure 4). Hao and Chen (2012) highlighted that *C. carpio* exposed for 14 d to ZnO NPs presented decreasing GSH activity. Conversely, Völker et al. (2015) showed that after 28 d exposure to Ag NPs, GSH activity increased. Masella et al. (2005) and Nordberg and Arner (2001) evidenced that high GSH values

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can increase lipid peroxidation (LPO) disturb the antioxidant balance. The end products of LPO are reactive aldehydes, such as MDA.

MDA like GSH is another non-enzymatic antioxidant and has been used extensively as a biomarker of oxidative stress (Xiong et al., 2011; Zhao et al., 2013b). MDA activity in the Caspian roach significantly increased in both of acute and sub-acute concentrations ($p < 0.05$) in a similar way indicating Zn oxidative effects. Significant increase in MDA activity was detected in *D. rerio* exposed to ZnO NPs (Zhao et al., 2013b) and to TiO_2 NPs (Xiong et al., 2011), but Gül et al. (2004) reported a decreasing MDA trend for Cyprinidae, suggesting the presence of species specific related factors, besides of exposure protocols and type of NPs.

After 14 d depuration, results showed that almost all antioxidant biomarkers significantly decreased compared to treatments being the differences with negative controls not significant suggesting the reversibility in the Zn related metabolism. Nevertheless, after the depuration period biomarkers went back to background levels, Zn content in gill, kidney, and liver was significantly greater than negative controls and this could be explained with the self-scavenging capacity of antioxidant defence systems or increased adaptation in response to the new condition, to be further investigated. In summary, with the exception of GSH, all biomarkers responded to the presence of Zn; most biomarkers were more sensitive to long term exposure*4.3. Blood biochemical factors*

Cortisol and glucose are two important general stress markers used to in various studies (Ates et al., 2013; Katuli et al., 2014b). Under hypothalamus-pituitary-internal (HPI) axis stimulation, cortisol secreted from the teleost head kidney increases the energy availability during stress conditions (Katuli et al., 2014a). Results showed that cortisol and glucose levels significantly increased compared to negative controls (Figure 5). Similarly, Katuli et al. (2014b) after exposing *D. rerio* to acute and sub-acute concentrations of Ag NPs, cortisol and glucose levels significantly increased than control like for Murray (2016) in the case of rainbow trout exposed to Ag NPs. In this study, cortisol levels increased more after acute exposure than sub-acute one being probably due to the

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adaptive response of cortisol (Fast et al., 2008) that tends to reduce after long-term exposure to stress condition thus gradually reducing its sensitivity. Fast et al. (2008) showed that after short-term stress cortisol levels significantly increased compared to negative controls and long-term stress groups. Glucose levels significantly increased in the exposed groups to ZnO NPs and this is related to cortisol role in gluconeogenesis process resulting in glucose production (Saravanan et al., 2011; Sheridan, 1989). Also Ates et al. (2016) and Lee et al. (2014) reported an increase of glucose levels in fish exposed to Fe NPs (*O. niloticus*) and ZnO NPs (*C. carpio*), respectively. Highest glucose levels showed after 28 d exposure to sub-acute concentration of $ZnSO_4$. It can be said that at the high concentrations, both forms had equal impact on glucose, but at low concentrations, $ZnSO_4$ had more effects. Similarly, Massarsky et al. (2013) reported the same effect for Ag NPs and $AgNO_3$ about the hatching delay in *D. rerio*.

After 14 d depuration, results showed that cortisol and glucose levels were back to the initial ones with no statistical difference compared to control groups (Figure 5). Katuli et al. (2014a) showed that after 96 h recovery period, cortisol and glucose levels significantly decreased after exposure of Caspian roach to diazinon. This suggests that the effects of ZnO NPs and $ZnSO_4$ on cortisol and glucose can be really like other toxic agents and that recovery is possible after the removal of pollutants.

4.4. Histopathological alteration

The panel of potential histopathological alterations can be an interesting tool to evaluate the effects of substances due to simplicity, early warning and ranking of different substances (Cengiz, 2006). Histopathological results (after qualitative assessment, but with high repeatability) after exposure to acute and sub-acute concentrations, in Caspian roach gill were reported Figure 6 (A-C), Figure 2s (A-D) and Table 1. Most observed lesions include shortening, collapse and epithelial lifting of secondary lamellae, epithelial hyperplasia and lamellar fusion. Some of structural alterations can originate an improved resistance to pollutants' exposure like for epithelial lifting causing an

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increase in the distance and time for contaminants to reach blood stream. As well as gill hyperplasia causes a decrease in gill surface area and a subsequent increase in the toxicant-blood diffusion distance (Cengiz, 2006; Katuli et al., 2014a). As mentioned previously (section 4.1) these lesions can be possible reason for high concentration of Zn in gill. The hyperaccumulation of Zn in gill (Figure 3) damaged its structure and the increased mucus production could have cause an increased gill filament capacity to absorb NPs (Bilberg et al., 2010) contributing to the general disruption of gill activity. Similarly, Rajkumar et al. (2016), Griffitt et al. (2007) and Smith et al. (2007) exposed *L. rohita*, *D. rerio*, *Oncorhynchus mykiss* to Ag NPs, CuO NPs and SWCNT, respectively, evidencing several lesions in gills.

Liver plays an important role in metabolism and detoxification of pollutants (Haschek and Rousseaux, 2013) and its pathology is used as an indicator of health status alteration (Federici et al., 2007; Rajkumar et al., 2016). After gill, liver was the second organ that hyperaccumulated Zn (Figure) and presented some visible lesions like nuclear conjunction and hypertrophy of hepatocytes (Figure 6, D-F and Figure 2s C-D; Table 2). Similarly, *L. rohita* exposed to Ag NPs presented liver lesions like formation of vacuolation and vacuolar degenerations (Rajkumar et al., 2016), while Federici et al. (2007) reported loss of sinusoid space and lipolysis in *O. mykiss* exposed to TiO_2 NPs, and Lee et al. (2012) observed hyperplasia, cytoplasm vacuolation in *C. carpio* after exposure to Ag NPs.

Kidney is an important organ to keep organism homeostasis via ions exchange and secretion of metabolic products and water and its impairment can damage fish physiology and ultimately survival (Katuli et al., 2014a). Several lesions such as degeneration of glomerulus and Bowman's capsule were observed in kidney after exposure to ZnO NPs (Figure 6, G-I and Figure 2s E-F; Table 3) also in relation to the significant Zn accumulation (Figure 3). Similarly, Lee et al. (2014) showed *C. carpio* exposed for 12 weeks to ZnO NPs presented several lesions in kidney like number and shape of the lysosomes and the renal tubule.

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In the case of muscle, no histopathological alterations were found after both acute and sub-acute exposure to zinc. This result was in accordance to Zn partitioning and accumulation in Caspian roach, so that there could be a proportionatility between the amount of Zn accumulated in tissue and the presence of lesions. Overall, it was observed that more histopathological alterations were present after sub-acute exposure (28 d at 4.8 mg/L of ZnO NPs).

After 14 d depuration, fewer lesions were observable and tissue health generally improved. Most lesions were detected in gill probably due to the high amount of Zn stored in this organ even after depuration.

5. Conclusion

In this study, the acute and sub-acute effects of ZnO NPs on the accumulation and fate of NPs, induction of antioxidant response and stress blood parameters, as well as histopathological effects in target tissues were investigated after treatment and depuration. $ZnSO_4$ was used to define the background Zn effects. Especially sub-acute concentration and both ZnO NPs and $ZnSO_4$, accumulated in target tissues; after depuration, the accumulated Zn decreased, but only sometimes down to background levels (control group). Zn bioaccumulation was accompanied by histopathological disorders in target tissues; after depuration, tissues recovered only partially. Also, most antioxidant biomarkers were overexpressed for both ZnO NPs and $ZnSO_4$; most effects were related to acute concentrations and $ZnSO_4$; after deportation, almost all biomarkers were back to the initial level suggesting the reversibility of the effects once the exposure to Zn is eliminated.

Results from this study could help modelling ZnO NPs effects and compartmentalization mechanisms suggesting the need for future research considering environmentally relevant concentrations as well as assessing the environmental risks associated to the release of ZnO NPs into the aquatic environment also considering the potential mixture effect in presence of other contaminants.

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Declaration of interest

The authors report no conflicts of interest related to this study.

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Table 1. Summary of histopathological effects in gill of *R. rutilus caspicus* after acute (48 mg/L, 4 d) and sub-acute (4.8 mg/L, 28 d) exposure to ZnO NPs and $ZnSO_4$ (Scenario B) and subsequent depuration (14 d) (Scenario C).

		Lesions			
Exposure scenario	Duration	Shortening of secondary lamellae	Collapse of secondary lamellae	Curling of secondary lamellae	Epithelial lifting
Control	4 and 28 d	-	-	-	-
Acute (48 mg/L)	4 d-ZnO NPs	+	++	+	++
	4 d-$ZnSO_4$	++	++	++	++
Sub-acute (4.8 mg/L)	28 d- ZnO NPs	+++	+++	+++	+++
	28 d-$ZnSO_4$	+++	+++	+++	+++
Depuration scenario					
Control	4 and 28 d	-	-	-	-
Acute	4 d-ZnO NPs	-	+	-	+
	4 d-$ZnSO_4$	+	++	-	+
Sub-acute	28 d- ZnO NPs	++	+	+	+
	28 d-$ZnSO_4$	++	++	+	++

Score value: None (−), mild (+), moderate (++) and severe (+++).

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Table 2. Summary of histopathological effects in liver of *R. rutilus caspicus* after acute (48 mg/L, 4 d) and sub-acute (4.8 mg/L, 28 d) exposure to ZnO NPs and $ZnSO_4$ (Scenario B) and subsequent depuration (14 d) (Scenario C).

Exposure scenario	Duration	Lesions			
		Nuclear conjunction	Hypertrophy of hepatocytes	Hepatic lipolysis	Focal necrosis
Control	4 and 28 d	-	-	-	-
Acute (48 mg/L)	4 d-ZnO NPs	-	+	+	-
	4 d-$ZnSO_4$	+	+	-	+
Sub-acute (4.8 mg/L)	28 d- ZnO NPs	+	++	+	++
	28 d-$ZnSO_4$	++	++	++	+
Depuration scenario					
Control	4 and 28 d	-	-	-	-
Acute	4 d-ZnO NPs	-	-	-	-
	4 d-$ZnSO_4$	-	+	-	-
Sub-acute	28 d- ZnO NPs	+	+	-	-
	28 d-$ZnSO_4$	+	+	+	+

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Table 3. Summary of histopathological effects in kidney of *R. rutilus caspicus* after acute (48 mg/L, 4 d) and sub-acute (4.8 mg/L, 28 d) exposure to ZnO NPs and $ZnSO_4$ (Scenario B) and subsequent depuration (14 d) (Scenario C).

Exposure scenario	Duration	Lesions		
		Degeneration of Bowman's capsule	Degeneration of glomerulus	Degeneration of renal tubule
Control	4 and 28 d	-	-	-
Acute (48 mg/L)	4 d-ZnO NPs	+	+	-
	4 d-$ZnSO_4$	+	+	+
Sub-acute (4.8 mg/L)	28 d- ZnO NPs	++	+++	-
	28 d-$ZnSO_4$	++	++	++
Depuration scenario				
Control	4 and 28 d	-	-	-
Acute	4 d-ZnO NPs	-	+	-
	4 d-$ZnSO_4$	-	-	+
Sub-acute	28 d- ZnO NPs	-	+	-
	28 d-$ZnSO_4$	+	-	+

Score value: None (−), mild (+), moderate (++) and severe (+++)

Figure
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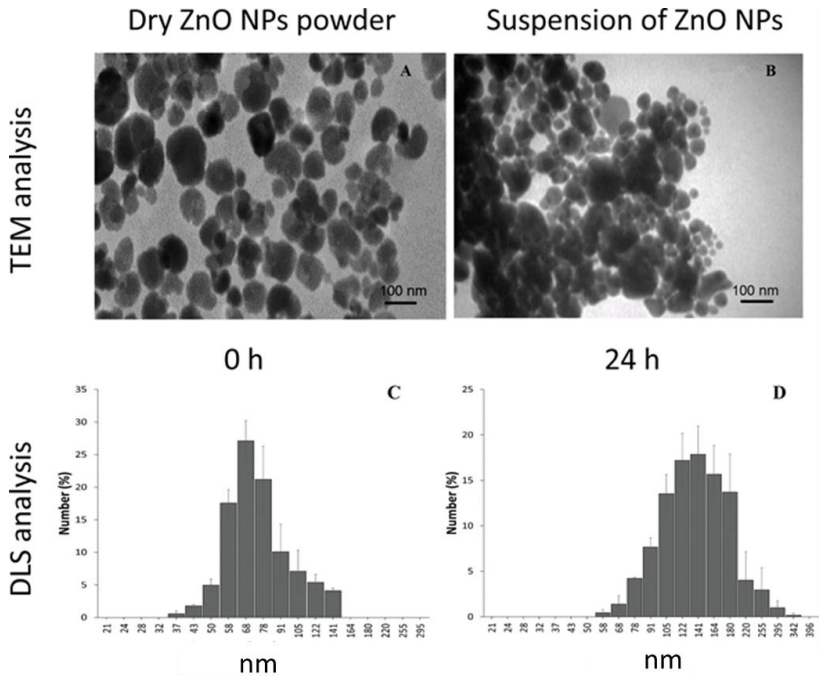

Figure 1. A) ZnO NPs dry powder; B) ZnO NPs in a water sample collected from fish tank (with fishes); C and D) hydrodynamic radius of ZnO NPs in a water sample collected from fish tank after 0 h (C) and 24 h (D) from dosing (48 mg/L ZnO NPs).

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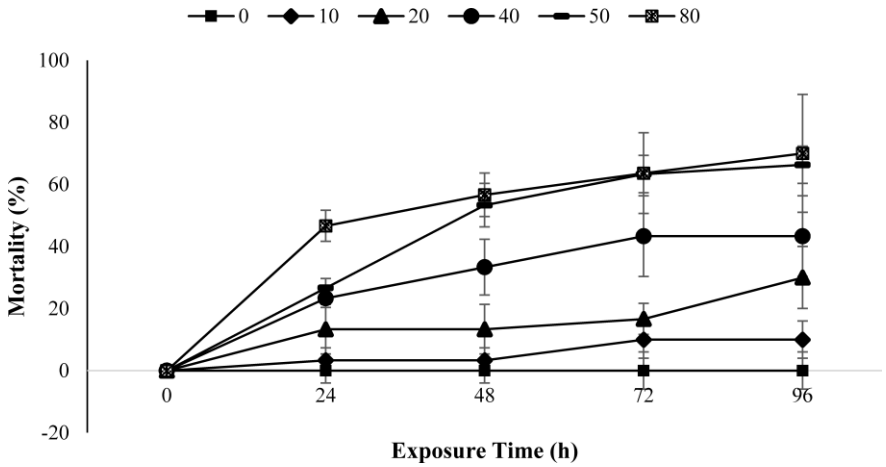

Figure 2. Mortality (%) in *R. rutilus caspicus* exposed to increasing ZnO NPs concentrations (0, 10, 20, 40, 50 and 80 mg/L ZnO NPs) (Scenario A).

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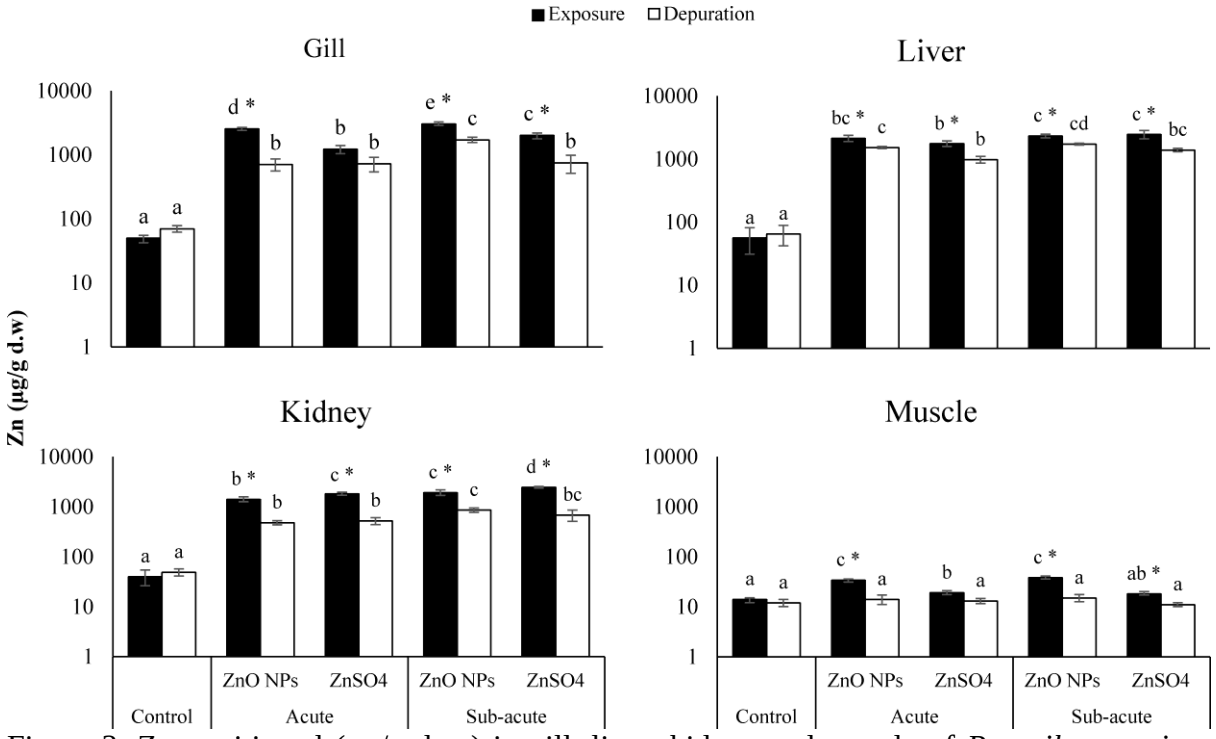

Figure 3. Zn partitioned (µg/g d.w.) in gill, liver, kidney and muscle of *R. rutilus caspicus* after acute (48 mg/L, 4 d) and sub-acute (4.8 mg/L, 28 d) exposure to ZnO NPs and $ZnSO_4$ (Scenario B) and subsequent depuration (14 d) (Scenario C). Letters indicate statistically significant differences between treatments in each scenario ($p < 0.05$); asterisk (*) indicates statistically significant differences between scenario B and C for both ZnO NPs and $ZnSO_4$ at each exposure concentrations ($p < 0.05$).

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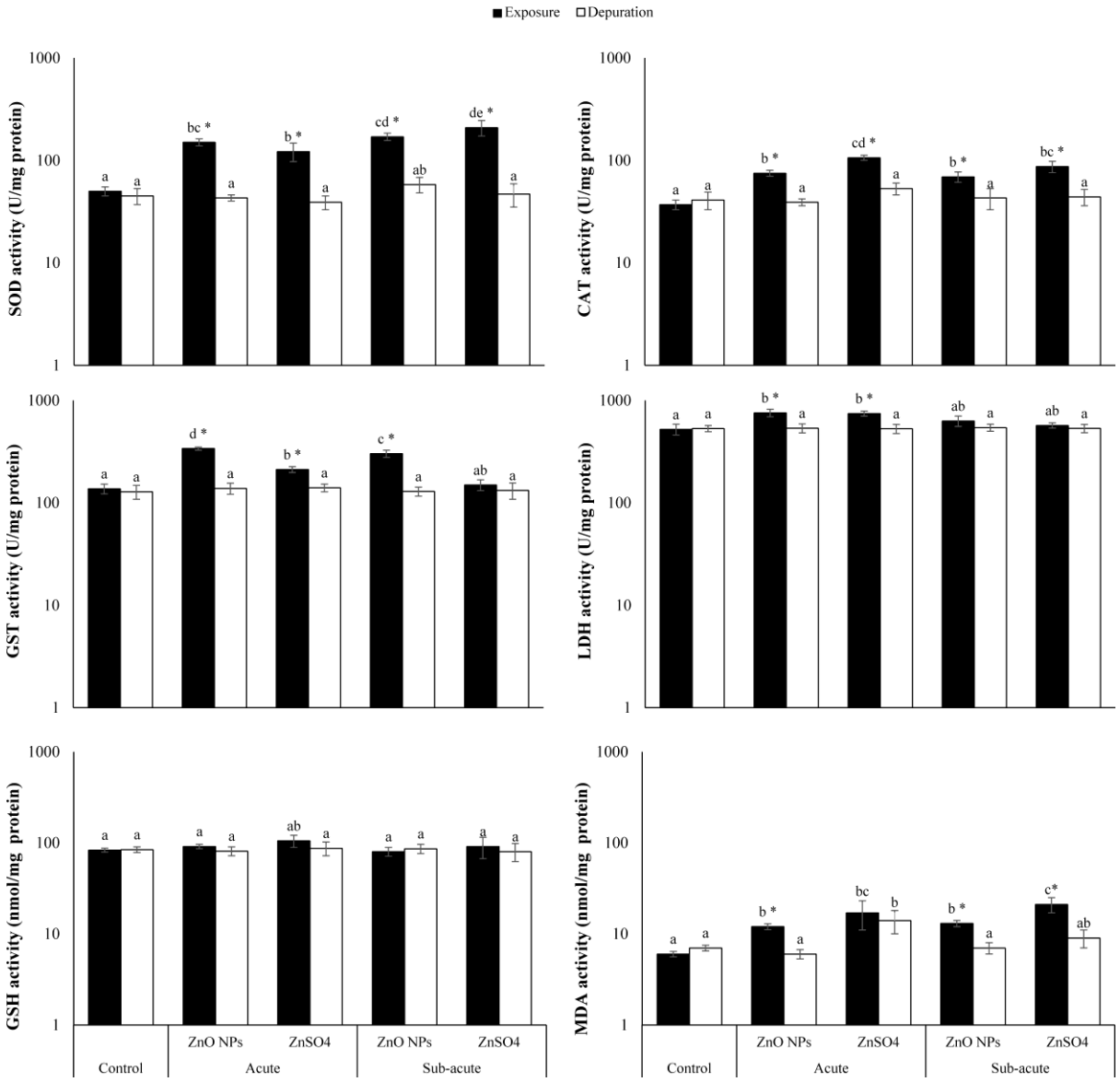

Figure 4. Biochemical parameters in liver of *R. rutilus caspicus* after acute (48 mg/L, 4 d) and sub-acute (4.8 mg/L, 28 d) exposure to ZnO NPs and $ZnSO_4$ (Scenario B) and subsequent depuration (14 d) (Scenario C). Letters indicate statistically significant differences between treatments in each scenario ($p < 0.05$); asterisk (*) indicates statistically significant differences between scenario B and C for both ZnO NPs and $ZnSO_4$ at each exposure concentration ($p < 0.05$) within the same target organ; U = 1 μmol/min.

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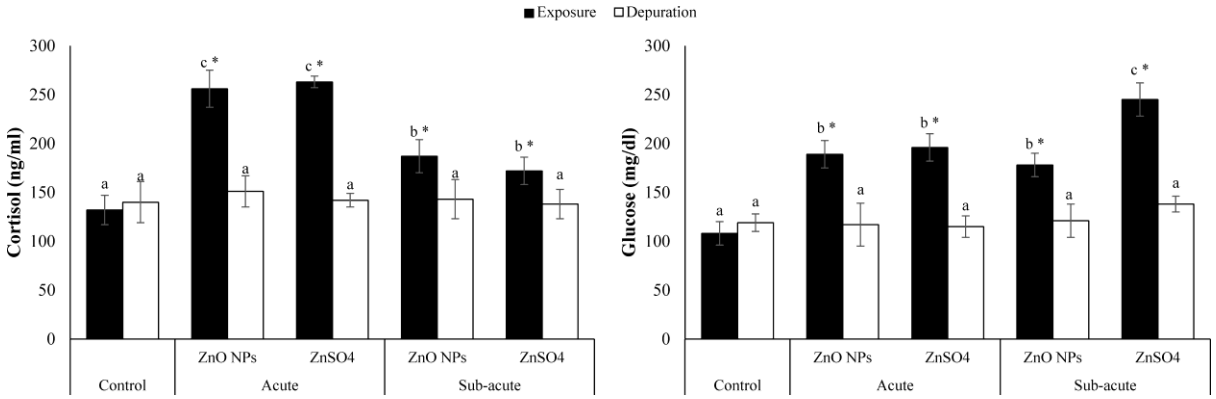

Figure 5. Cortisol and glucose levels in blood of *R. rutilus caspicus* after acute (48 mg/L, 4 d) and sub-acute (4.8 mg/L, 28 d) exposure to ZnO NPs and $ZnSO_4$ (Scenario B) and subsequent depuration (14 d) (Scenario C). Letters indicate statistically significant differences between treatments in each scenarios ($p < 0.05$); asterisk (*) indicates statistically significant differences between scenario B and C for both ZnO NPs and $ZnSO_4$ at each exposure concentration ($p < 0.05$).

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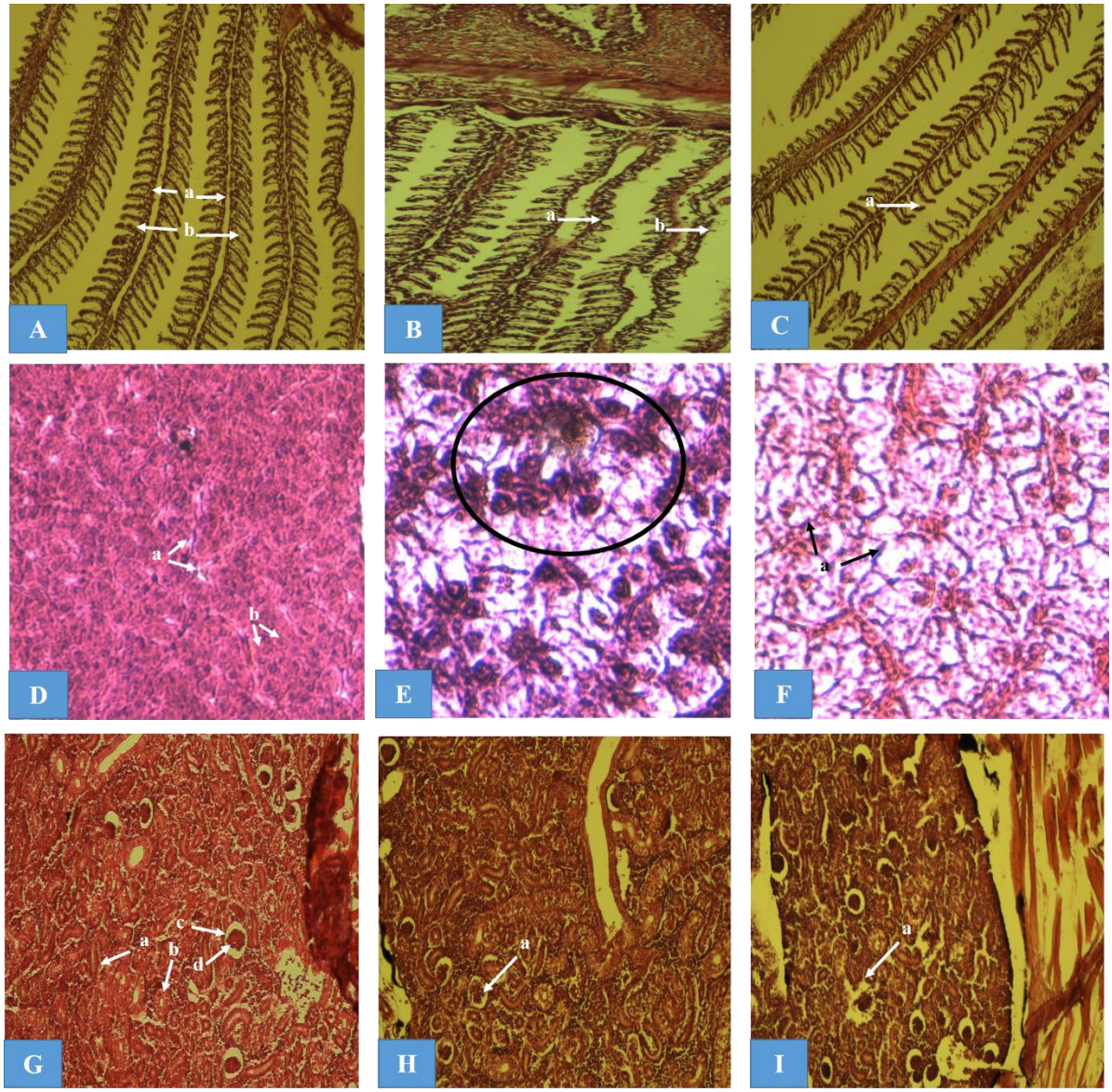

Figure 6. Histopathology of *R. rutilus caspicus* gill (A-C), liver (D-F) and kidney (G-I) tissues after hematoxylin and eosin staining considering acute or sub-acute exposures after depuration period. (A) control fish: (a) primary lamella, (b) secondary lamellae (100x); (B) sub-acute exposure to ZnO NPs (4.8 mg/L; 28 d): (a) shortening of secondary lamellae, (b) collapse of secondary lamellae (100x); (C) after depuration of sub-acute exposure (14 d): (a) curling of secondary lamellae (100x); (D) control fish: (a) sinusoidal portal blood, (b) hepatocytes (400x); (E) acute exposure to ZnO NPs (48 mg/L; 4 d): (circles) nuclear conjunction (1000x); (F) after depuration of sub-acute exposure (14 d): (a) hypertrophy of hepatocytes (1000x); (G) control fish: (a) longitudinal tubule, (b) tubule, (c) Bowman's capsule, (d) glomerulus (400x); (H) acute exposure to ZnO NPs (48 mg/L; 4 d): (a) degeneration of glomerulus (400x); and (I) sub-acute exposure to ZnO NPs (4.8 mg/L; 28 d): (a) degeneration of Bowman's capsule (400x).

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