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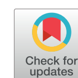
Acute toxic effects caused by the co-exposure of nanoparticles of ZnO and Cu in rainbow trout



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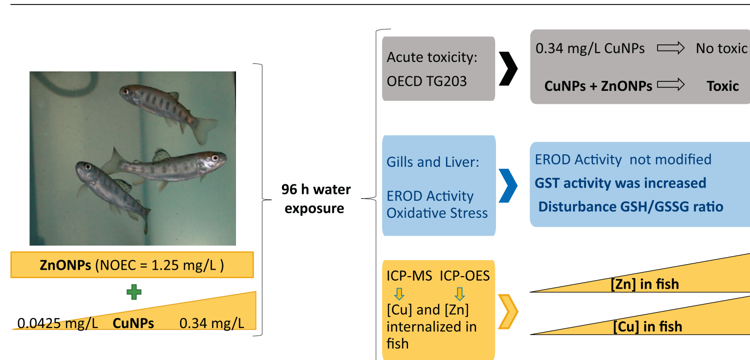
HIGHLIGHTS

- Co-exposure to low concentrations of CuNPs and ZnONPs produces lethal effects.
- The lethal effect was inversely concentration-related.
- Increasing concentrations of CuNPs favour the accumulation of Zn in fish.
- Higher levels of Zn in fish were correlated with a lower mortality.
- Co-exposure leads to altered GST activity and GSH/GSSG ratio in gill and liver.

GRAPHICAL ABSTRACT



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ABSTRACT

The toxic effects produced by the co-exposure to low- and non-toxic concentrations of zinc oxide (ZnONPs) and copper nanoparticles (CuNPs) was assessed in rainbow trout following the OECD Test Guideline 203. Four groups of trouts were exposed for 96 h to a range of concentrations (0.0425–0.34 mg/L) of CuNPs (50 nm) in combination with a fixed non-toxic concentration (1.25 mg/L) of ZnONPs (25 nm) determined from an independent concentration-response study. One additional group was exposed to the highest concentration of CuNPs alone. Behaviour and mortality were observed during the experiment. After 96 h exposure, accumulated levels of Cu and Zn in the fish were measured by ICP-MS and ICP-OES, respectively. The induction of oxidative stress in liver and gills was evaluated by the glutathione-S-transferase (GST) activity and the reduced glutathione (GSH) / oxidized glutathione (GSSG) ratio. The ethoxyresorufin-*O*-deethylase (EROD) activity was also assessed. The results showed that CuNPs at the highest tested concentration do not cause acute toxicity, whereas exposure to all mixtures caused mortality, which was inversely proportional to the concentration of CuNPs (from 28% to 86% survival). Accumulated levels of Cu and Zn in the fish increased with the increasing concentrations of CuNPs, suggesting that the presence of CuNPs favours the entry of Zn. In general, the GST activity increased significantly in the gills of co-exposed groups, whereas the GSH/GSSG ratio was altered in the liver. The EROD activity was not modified. In conclusion, the co-exposure to these NPs potentiates their toxicity, observing an alteration of the GST activity and GSH/GSSG ratio in gill and liver, which was more pronounced at the lowest concentration of CuNPs. The lower toxic effect observed with the highest concentrations of CuNPs coincides with a greater internalization of Zn.

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1. Introduction

In the last two decades, the continuous increase in the production and application of nanoparticles (NPs) has resulted in the release of these materials to aquatic ecosystems (Bystrzejewska-Piotrowska et al., 2009). Metal-based NPs are used in several applications because of their unique properties. In this work, we focus on the zinc oxide NPs (ZnONPs) and copper NPs (CuNPs). ZnONPs are used for instance in tires to extend product life, in sunscreens as UV absorbers (Osmond and McCall, 2010) as well as in various products for their antibacterial properties (Perelshtein et al., 2015). CuNPs are applied in batteries, ink, water regeneration systems and as bactericides (Ren et al., 2009; Rubilar et al., 2013). The annual production of ZnONPs reached the 550 tons in 2012 (Bondarenko et al., 2013) whereas the production of CuNPs was estimated in 200 tons in 2010 (Ho et al., 2017).All these produced NPs will reach the environment during several steps along their life-cycle.

The divalent ion forms of Zn and Cu are nutritionally essential for all living organisms as they are cofactors for a number of enzymes. However, when present in excess, these metals can cause adverse effects. They can interfere with homeostasis of other metals, cause DNA damage, and generate reactive oxygen species (ROS) that can adversely modify proteins, lipids, and DNA (Aruoma et al., 1991; Banci et al., 2010; Boyles et al., 2016; Halliwell and Gutteridget, 1984; Xie et al., 2006). The *in vitro* toxicity of CuNPs and ZnONPs has been well characterised by our research group (Bermejo-Nogales et al., 2017; Connolly et al., 2015, 2016; Fernandez-Cruz et al., 2013; Galbis-Martínez et al., 2018; Hernández-Moreno et al., 2016; Li et al., 2015a; Song et al., 2014). These studies revealed the complexity of the toxic effects of these nanomaterials and showed that although the toxicity is influenced by the metal (Zn or Cu) ions, the NPs themselves greatly contributes to the observed toxicity.

In addition to *in vitro* studies, the *in vivo* individual toxicity of ZnONPs and CuNPs has also been assessed. ZnONPs toxicity has been evaluated in studies across a wide range of taxonomic groups from different trophic levels, being algae and crustacea the most sensitive to ZnONPs (Adam et al., 2014, 2015; Bondarenko et al., 2013; Ma et al., 2013). It has been, in general, considered that the toxicity was mainly caused by dissolved ions. On the contrary, a study in *Daphnia magna* exposed to CuNPs or to ZnONPs suspensions found that the toxicity was caused by the particle fraction rather than the ion fraction (Xiao et al., 2015). Zn also tends to accumulate in fish exposed to ZnONPs as observed by Connolly et al. (2016), who evidenced by means of a bioaccumulation assay in rainbow trout (*Oncorhynchus mykiss*) exposed to ZnONPs that Zn levels were not eliminated during the depuration phase. Oxidative stress responses were observed in gills after ZnONPs bioaccumulation in this organ. Furthermore, exposures to high doses of ZnONPs resulted in an enhancement of ethoxy-resorufin-*O*-deethylase (EROD) associated with oxidative stress responses in liver. The impact of ZnO nanoparticle has been also assessed on the embryonic development of zebrafish (*Danio rerio*). In this case an increment in ROS, and therefore a higher level of cellular oxidative stress, was found in embryos exposed to ZnONPs (Zhu et al., 2009).

The adverse effects of CuNPs have also been documented. CuNPs induced serious toxicological effects and important injuries on kidney, liver, and spleen of mice (Chen et al., 2006). The individual CuNPs toxicity has also been assessed in diverse fish species that exhibit important differences in sensitivity to these NPs, being zebrafish (*Danio rerio*) more sensitive than fathead minnow (*Pimephales promelas*) and this species more than rainbow trout (Song et al., 2015). Other studies had evaluated the CuNPs toxicity in different stages of the development of zebrafish: embryos (Hua et al., 2014), larvae (Chen et al., 2011) and adults (Griffitt et al., 2007).

Once the NPs are released to the environment, they may interact with numerous pollutants, including other NPs. This fact has provoked a considerable concern about the potential adverse effects of NPs co-exposures. However, studies reporting the effects of NPs' combinations are scarce. Previous *in vitro* studies in our laboratory had assessed the toxicity of the co-exposure of ZnONPs and CuNPs at low- and non-toxic concentrations. An enhancement of CuNP's toxicity was found with the addition of ZnONPs in HepG2 cells (Li et al., 2015a) and PLHC-1 cells (Hernández-Moreno et al., 2016). The toxicity was attributed to the ZnONPs in both cases. Moreover, the results demonstrated that internalization of Zn ions protected the cell against the toxicity of the internalized CuNPs and ZnONPs. Higher levels of internalized Zn were found with increasing concentrations of CuNPs, during the co-exposure experiments.

In addition, *in vivo* studies performed in bacteria, algae and mouse showed that the effects induced after co-exposure of different NPs were different from those observed after exposure to individual NPs. Some studies suggested that the effects of NPs mixtures are weaker than expected when the added effects of the individual particles were considered. This was the case of the co-exposures to TiO_2NPs and ZnONPs in bacteria and zebrafish embryos (Hua et al., 2016; Tong et al., 2015). On the other hand, the effects of ZnONPs and graphene oxide NPs were additive in *Scenedesmus obliquus* and *Daphnia magna* but antagonistic to zebrafish (Ye et al., 2018). A recent work demonstrated a reduction of toxicity after the combination of CuONPs and ZnONPs to freshwater algae (Ye et al., 2017).

Taking into account all of the above, the toxic potential of multiple NPs mixtures need more studies to elucidate the possible adverse effects and the mechanisms of action. Thus, the main goal of the present work was to investigate, in rainbow trout fingerlings, the potential modulation of the acute toxic effect caused by CuNPs in the presence of ZnONPs, at concentrations previously estimated as non-toxic. In addition, in an attempt to elucidate the mechanisms underlying the observed toxicity, some biomarkers of the antioxidant defence system have been measured such as Glutathione-S-transferase (GST) activity and the ratio of reduced/oxidized glutathione (GSH/GSSG). The induction of EROD as a biomarker of chemical exposure and toxic effects in fish (Whyte et al., 2000) has also been studied. The liver and gill were selected to study these activities, since the first organ is responsible for metabolizing xenobiotics and the second one is the first barrier in contact with the NPs dispersed in medium. Furthermore, the levels of Zn and Cu accumulated in fish after 96 h exposure have been measured.

2. Materials and methods

2.1. Chemicals and nanomaterials

Uncoated CuNPs (50 nm, powder) were purchased from IoLiTec, Inc. (Heilbronn, Germany) and uncoated ZnONPs (25–30 nm, powder) were acquired from Tecnan (Los Arcos, Navarra). The size of the pristine NPs was characterised by transmission electron microscopy (TEM) in previous studies (Li et al., 2015b) using a JEOL 2100 HT (JEOL Ltd., Japan) operated at an accelerating voltage of 200 kV with integrated energy dispersive X-ray (EDX) spectroscopy (Oxford Inca). The mean diameters obtained were 63 ± 16 nm for CuNPs and 19 ± 4 nm for the ZnONPs. All chemicals and reagents used were purchased from Sigma Aldrich (Madrid, Spain) unless otherwise stated.

2.2. Fish exposure

Acute toxicity studies were carried out according to the OECD Test Guideline (TG) n° 203 (OECD, 1992). Rainbow trout (*O. mykiss*) fingerlings (mean initial weight and length of 0.3–0.7 g and 4 ± 1 cm) were kept in $0.59 \times 0.24 \times 0.23$ m^3 rectangular 30 L tanks supplied with semi-static flow water taken from a tank with dechlorinated and filtered tap water supplemented with a mixture of salts (Aquadur ®, JBL GmbH & Co. KG, Neuhofen, Germany) that allowed reaching an appropriate hardness (see conductivity values below). Before initiating the experiments, fish were acclimated for 10 days in the same aquaria

where the exposures took place. Fish were maintained under 16 h light, 8 h dark photoperiod. Physicochemical properties were measured every day in water, obtaining values of 7.46 ± 0.04, 12.95 ± 0.15 °C, 413.4 ± 3.01 mS/cm and >90% O2, for pH, temperature, conductivity and dissolved oxygen, respectively. The faeces were cleaned every day. Fish were fed daily at a rate of 2% of their body weight during the acclimation period. 24 h before starting the exposure period, fish were fasted as well as during the 96 h test. A commercial diet for trout, Inicio Plus 887 (BIOMAR Iberia, S.A., Dueñas, Spain), with pellets of 1.9 mm in diameter was used.

Two assays were performed. According to OECD TG 203 the study was developed with one single aquarium for each tested concentration, having the minimum number of animals required to evaluate the acute toxicity of a substance. In a first assay, animals were divided in six tanks (7 animals each) from which five were used to expose fish to ZnONPs at a range of nominal concentrations going from 0.625 to 50 mg/L (5 concentrations). An additional tank was the control group; fish were maintained in the same conditions and without any treatment. The NPs were suspended in 1 L water from the aquarium, vortexed for 1 min and then progressively added to the aquarium under agitation. Due to the precipitation observed, the water from the exposure tanks, was agitated periodically. The second assay consisted in the exposure of fish to a range of concentrations of CuNPs in combination with the no-observed effect concentration (NOEC) estimated for ZnONPs in the first experiment (1.25 mg/L, corresponding to 1.0 mg/L of Zn). In previous assays, an LC_{50} of 0.62 ± 0.15 mg/L and a lowest observed effect concentration (LOEC) of 0.17 mg/L for rainbow trout exposed to the same CuNPs as used in the present work were reported (Song et al., 2015). According to these results, CuNPs concentrations of 0.0425, 0.085, 0.17 and 0.34 mg/L were selected for the co-exposures. CuNPs in powder were dispersed in 1 L of water from the aquarium and sonicated in a waterbath for 10 min (S 40H Elmasonic water bath sonicator; Elma Schmidbauer GmbH). ZnONPs were added to the different CuNPs preparations, being agitated previously to its addition to the aquaria. Fish were also exposed to the highest concentration of CuNPs alone (0.34 mg/L). In this second assay, a control group was also included. Water from the aquaria was agitated periodically to mitigate the precipitation observed with the mixture of NPs. NPs in the exposure medium were characterised by DLS (size) and by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Cu and Zn concentrations) as indicated in the following sections.

All the experiments were performed according to the EU and national legislation for the use of laboratory animals after receiving a favourable report of the INIA ethical committee for animal experimentation and the corresponding authorization from the competent authority at the Community of Madrid regional government.

2.3. Characterisation of NPs in the exposure media

The hydrodynamic size and zeta potential of the NPs in the aquaria waters was determined by dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK). Size measurements were performed in each of the aquaria directly after preparation and every 24 h. Four independent measurements were taken with each measurement consisting of six runs, each of 20 s duration. Zeta potential was evaluated in each working solution directly after preparation, performing 3 measurements with a minimum of 10 and maximum of 100 runs each one.

2.4. Zn and Cu concentrations in aquaria water and in fish

Water samples from each aquarium were taken every 24 h (time 0, 24, 48, 72 and 96 h) to measure the Zn and Cu concentrations. In order to establish the contribution of Cu and Zn ions to the toxicity, according to Hernández-Moreno et al. (2016), duplicated samples were centrifuged (4600 ×g for 2 h at 4 °C), using a Gyrozen 1248R centrifuge (GYROZEN, Korea) and the supernatants were used to evaluate the ionic dissolved fraction. Four whole-body fish from each treatment were used to determine metal levels accumulated in fish after the exposure period. Fish bodies were stored at −30 °C until analysis. Fish samples were digested in a DigiPrep block (SCP SCIENCE, Canada) after drying at 100 °C during 24 h, crushing and grinding in a mortar, and taking 50 mg of this homogenate for digestion. Together with the homogenate, 0.5 mL of milliQ water, 2 mL of nitric acid, 0.5 mL of hydrofluoric acid were added to a 15 mL polyethylene DigiTUBEs (SCP SCIENCE, Canada). It was heated to 75 °C and held for 10 min, during which 0.5 mL of H_2O_2 drop-by-drop were added, raising the temperature to 120 °C and holding it for 15 min. Water samples were processed similarly, adding the same amounts of nitric acid, hydrofluoric acid and H_2O_2 to 10 mL of sample. The digestion was done with a similar temperature ramp. The Cu and Zn concentrations in water and Zn concentrations in fish were determined by ICP-OES and, due to the low Cu concentration in fish, Cu was determined by inductively coupled plasma – mass spectrometry (ICP-MS) in fish. An Agilent 5100 Synchronous Vertical Dual View with a vertical torch and charge coupled device CCD detector was used for ICP-OES. A Thermo iCAP-RQ (Thermo Scientific, Bremen, Germany) equipped with a quadrupole mass analyser and an electron multiplier detector was used for ICP-MS. For this technique, analyses were performed in collision cell (kinetic energy discrimination) mode with helium gas, in order to overcome isobaric and polyatomic interferences. OneNeb and Meinhard nebulisers (for ICP-OES and ICP-MS, respectively) with a baffled cyclonic spray chamber and a peristaltic pump were used for sample introduction. To validate the assay a certified reference material and spiked samples were analyzed by ICP-MS and ICP-OES. The certified reference material LGC6019 (River Water) was purchased from LGC (UK). Water and fish digested samples were spiked with 1 mg/L of Zn and 10 μg/L of Cu. The assay samples, the certified reference material and the spiked samples were prepared in triplicate, and from each replicate three measurements were taken. The measurement error was under 5% in all cases. Limits of detection (LOD) and limits of quantification (LOQ) were calculated as being 3 and 10 times the standard deviation of the blank, respectively. The LOQ obtained for both Zn and Cu were 30 ng/L for waters and 0.3 mg/kg for fish.

2.5. Oxidative stress studies

The livers and gills of three fish from each treatment were removed the last day of the experiment and stored at −80 °C until the development of the corresponding assays. EROD and glutathione-S-transferase (GST) activities and the ratio of reduced glutathione (GSH) and oxidized glutathione (GSSG) (GSH/GSSG) were monitored as biomarkers of toxicity and oxidative stress in both liver and gill tissues. Liver and gill samples were extracted and weighed (0.033 g ± 0.005 and 0.14 g ± 0.016, respectively). The entire liver or gills were used for EROD, GST, total glutathione (tGSH) and GSSG analysis. Each liver was homogenised in 150 μL and each gill in 350 μL of ice cold homogenisation buffer. The buffer was prepared with 0.1 M Tris HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.25 M sucrose, 150 mM KCl, 20% v/v glycerol, dithiothreitol (DTT) 1 mM and protease inhibitor cocktail (phenylmethylsulfonyl fluoride (PMSF) 0.125 mM, and 5 μg/mL of pepstatin A, aprotinin and leupeptin). Tissues were homogenised in buffer and sonicated using water bath sonication (S 40H Elmasonic, Elma, Germany) for 15 s to create a tissue homogenate. The total volume of homogenate was divided in three equal fractions. One fraction was used to perform EROD and GST activities and was centrifuged at 6704 ×g in a 5415 R series Eppendorf centrifuge for 10 min at 4 °C. The resulting supernatant was then centrifuged at 15,682 ×g for 60 min also at 4 °C using the same centrifuge. The supernatant was used for GST analysis and the resulting pellet for EROD analysis (Valdehita et al., 2012). The two remaining fractions of tissue homogenates were used for tGSH and GSSG analysis (one fraction for each assay). The homogenates were incubated for 1 h with a solution of 5-sulfosalicyclic

acid (5%) to remove interfering proteins (dilution1:2). Then they were centrifuged at 15,682 ×g for 30 min at 4 °C to obtain the supernatant used for analysis. For each of the assays, all samples were evaluated at the same time using 96-well plates (Greiner Bio-One GmbH, Germany) following the corresponding protocols described below.

2.5.1. EROD activity

EROD activity was quantified following the method of Burke and Mayer (1974). Fluorescence of the product formed, resorufin, was measured at 530 nm excitation and 590 nm emission in a Tecan-Genios microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Microsomal protein concentrations were quantified using a fluorescamine-based assay (Udenfriend et al., 1972) and bovine serum albumin as a standard. EROD activities were expressed as pmol resorufin/min/mg protein.

2.5.2. GST activity

GST activity was measured according to the method described by Habig et al. (1974) with slight modifications for a 96-well plate format (Tiwari et al., 2011). The total reaction volume per well was 200 μL consisting of 20 μL of sample (diluted 1:25 in potassium phosphate buffer (0.1 M) pH 6.5) and 180 μL of a reaction mixture of 1-chloro-2,4-dinitrobenzene (CDNB) (1 mM) and GSH (10 mM) prepared in potassium phosphate assay buffer (pH 6.5). The product of conjugation of GSH with CDNB was measured at 340 nm absorbance in a Tecan-Genios microplate reader. Changes in absorbance per min were converted into nanomoles of CDNB conjugated per min and presented as nmoles/min/mg prot.

2.5.3. GSH and GSSG levels

Both tGSH and GSSG were measured according to the modified method of Allen et al., (2000). The assay is based on the sequential oxidation of GSH by 5,5′-dithio bis 2-nitrobenzoic acid (DTNB) and reduction by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of glutathione reductase (GR) (Griffith, 1980). Samples were diluted in assay buffer (sodium phosphate buffer/EDTA (143 mM/6.3 mM)) using a 1:50 and 1:25 dilution factor for tGSH and GSSG analysis, respectively. The total reaction volume per well was 150 μL consisting of 25 μL sample/standard and 125 μL of a reaction mixture of GR (229 U/mL), NADPH (2.39 mM) and DTNB (0.01 M) prepared in assay buffer. The reaction was monitored by measuring the 5′-thiol-2-nitrobenzoic acid (TNB) product formation at 405 nm absorbance every min over a 10 min time frame in a Tecan-Genios microplate reader. The concentration of tGSH was calculated from a GSH standard curve. Levels of GSSG were measured in the same way with 1 h pre-incubation of samples and standards with 2 μL of 4-vinyl pyridine solution per 100 μL. Levels of reduced glutathione (GSH) were calculated by subtracting the amount of GSSG from tGSH content and the ratio of GSH/GSSG was calculated.

2.6. Statistical analysis

The estimation of the concentration-response function and the calculation of the LC_{50} (concentration causing a 50% of death with respect to the controls) for ZnONPs and mixture of NPs treatments were done by fitting the assay results to a regression model equation for a sigmoid curve:

$$y = \max/(1 + e{-}[(x{-}IC_{50})/b]) + \min$$

where max is the maximal response observed, b is the slope of the curve and min the minimal response. The results from the bioassays and metal levels measurements are expressed as the mean ± standard error of the mean (SEM), n = 3 or n = 4, respectively. The normality and homoscedasticity of all data were checked by the Shapiro–Wilk test and Bartlett's test, respectively. A parametric one-way analysis of variance (ANOVA) followed by a Holm-Sidak *post hoc* test was applied ($p < 0.05$). All the analyses were performed with SigmaPlot 12.5 (Systat Software Inc., Chicago, IL, USA).

3. Results

3.1. Characterisation of nanoparticles in the exposure medium

Nanoparticle size distribution in water was evaluated by DLS. However, measurements were not conclusive, since polydispersity indexes presented values higher than 0.6. Results showed unstable suspensions, which did not meet quality criteria for a correct measurement, probably due to the low concentrations used in the present study. All the NPs (mixture or alone) suspensions showed negative charged zeta potential values. *Z*-potential was found −6.41 ± 0.205 mV for ZnONPs (1.25 mg/L) and −12.5 ± 0.1 mV for CuNP (0.34 mg/L), whereas values for the mixtures were −10.1 ± 1.98, −9.69 ± 1.15, −13.4 ± 1.3 and −14.3 ± 0.9 from the lowest to the highest CuNPs concentrations, respectively. These values indicated that suspensions were not stable.

Metal (Cu and Zn) content was evaluated in the water samples of all exposure groups before and after centrifugation to evaluate the ion release during the exposure period (Fig. 1). Levels of Cu in the three lower co-exposure groups were similar to those of the control. Unexpectedly, Cu levels were higher in the waters treated with the mixture of CuNPs 0.34 mg/L and ZnONPs than in those containing only CuNPs (0.252 mg/L and 0.145 mg/L after 96 h exposure, respectively). Real concentrations were in all cases lower than nominal concentrations. When the centrifuged samples were analyzed, Cu ion levels increased linearly with time and concentration from 24 h exposure (Fig. 1). The percentage of ion release related to nominal concentration after 96 h exposure were 14, 13 and 17% for the waters treated with 0.0425, 0.085, and 0.17 mg CuNPs/L, and 24 and 26% for the treatments with CuNPs at 0.34 mg/L with and without ZnONPs, respectively. On the other hand, no differences were found between the different treatment groups in relation to Zn concentrations (Fig. 1). In fact, this was expected since ZnONPs nominal concentration was fixed at 1.25 mg/L (1 mg/L of Zn) for all the co-exposure groups. Real concentration of Zn reached around 50% of the nominal concentration. The percentage (related to nominal concentrations) of Zn ions release in waters from the co-exposure groups after 96 h was 42.6 ± 3.6% (mean ± SD). The loss of concentration of Zn was not observed in the waters of the different groups of fish treated with the ZnONPs alone (data not shown).

3.2. Acute toxicity assay

All the requirements cited in the OECD 203 were fulfilled, observing no death in control fish during both experiments. Records were kept of visible abnormalities. No loss of equilibrium or abnormal swimming behaviour was observed. In addition the respiratory rate, posture in the water column and pigmentation were normal in all the groups. Measurement of pH, dissolved oxygen and temperature were carried out daily.

As result of the 96 h exposure to a range of concentrations of ZnONPs (Fig. 2), a NOEC of 1.25 mg/L and an LC_{50} of 3.03 mg/L were derived from the dose-response curve. Once established the NOEC for ZnONPs, this concentration was used to perform the co-exposure experiments. As well, CuNPs were tested alone at the highest concentration. No mortality was recorded (Fig. 2). However, once fish were exposed to the combination of both NPs, toxic effects were observed (Fig. 2). There was an inverse relationship between the mortality observed and the concentration of Cu. Fish co-exposed to the lowest concentration (0.0425 mg/L) of CuNPs started to die after 24 h exposure (1 fish). After 48 h, only one animal was affected from the 0.085 mg/L tank. The third day of the experiment (72 h) the lethal effect was more notable affecting to all the treated groups (4, 2, 2 and 1 fish from the tanks treated with 0.0425, 0.085, 0.17 and 0.34 mg CuNPs/L 1.25 mg ZnONPs/L, respectively). In

Cu levels in exposure water
Zn levels in exposure water
Cu levels in exposure water after centrifugation
Zn levels in exposure water after centrifugation
% Cu ion release
% Zn ion release
Concentration (mg/L)
% ion release
Time (h)
CTRL
Cu0.34
Cu0.34+Zn1.0
Cu0.17+Zn1.0
Cu0.085+Zn1.0
Cu0.042+Zn1.0

Fig. 1. Cu and Zn levels (mg/L) in the different water treatments along the experiment period and identification of ion release from NPs. Values are presented as mean ± SD of two measurements.

total, 5, 3, 2 and 1 fish died after co-exposure to 0.0425, 0.085, 0.17 and 0.34 mg CuNPs/L. The last day of exposure, there were no casualties.

3.3. Cu and Zn levels in fish

Cu and Zn levels were determined in fish bodies at the end of the experiment and are presented in Figs. 3 and 4, respectively. Cu content in body fish showed an increased trend associated to the increased levels of CuNPs in the exposure medium. In fact, significant differences related to the control group were found in those fish exposed to CuNPs 0.17 mg/L and ZnONPs, CuNPs 0.34 mg/L and ZnONPs and CuNPs 0.34 mg/L alone. The measured concentrations (mean ± SEM, n = 4) were 19 ± 6 mg/kg ($p < 0.05$), 27 ± 3 mg/kg ($p < 0.01$) and 25 ± 5 mg/kg ($p < 0.01$), respectively. Zn levels in fish also increased

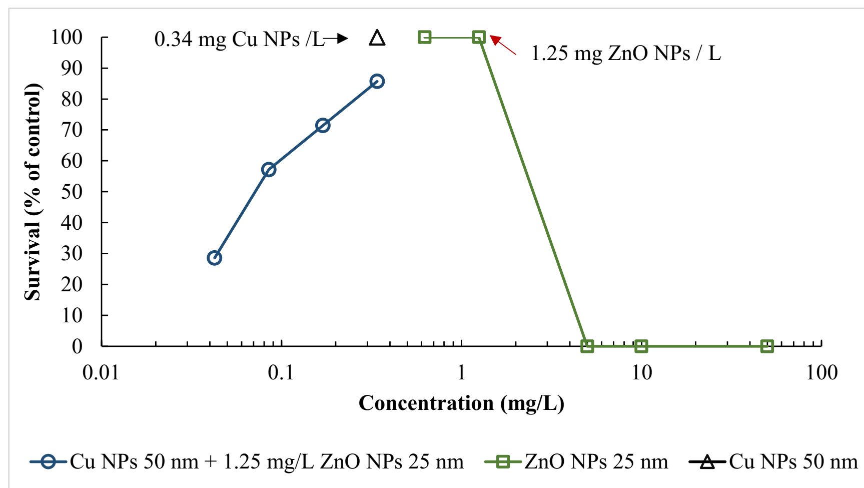

Fig. 2. Percentage of fish survival related to the control group after 96 h exposure to a concentration range of ZnONPs (0.625–50 mg/L), to a concentration range of CuNPs (0.0425–0.34 mg/L) in co-exposure with a fixed concentration of ZnONPs (1.25 mg/L) and to the highest concentration tested of CuNPs (0.34 mg/L).

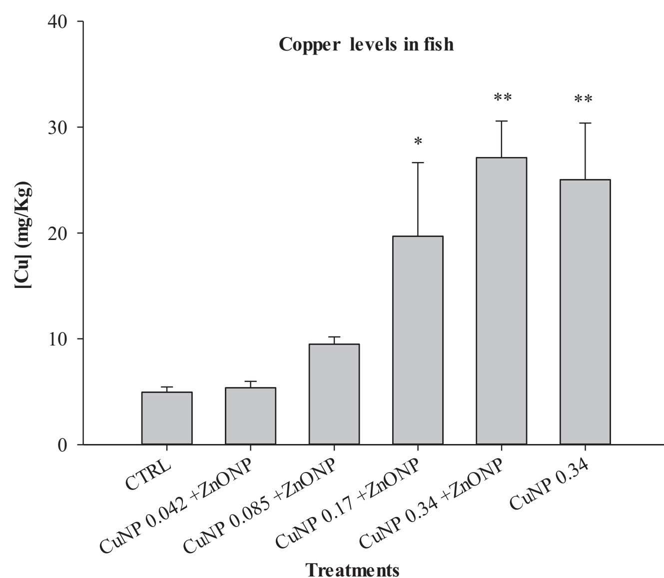

Fig. 3. Copper levels (mg/kg) in the different exposed fish groups (control, Cu NPs 0.34 mg/L, ZnONPs 1.25 mg/L and co-exposure varying CuNP concentrations (0.0425, 0.085, 0.17 and 0.34 mg/L).. Bars represent the mean ± SEM (n = 4). Significant differences between the control and treatment groups are represented as * ($p < 0.05$) or ** ($p < 0.01$) according to a one-way ANOVA, Holm-Sidak *post hoc* test.

significantly in those fish exposed to CuNPs 0.17 mg/L and ZnONPs (247 ± 40 mg/kg, $p < 0.01$) or CuNPs 0.34 mg/L and ZnONPs (262 ± 50 mg/kg, $p < 0.01$). As expected, fish exposed to CuNPs alone presented similar levels of Zn that control fish. In addition, fish exposed to ZnONPs at a NOEC showed levels of Zn close to those found in the controls.

3.4. Biomarkers

3.4.1. EROD activity

The basal value of EROD activity in liver and gill of the control group was 146.6 ± 8.9 and 9.5 ± 0.9 pmol/min/mg protein (mean ± SEM, n = 3), respectively. The EROD activity was not modified neither in liver nor in gill of rainbow trout after the exposure with CuNPs (0.34 mg/L), ZnONPs (1.25 mg/L), or after the different co-exposure treatments (data not shown).

3.4.2. Glutathione S transferase (GST) activity

The basal value of GST activity in the liver tissues of the control group was 837.1 ± 39.4 nmol/min/mg prot (mean ± SEM, n = 3) (Fig. 5a). Following exposure to 1.25 mg/L of ZnONPs or after 0.34 mg/L of CuNPs, the GST activity was not significantly modified. However, when fish were co-exposed to different CuNPs concentrations in combination with 1.25 mg/L of ZnONPs, an important decrease but not significant in GST activity in liver was observed at the lowest concentration of CuNPs used (0.0425 mg/L), reaching 522.7 ± 12.1 nmol/min/mg prot. The co-exposure with CuNPs at 0.085 mg/L caused a significant enhancement (1383.13 ± 16.3 nmol/min/mg prot) in the GST activity in this organ. In the other co-exposed groups, no relevant changes in GST liver activity were produced with respect to controls. On the other hand, the GST activity in the gill (Fig. 5b) was neither modified after CuNPs (0.34 mg/L) or ZnONPs (1.25 mg/L) exposures. But the co-exposure with ZnONPs (1.25 mg/L) and CuNPs increased significantly the GST activity at all CuNPs concentrations tested except at 0.17 mg/L, probably due to the small number of animals (n = 3) and high variability among them. The GST activity in the control group was 613.9 ± 46.8 nmol/min/mg protein and it reached 1338.4 ± 141.8, 1010.62 ± 117.4, 821.8 ± 108.4 and 946.7 ± 66.1 nmol/min/mg protein in the groups co-exposed to 0.0425, 0.085, 0.017 and

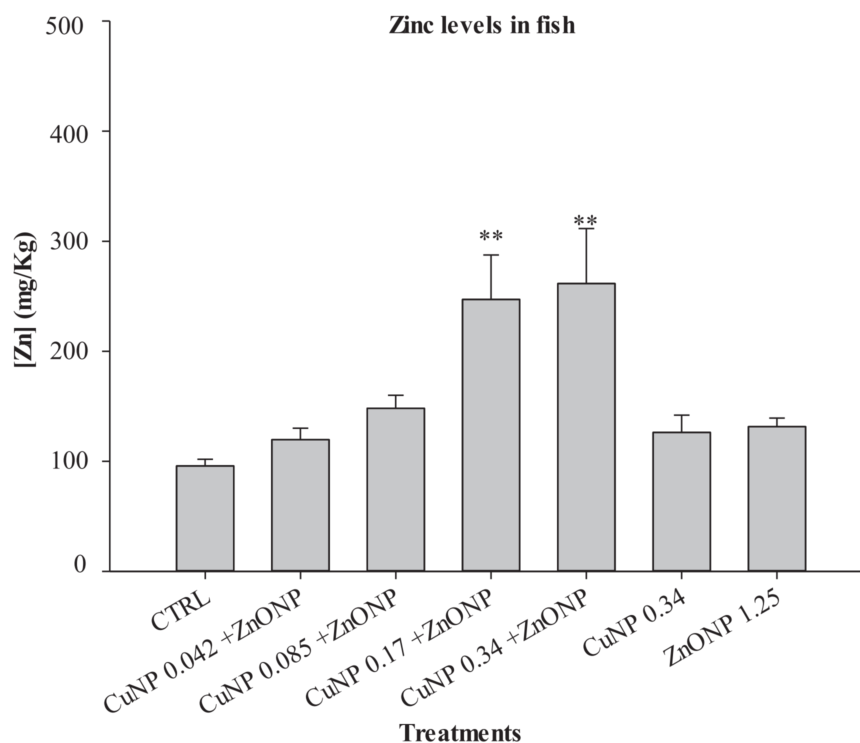

Fig. 4. Zinc levels (mg/kg) in the different exposed fish groups (control, Cu NPs 0.34 mg/L, ZnONPs 1.25 mg/L and co-exposure varying CuNP concentrations (0.0425, 0.085, 0.17 and 0.34 mg/L). Bars represent the mean ± SEM (n = 4). Significant differences between the control and treatment groups are represented as * ($p < 0.05$) or ** ($p < 0.01$) according to a one-way ANOVA, Holm-Sidak *post hoc* test.

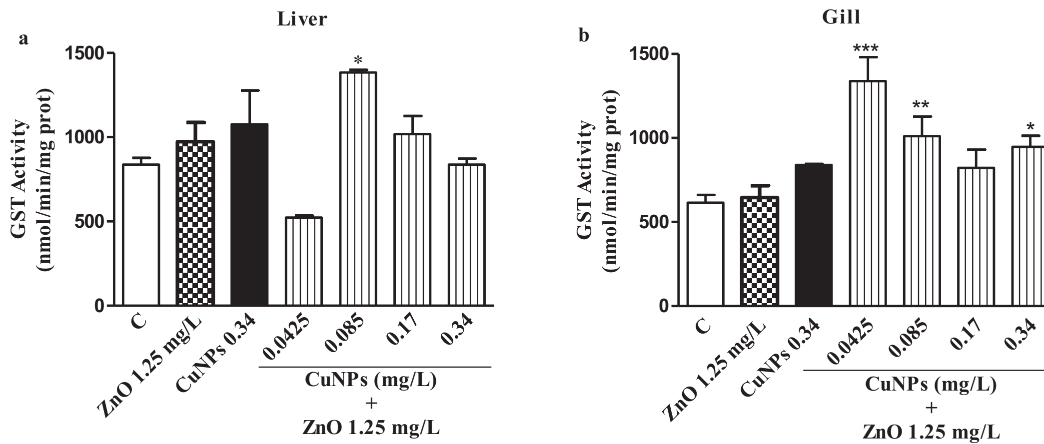

Fig. 5. GST activity in samples of cytosolic fractions prepared from homogenised tissues of the livers (a) and gills (b) of control and treatment groups (Cu NPs 0.34 mg/L, ZnONPs 1.25 mg/L and co-exposure varying CuNP concentrations (0.0425, 0.085, 0.17 and 0.34 mg/L). Bars represent the mean ± SEM (n = 3). Significant differences between the control and treatment groups are represented as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$) according to a one-way ANOVA, Holm-Sidak *post hoc* test.

0.34 mg/L, respectively ($p \leq 0.001$, $p < 0.01$, $p > 0.05$ and $p < 0.05$, respectively).

3.4.3. GSH/GSSG ratio

The GSH/GSSG ratio in the liver tissues of the control group was 2.05 ± 0.2 (mean ± SEM, n = 3) (Fig. 6a). For the animals receiving CuNPs (0.34 mg/L) or ZnONPs (1.25 mg/L), there was no significant difference in GSH/GSSG ratios with respect to controls. However, after the co-exposure with ZnONPs (1.25 mg/L) and CuNPs (0.0425 mg/L) the ratio increased significantly (4.13 ± 0.11, $p < 0.001$). Following exposure to higher concentrations of CuNPs the response was the opposite, thus the GSH/GSSG ratio begins to decrease significantly reaching values of 0.21 ± 0.01 and 1.29 ± 0.02 ($p < 0.001$ and $p < 0.05$, respectively) at the two highest concentrations of CuNPs (0.17 and 0.34 mg/L), respectively. The GSH/GSSG ratio in the gill tissues of the control group was 1.16 ± 0.1 (Fig. 6b). In this tissue, only the co-exposure with ZnONPs and the lowest CuNPs concentrations (0.0425 mg/L) increased significantly the GSH/GSSG ratio to 1.59 ± 0.1 ($p < 0.05$).

4. Discussion

The present study establishes hazard values for rainbow trout after 96 h exposure to ZnONPs of 25 nm, reporting a NOEC of 1.25 mg/L and an LC_{50} of 3.03 mg/L. Our values are in accordance with those reported for zebrafish exposed to ZnONPs of 14 nm, showing an LC_{50} of 2.9 mg/L and a LC_{10} of 1.6 mg/L (Ye et al., 2018).

The adverse effects of CuNPs were also documented in diverse fish species (Barjhoux et al., 2012; Chen et al., 2011; Griffitt et al., 2007; Hua et al., 2014; Song et al., 2015; Wang et al., 2014). Indeed, Song et al. (2015) identified hazard values for *O. mykiss* after a 96 h exposure to the same CuNPs, of 0.68 ± 0.15 mg/L and 0.17 mg/L as LC_{50} and LOEC, respectively. Based on these values, our experiments were developed taking a maximum concentration of 0.34 mg/L (1/2 LC_{50}) expecting to observe mild toxic effect. Nevertheless, when rainbow trout fingerlings were exposed to 0.34 mg/L of these CuNPs individually, no mortality was registered in our laboratory. The co-exposure to CuNPs at concentrations ≤0.34 mg/L and ZnONPs at a NOEC concentration of 1.25 mg/L resulted in non-expected lethal effects, which were inversely –related to the CuNPs concentration. In previous *in vitro* studies in a fish and a

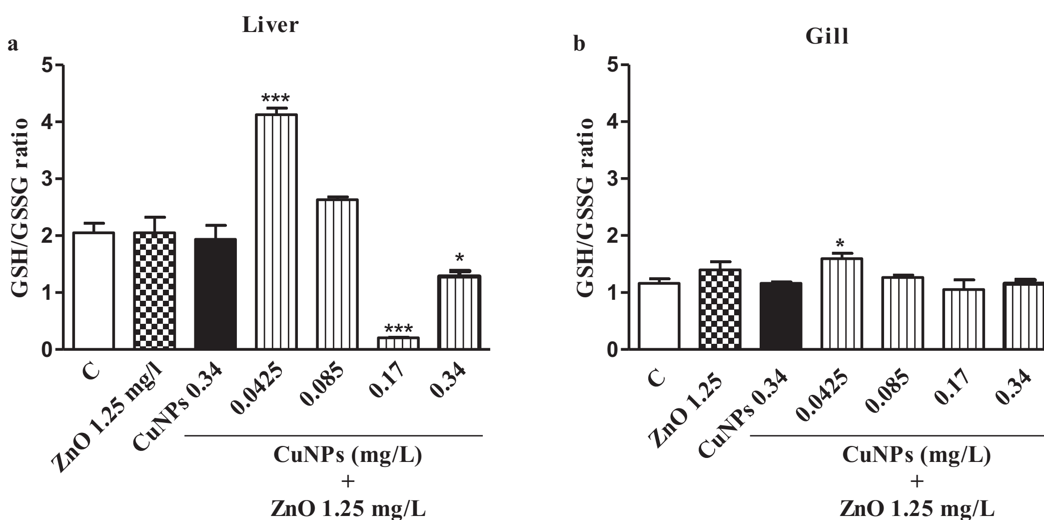

Fig. 6. GSH/GSSG ratios in samples of cytosolic fractions prepared from homogenised tissues of the livers (a) and gills (b) of control and treatment groups (Cu NPs 0.34 mg/L, ZnONPs 1.25 mg/L and co-exposure varying CuNP concentrations (0.0425, 0.085, 0.17 and 0.34 mg/L). Bars represent the mean ± SEM (n = 3). Significant differences between the control and treatment groups are represented as * ($p < 0.05$) or *** ($p < 0.001$) according to a one-way ANOVA, Holm-Sidak *post hoc* test.

mammalian cell lines we also observed an increase of the toxicity of the CuNPs in presence of a non-toxic concentration of the ZnONPs, but the response was dose-related (Li et al., 2015a; Hernández-Moreno et al., 2016). In a recent work performed in freshwater algae the co-exposure to CuONPs and ZnONPs resulted in a reduction of toxicity (Ye et al., 2017). To our knowledge, there are no studies about the acute toxicity induced in fish by the co-exposure of CuNPs and ZnONPs.

In an attempt to elucidate the underlying mechanisms of toxicity, we have measured the accumulated levels of Cu and Zn in fish after 96 h exposure, and we have measured oxidative stress related parameters in liver and gills. In addition, the levels of Cu and Zn in the exposure waters were measured daily along the assay to identify the NPs concentrations and the ion release. After 24 h of co-exposure to CuNPs and ZnONPs, the first lethal effects were recorded at the lowest CuNPs concentrations. The highest lethal effects were observed after 72 h exposure in all groups co-exposed to both NPs, with a higher mortality detected for the lower CuNPs concentrations. At this time point, the percentage of Cu ions released to water in the group co-exposed to ZnONPs and 0.0425 mg CuNPs/L were lower than those measured in the group co-exposed to ZnONPs and the highest CuNPs concentrations (0.34 mg/L); 6.5% and 16%, respectively. At the same time point, the percentage of Zn ions released was the same in all the co-exposed groups ($36 \pm 2\%$, mean $\pm$ SD, $n = 4$ groups). These results indicate that fish have been mainly exposed to CuNPs and ZnONPs, and that the ion release does not explain the higher percentage of lethality registered with the lower concentrations of CuNPs. A previous study of acute toxicity performed in zebrafish with CuNPs or with the corresponding Cu ion dissolved fraction demonstrated that the effects of nanocopper on fish were not mediated solely by dissolution of the particulate copper (Griffitt et al., 2007). Something curious from the study of Cu levels in water along the exposure time is the higher Cu levels measured in the group exposed to CuNPs at 0.34 mg/L in combination with ZnONPs in relation to those measured in the group treated only with CuNPs (0.34 mg/L). It could be that the presence of CuNPs and ZnONPs avoid the aggregation of CuNPs, thus favouring the maintenance of the NPs in suspension, whereas when the CuNPs are alone they could aggregate and deposit on the walls of the aquaria (whereas ZnONPs seem to be less aggregated when they are alone). This could also contribute to explain the differences in mortality between the groups exposed to 0.34 mg/L of CuNPs alone or co-exposed to 0.34 mg/L of CuNPs and ZnONPs (no mortality *versus* a 15% mortality, respectively).

The accumulated levels of Cu in the fish increased with the exposure concentration of CuNPs. These increases were significantly different from the control group in the groups treated with the two highest concentrations of CuNPs. The levels reached were similar in the animals treated with 0.34 mg CuNPs/L alone or in co-exposure with ZnONPs. For the measured Zn levels in fish, despite having been exposed to the same concentration of ZnONPs, the concentration increased as the concentration of CuNPs administered increased being significantly higher than the controls at the two highest concentrations. The presence of CuNPs would, therefore, favour the accumulation of Zn. This was also observed in our previous studies in cell lines (Li et al., 2015a; Hernández-Moreno et al., 2016). Moreover, the highest levels of Zn could be correlated with the lowest mortality of animals. This fact could be explained from a physiological point of view by the essential nature of Zn. In fact, this protective effect was also observed in our previous two works in cells.

Other authors have studied the effects of waterborne CuNPs and $CuSO_4$ on rainbow trout by measuring copper levels in tissues (Shaw et al., 2012). They found that copper accumulated in the gills more quickly with the salt but an accumulation of Cu in intestine was only observed with the NPs. These authors also studied the effects on haematology and biochemistry and they found that CuNPs have similar types of toxic effects to $CuSO_4$, which can occur at lower tissue Cu concentrations than expected for the salt. They suggested that CuNPs are an ionoregulatory toxicant to rainbow trout showing important decreases in branchial, brain and intestine Na/ K- ATPase activity with depletion of plasma and carcass ion concentrations. They also found that CuNPs increased thiobarbituric acid reactive substances (TBARS) levels in gills and intestine.

Mansouri et al. (2018) also studied the impact of ZnONPs *versus* Zn ions on the gills of rainbow trout following a 14 days waterborne exposure. They found that although the accumulation capability of Zn ions was higher than ZnONPs, the NPs caused more structural damages to gills compared to ions. Zn accumulation in the gills, liver and intestine of rainbow trout with alterations of oxidative stress biomarkers (EROD, GST and GSH/GSSG ratio) after a 10 days dietborne exposure to ZnONPs has also been reported (Connolly et al., 2016). In a study (Abdelazim et al., 2018) performed with Nile tilapia (*Oreochromis niloticus*) exposed to ZnONPs, GSH levels and GST activity were decreased in the muscle.

Results from the present study also indicated effects on the GST and GSH/GSSG ratio in gill and in liver following the co-exposure to CuNPs and ZnONPs, but not in those groups exposed to the CuNPs or ZnONPs alone. GST was slightly increased in liver of fish exposed to the second lower concentration of CuNPs (0.085 mg/L) and ZnONPs. However higher effects were observed in the gills with significant increases of GST after co-exposure to ZnONPs with the lowest CuNPs concentration (0.0425 mg/L) and with 0.085 and 0.34 mg/L of CuNPs. Mwaanga et al. (2014) reported results for CuONPs and ZnONPs on GST enzyme activity in *Daphnia magna*, indicating a concentration dependent decrease in the enzyme activity for both metal oxide NPs. These results contrast with those from the present study, where it appears an increase in GST activity in fish. Copper binds thiol-containing molecules such as glutathione and, it has been observed a reduction of tGSH in the livers of three-spined stickleback, *Gasterosteus aculeatus*, exposed to $CuSO_4$ (Sevcikova et al., 2011). Some authors described that the accumulation of copper in hepatocytes, as a time-related process, may exert a negative effect on EROD activity by lowering the available GSH due to inhibited synthesis or higher oxidation to GSSG (Ghosh et al., 2001). However, in our study with fish, EROD activity was not modified in any of the evaluated tissues, even when the ratio GSH/GSSG suffered a decrease in the livers of fish co-exposed to ZnONPs with CuNPs at 0.17 and 0.34 mg/L. The ratio GSH/GSSG increased in liver and gills with the lowest concentration of CuNPs, neither influencing the EROD activity. A reduction of tGSH levels similar to this reported for the three-spined stickleback exposed to $CuSO_4$ was also observed in the present study in fish co-exposed to the highest concentrations of CuNPs, but not to CuNPs alone.

The effects observed on the biomarkers of the antioxidant defence system (GST and GSH/GSSG ratio) indicated that the co-exposure to these NPs induce oxidative stress at all concentrations tested. The higher toxicity produced at the lowest co-exposure concentration could be explained by the highest induction of oxidative stress in liver and gill at this dose level as it can be deduced from the high levels of GST measured and the unbalance on the GSH/GSSG ratio. The accumulation of Zn ions at the highest co-exposed concentrations could explain the protection observed, as it has been discussed previously (Li et al., 2015a; Hernández-Moreno et al., 2016).

Nowadays, it is unclear the range of concentrations of Cu and Zn coming from the nanoparticles that are present in the environment. Different models have been developed to predict these concentrations. Gottschalk et al. (2009) indicated concentrations of ZnONPs in surface water ranging from 0.008 to 0.055 μg/L, being 0.010 μg/L the most frequent value considered by the model. Pu et al. (2016) estimated that ZnONPs and CuNPs, among other metal NPs, appear at concentrations lower than 1 μg/L at aquatic settings. These estimations indicated lower concentrations than those assayed in this work. However, it should be noticed that nanomaterial's production is exponentially increasing, making possible higher concentrations in water in the future. In addition, ZnO (non-nanomaterial) is used at concentrations of 20 mg/kg in the rainbow trout feed as a supplement of Zn (EFSA, 2014). In the future, it is possible the use of ZnONPs in fish feed as it has being proposed for pigs (Wang et al., 2017).

In conclusion, the co-exposure of rainbow trout to non-toxic concentrations of CuNPs and a fixed non-toxic concentration of ZnONPs resulted in lethal effects inversely related to the concentration of CuNPs. The higher accumulation of Zn in the fish exposed to higher concentrations of CuNPs could protect from oxidative stress or other toxicity mechanisms, so that the higher toxicity and oxidative stress levels were observed with the lower CuNPs concentrations. One remaining issue is the reason of the higher toxicity caused by the lower CuNPs concentrations in the co-exposure treatments with respect to the lower toxicity observed in fish exposed to CuNPs alone at the highest concentration. This must be clarified in further studies, but we can hypothesize that the co-exposure to CuNPs and ZnONPs causes an interaction of them resulting in an unexpected toxic effect, which is progressively reversed by a higher accumulation of Zn ions in fish in presence of increasing concentrations of Cu. This was also observed *in vitro* in cell lines (Li et al., 2015a; Hernández-Moreno et al., 2016). In addition, as it has been reported previously (Shaw et al., 2012; Mansouri et al., 2018), lower concentrations of CuNPs and ZnONPs are capable of producing more severe effects than the corresponding ions. Therefore, it could be that the higher measured concentrations of Cu and Zn in fish correspond to ions and the observed effects at lower concentrations are due to NPs, as happened in previous studies developed *in vitro*.

CRediT authorship contribution statement

David Hernández-Moreno: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Ana Valdehita:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Estefanía Conde:** Methodology, Formal analysis, Investigation. **Isabel Rucandio:** Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Supervision. **José-María Navas:** Resources, Formal analysis, Writing - original draft. **María-Luisa Fernández-Cruz:** Conceptualization, Methodology, Formal analysis, Resources, Writing - original draft, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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