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RESEARCH PAPER

# Comparative toxicity of metal oxide nanoparticles (CuO, ZnO and $TiO_2$) to developing zebrafish embryos

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**Abstract** Increasing use of nanomaterials is resulting in their release into the environment, making necessary to determine the toxicity of these materials. With this aim, the effects of CuO, ZnO and $TiO_2$ nanoparticles (NPs) on zebrafish development were assessed in comparison with the effects caused by the ionic forms (for copper and zinc), bulk counterparts and the stabilizer used for rutile $TiO_2$ NPs. None of the NPs caused significant embryo mortality. CuO NPs were the most toxic affecting hatching and increasing malformation prevalence ($\geq$1 mg Cu/L), followed by ZnO NPs that affected hatching at $\geq$5 mg Zn/L and stabilized $TiO_2$ NPs that caused mortality and decreased hatching at 100 mg Ti/L. Exposure to the stabilizer alone provoked the same effect. Thus, toxicity of the $TiO_2$ NP suspension can be linked to the surfactant. For all the endpoints, the greatest effects were exerted by the ionic forms, followed by the NPs and finally by the bulk compounds. By autometallography, metal-bearing deposits were observed in embryos exposed to CuO and ZnO NPs, being more abundant in the case of embryos exposed to CuO NPs. The largest and most abundant metal-bearing deposits were detected in embryos exposed to ionic copper. In conclusion, metal oxide NPs affected zebrafish development altering hatching and increasing the prevalence of malformations. Thus, the use and release of metal oxide NPs to the environment may pose a risk to aquatic organisms as a result of the toxicity caused by NPs themselves or by the additives used in their production.

**Keywords** Zebrafish development · Metal oxide nanoparticles · Fish embryo test · Nanotoxicology · Environmental and health effects

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## Introduction

In recent years, our ability to engineer matter at the nanoscale has been developed to a point in which new products or enhanced materials arising from nanotechnology have been introduced in the markets worldwide (Maynard et al. 2006).

Due to their small size and consequent large number of surface atoms per mass unit, nanoparticles (NPs) possess unique mechanical, catalytic and optical properties as well as electrical conductivity when compared with their bulk counterparts (Niemeyer 2001; Oberdörster et al. 2005). These unique properties make them suitable for many industrial processes, and consequently, manufactured NPs are currently used in different areas, such as electronics, biomedicine, pharmaceuticals, cosmetics, environmental analysis and remediation, catalysis and material sciences (Nowack and Bucheli 2007; Ju-Nam and Lead 2008). Concomitantly with the increasing integration of nanomaterials (NMs) in outstanding technological applications and their introduction in mass produced commercial goods, concerns are rising due to nano-specific properties potentially leading to unforeseen health or environmental hazards (Maynard et al. 2006). The distinctive behaviour of nanometre-scale particles when compared to their larger counterparts has led to the development of nanotoxicology, the branch of toxicology responsible for assessing the effects of these NMs on living organisms (Donaldson et al. 2004). It would be appropriate to ensure that when novel chemical substances, such as NPs, are produced for commercial use, a risk assessment is carried out prior to mass production and use, in case such applications bring forth negative impacts on the environment (Lee et al. 2010; Thomas et al. 2011; Warheit et al. 2008). Toxicological evaluation of the biological effects plays a key role in the assessment of chemicals safety.

As the most numerous and phylogenetically diverse group of vertebrates, fish have been valuable models for the understanding of fundamental processes in vertebrate evolution, development and disease (Spitsbergen and Kent 2003). In this context, over the past decades, fish have become model organisms for toxicological studies (Spitsbergen and Kent 2003; Teraoka et al. 2003). Among fish species, the zebrafish (*Danio rerio*) is one of the most used models. Studies using zebrafish have increased exponentially due to the multiple advantages it offers as a toxicological model (Hill et al. 2005). The deleterious effects of different substances, including manufactured NPs, have been assessed in studies conducted with adult zebrafish (Griffitt et al. 2007; Hill et al. 2005; Xiong et al. 2011; Yu et al. 2011). In this scenario, the increasing efforts on seeking alternatives to traditional animal tests make zebrafish embryos even more of a suitable choice when toxicity screenings have to be performed (Busch et al. 2011; Lin et al. 2011; Yang et al. 2009). Their high reproductive output and the optical clarity of zebrafish embryos allow the performance of different bioassays and the direct observation of embryo development and alterations (Teraoka et al. 2003). Several toxicity assays have been developed using zebrafish embryos (Hallare et al. 2006; Lammer et al. 2009; Nagel 2002). Viability and morphological assessment are the typical endpoints used in these studies to assess developmental toxicity (Augustine-Rauch et al. 2010). Interestingly, zebrafish embryos have been proposed as a predictive model for NMs toxicity assessment (Fako and Furgeson 2009), and several studies have already been conducted (Asharani et al. 2008; Lee et al. 2007; Zhu et al. 2007, 2008). Results indicate that nano-sized metals can cause both lethal and sublethal effects on developing fish, which include increased mortality, abnormal development and hatching rate reduction (Shaw and Handy 2011). Specifically, metal oxide NPs cause different toxic effects, including tissue damage, acute lethality or induction of ROS production (Lin et al. 2011; Zhao et al. 2013; Zhu et al. 2008, 2009). Among the range of nanotechnology products, in this work, we have focused our attention to three metal oxide NPs (CuO, ZnO and $TiO_2$) that are widely implemented in consumer products. The soluble and bulk forms of these metals are toxic to aquatic organisms (Bernardeschi et al. 2010; Clearwater et al. 2002; Grosell et al. 2007; Larsson et al. 1980; Stohs and Bagchi 1995), implying that the potential adverse effects over biota could also exist for the nanoparticulate forms; additionally, such effects, given NPs unique properties, could be different from those caused by the ionic and bulk forms. Thus, to further characterize the toxicity of metal oxide NPs, in this study, we employed zebrafish embryo testing to analyse the toxic effects of CuO, ZnO and $TiO_2$ NPs in comparison with their bulk and ionic counterparts (in the case of copper and zinc) and when necessary with other substances present in the NPs suspensions.

CuO NPs are used as additives in lubricants, in computer processors, conductive coatings, printer inks and cosmetics, and as antimicrobial agent (Ren et al. 2009; Shaw and Handy 2011). These NPs have already been shown to be toxic to different organisms producing oxidative stress and DNA damage (Lee et al. 2010). In zebrafish, embryo exposure to CuO NPs reduces hatching rate at concentrations higher than 0.5 mg CuO/L (Lin et al. 2012).

ZnO NPs are applied in electronic sensors, solar voltaic devices as well as in the production of sunscreens, cosmetics, paints, ceramics and fungicides or in wastewater treatment (Lee et al. 2010; Vaseem et al. 2010). Previous studies in zebrafish embryos have shown that ZnO toxicity is dose-dependent and similar for the nanoparticulate and bulk forms (Zhu et al. 2008), although inconsistent results appear in the literature. Specifically, Zhu et al. (2008) reported LC50 values of 1.793 mg/L and 1.55 mg/L for the ZnO NPs and bulk ZnO, respectively. However, in other works, mortality was only observed at concentrations higher than 50 mg/L (Bai et al. 2010). Effects on embryo hatching have usually been reported at concentrations higher than 1–5 mg/L (Bai et al. 2010; Lin et al. 2012; Zhao et al. 2013; Zhu et al. 2008).

Finally, $TiO_2$ NPs, which are among the most widely used NPs, are very useful in photocatalysis, in environmental technology for the treatment of wastewater and ground water and for the degradation of air pollutants, and are often used in the production of sunscreens and cosmetics (Fries and Simko 2012; Macwan et al. 2011). Even though studies with adult zebrafish have shown that $TiO_2$ NPs seem to be less toxic than other metal oxide NPs, toxic effects have been reported after exposure to high doses and prolonged periods (Chen et al. 2011a; Wang et al. 2011). $TiO_2$ exists in different structural forms with different properties and consequent environmental impacts. Among them, anatase and rutile are considered the most likely to be found in the environment (Ju-Nam and Lead 2008). As reviewed by Auffan et al. (2009), anatase has proved to be biologically more active in terms of cytotoxicity or DNA damage. However, according to the existing literature, exposures of up to 500 mg/L of anatase $TiO_2$ either as NP or in its bulk form do not produce toxic effects on developing zebrafish embryos (Zhu et al. 2008).

Taken into account that the properties and, therefore, the potential toxicity of NPs may vary depending on many factors, such as synthesis method, size and coatings, with this study, we aimed to contribute to the existing knowledge on metal oxide NPs toxicity using the zebrafish embryo test. Endpoints assessed were survival, hatching and malformation prevalence. In addition, autometallography (Soto et al. 1996) has been applied for the first time as a potentially useful technique to determine the fate of metals in zebrafish embryos after exposure to metal oxide NPs.

## Materials and methods

### Fish maintenance and breeding

Adult zebrafish (*Danio rerio;* AB Tübingen) were maintained at 27 ± 1 °C with a 14 h light/10 h dark cycle in 100 L tanks following standard protocols. The day prior to the beginning of the experiment, one female and two male adult zebrafish were placed separately in the same breeding tramp which had previously been located in a 2 L tank containing conditioned water. Then, fish were left overnight, and just before turning on the light, they were allowed to gather. The resulting embryos were collected with Pasteur pipettes, and fertilized viable eggs were selected under a stereoscopic microscope (Nikon smz800, Kanagawa, Japan).

### Metal compounds used for experimental exposures

CuO (CuO-poly) and rutile/anatase $TiO_2$ ($TiO_2$-RUAN-poly) NPs were provided by Intrinsiq Materials. Disodium laureth sulfosuccinate (DSLS)-stabilized rutile $TiO_2$ ($TiO_2$-60-DSLS) NPs were produced at Dead Sea Laboratories. DSLS was provided by Zschimmer & Schwarz Italiana S.p.A. (Tricerro, Italy). Commercial rutile/anatase $TiO_2$ ($TiO_2$-RUAN-P25) NPs were obtained from Evonik Industries (Dusseldorf, Germany). ZnO NPs, bulk CuO, bulk ZnO, bulk rutile $TiO_2$ ($TiO_2$-BRU), bulk anatase $TiO_2$ ($TiO_2$-BAN), $CuCl_2$ and $ZnCl_2$ were purchased from Sigma-Aldrich (Madrid, Spain).

### Characterization

The main characteristics of NPs are shown in Table 1. For the CuO and $TiO_2$ NPs used in the present study, characterization data have been previously reported in the literature (Buffet et al. 2011, 2013; Katsumiti et al. 2014).

**Table 1** Main characteristics of NPs used in the present work

| NPs | Crystal structure | Synthesis Method | Size in nm | | Z potential in mV | Original form | Reference |
|---|---|---|---|---|---|---|---|
| | | | DLS | TEM SEM | | | |
| CuO-poly | Tenorite | Plasma | 40–500<br>Average 197 | – | 26.3 | Powder | Buffet et al. (2011, 2013) |
| ZnO | – | Plasma | 150–1,000 | <100 (TEM) | −22.2 | Powder | This work |
| $TiO_2$-RUAN-poly | 55 % rutile<br>45 % anatase | Plasma | – | <100 (TEM) | −19.8 | Powder | Katsumiti et al. (2014) |
| $TiO_2$-60-DSLS | 100 % rutile | Milling | – | 10–400<br>Average 60<br>(SEM) | −24.6 | Suspension | Katsumiti et al. (2014) |
| $TiO_2$-RUAN-P25 | 10 % rutile<br>70 % anatase<br>(20 % unidentifiable) | Unknown | – | Majority 10–20<br>Also >100<br>(TEM) | −25.1 | Powder | Katsumiti et al. (2014) |

*DLS* dynamic light scattering, *DSLS* disodium laureth sulfosuccinate, *SEM* scanning electron microscopy, *TEM* transmission electron microscopy

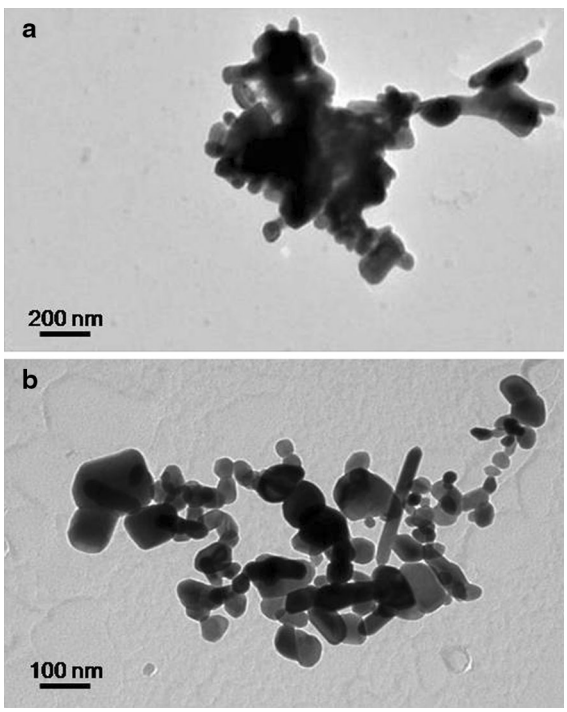


**Fig. 1** TEM micrographs of bulk ZnO (*top*) and ZnO nanoparticles (*bottom*)

For ZnO NPs, transmission electron microscopy (TEM) was used to characterize the size and shape of the nanoparticles (Fig. 1), using a Hitachi H7100 TEM (Tokyo, Japan) for Fig. 1a and a JEOL 1200EX TEM (Tokyo, Japan) for Fig. 1b, both operating at 100 kV. Dynamic light scattering (DLS) measurements were performed in a Malvern Zetasizer Nano ZS instrument (Worcestershire, UK).

## Preparation of exposure suspensions

In order to minimize particle aggregation and sedimentation, deionized water was used to prepare NP suspensions and used as control media, as it has previously been done in similar studies (Zhu et al. 2008), after corroborating that deionized water did not affect embryo survival and development. For the experimental exposure of zebrafish embryos, stock solutions (or suspensions in the case of more insoluble forms) of the chloride and oxide forms of copper and zinc were prepared at the highest nominal concentration evaluated (10 mg Cu or Zn/L). For that purpose, metal salts and metal oxide forms were added to deionized water, stirred for 3 h and sonicated for 10 min in a Selecta Ultrasons H 3000840 (50 Hz; Barcelona, Spain) sonication bath at 25 °C. The day of the beginning of the test, dilutions (0.01, 0.1, 1, 5 mg/L) were prepared. Once ready, they were manually shaken and sonicated again for 10 min. Prior to expose the organisms, pH and temperature were measured.

In the case of titanium dioxide, all the stock suspensions and dilutions (0.1, 1, 10, 50, 100 mg Ti/L) were prepared on the day of the experiment. Different protocols were used to prepare the stock suspensions. $TiO_2$-BAN was suspended in deionized water and hand-shaken before preparing the dilutions as well as before starting the exposures. In the case of $TiO_2$-RUAN-P25 NPs, $TiO_2$-RUAN-poly NPs and $TiO_2$-BRU, powders were gently hand-disaggregated using a pestle and mortar and adding a few drops of deionized water. Deionized water was then added until a homogeneous suspension was obtained. Afterwards, samples were diluted and sonicated for 1 min before starting the exposures. Finally, $TiO_2$-60-DSLS NPs suspension was vortexed before preparing the dilutions. Exposure media containing the equivalent concentration of surfactant present in the $TiO_2$-60-DSLS NPs suspensions (0.00834, 0.0834, 0.834, 4.17, 8.34 mg/L) were also prepared.

### Embryo toxicity study

For each compound and concentration, eight newly fertilized embryos were exposed for 120 h in 24-well plates. Two embryos were placed in each well containing 2 mL of the corresponding medium or deionized water (control) at a constant room temperature of 27 °C. Microplates were covered to avoid evaporation. Three replicates were run for each of the tested compounds and concentrations.

Daily and up to the end of the test, embryos were examined to determine survival and hatching rates and malformation prevalence at 120 hpf. Hatching time was calculated as the average time that embryos needed to hatch. In the case of alive individuals that had not hatched at 120 hpf when the test finalized, a hatching time of 144 h was considered to calculate the average hatching time. Criteria for normal zebrafish embryo development morphology were based on Kimmel et al. (1995). By means of a photographic camera attached to the stereoscopic microscope, photographs of malformed larvae were taken.

Autometallography has been previously employed to successfully localize copper and zinc in the tissues of aquatic species exposed to the soluble form of these metals (Soto et al. 1996). Thus, in the case of the exposures to the three forms of copper and zinc, at the end of the experiment (120 hpf), five unexposed larvae and five larvae exposed to 10 mg metal/L, as the highest concentrations tested of each compound, were fixed and processed for histology and further localization of metal by autometallography in paraffin sections.

### Histological processing and autometallography

Larvae were fixed in 10 % neutral buffered formalin for 24 h and processed for paraffin embedding and haematoxylin/eosin (H/E) staining.

For autometallography, the next sections coming after those stained with H/E were dewaxed and processed for autometallography as described by Soto et al. (1996). Abundance of autometallographical black silver deposits (BSDs) in the tissues was semi-quantified using the following criteria: no presence of BSDs (−), presence of homogeneously distributed small BSDs (+); homogeneously distributed small BSDs plus the presence of agglomerations of BSDs of larger size (++) and greater presence of homogeneously distributed BSDs plus the presence of abundant large deposits (+++). All the observations were done under the 100× objective.

### Data analysis

Statistical analyses were done using the SPSS/PC statistical package (SPSS Inc, Microsoft Co, WA, USA). For survival and hatching rates and malformation prevalence, significant differences were studied by Fisher's exact test ($p < 0.05$). In the case of hatching time, significant differences between pairs of means were studied by means of two-independent samples Mann–Whitney test ($p < 0.05$).

In order to determine the lethal concentration for the 50 % of the organisms exposed ($LC_{50}$), probit analysis was performed employing the same statistical package.

## Results

During the exposures, all the NPs tested tended to aggregate and sediment. In the case of CuO-poly NPs, whose brownish colour made it more evident, 24 h after adding the test suspension, a layer of sedimented NPs could be observed at the bottom of the wells as well as attached to the surface of the organisms (Fig. 2C).

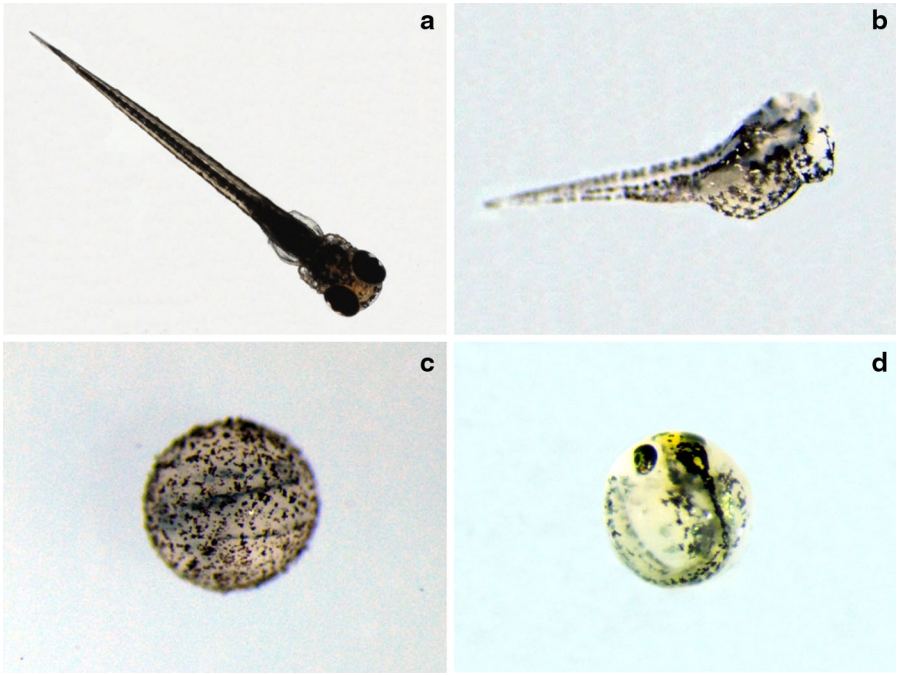


**Fig. 2** Zebrafish embryos at 120 hpf. **a** Control embryo. **b** Anencephalic embryo exposed to CuO NPs (5 mg Cu/L). **c** Non-hatched embryo covered by CuO NPs (10 mg Cu/L). **d** Non-hatched embryo exposed to ZnO NPs (0.01 mg Zn/L)

## Embryo toxicity of copper

Exposure to CuO-poly NPs or bulk CuO up to 10 mg Cu/L did not produce any significant decrease on embryo survival (Fig. 3a), and their $LC_{50}$ values were estimated to be above the highest tested concentration (Table 2). The ionic form was the most toxic for all the parameters analysed. A $LC_{50}$ value of 3.083 mg/L was calculated for ionic copper which increased significantly embryo mortality at 5 and 10 mg/L (Fig. 3a). According to the other parameters analysed, CuO-poly NPs showed higher toxicity than bulk CuO. Significantly decreased hatching rate was observed in embryos treated with CuO-poly NPs at 10 mg Cu/L (Fig. 3b). At this concentration, 73.34 % of the surviving embryos had not hatched, and a significant hatching delay was registered at concentrations of 1, 5 and 10 mg/L (Fig. 3c). Ionic copper affected hatching in a more severe manner, reducing the percentage of hatched embryos and delaying hatching at concentrations in the range 0.1–10 mg/L (Fig. 3b, c). At 0.1 mg Cu/L, only around 40 % of the embryos could hatch, while at higher concentrations, none of the surviving embryos were able to hatch. Bulk CuO exerted the lowest effect, affecting only the hatching time (Fig. 3c). CuO-poly NPs produced significant increase in the malformation prevalence (evaluated as the percentage of surviving embryos) in embryos exposed to 1, 5 and 10 mg Cu/L (Fig. 3d). Again, ionic copper provoked malformations at a lower concentration (0.1 mg Cu/L) than the other copper forms and affected 100 % of the surviving embryos at concentrations of 1 and 5 mg Cu/L. Yolk sac oedema and tail flexures were the predominant abnormalities. Even if the malformation prevalence did not show such a notable increase in the case of embryos exposed to CuO-poly NPs, severe malformations such as anencephaly (embryo exposed to 5 mg Cu/L) were observed in CuO-poly NPs-exposed embryos (Fig. 2b). No significant incidence on the malformation prevalence was observed in the case of bulk CuO-exposed embryos (Fig. 3d).

## Embryo toxicity of zinc

As described for copper, ZnO NPs were less toxic (LC50 > 10 mg Zn/L, Table 2) than the ionic form of zinc, which exerted the highest toxicity

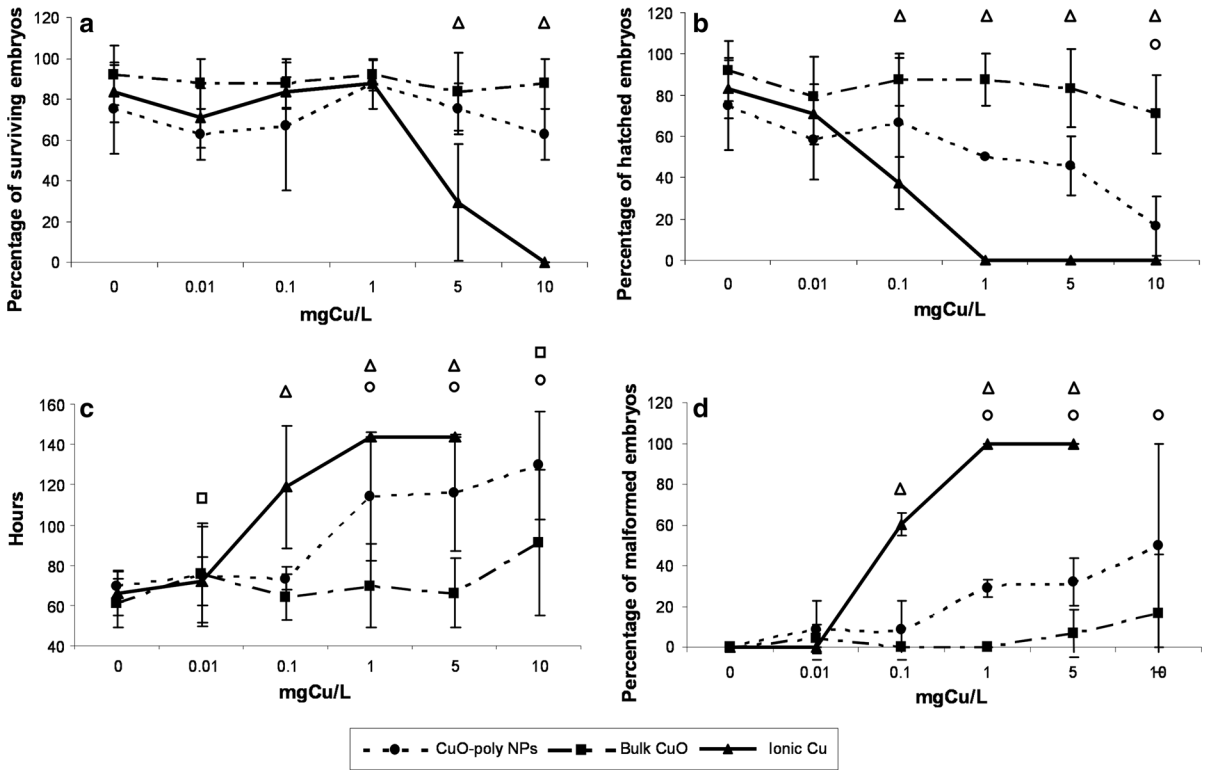


**Fig. 3** Results obtained for embryos exposed to CuO NPs (*circles*), ionic copper (*triangles*) and bulk CuO (*squares*). **a** Percentage of surviving embryos after 120 h of exposure. **b** Percentage of hatched embryos after 120 h. **c** Embryo hatching time. **d** Percentage of malformed embryos over surviving embryos after 120 h. Significant effects towards the unexposed controls are identified for each tested compound by empty forms

(LC50 = 3.004 mg Zn/L). None of the tested concentrations of ZnO NPs or bulk ZnO produced significantly increased mortality, while significantly reduced survival was observed in all the exposures to ionic zinc (Fig. 4a). Regarding the hatching parameters, ZnO NPs produced significant reduction of the percentage of hatched embryos only at 10 mg Zn/L (Fig. 4b) although this effect was also registered at lower concentrations (Fig. 2d). Again, ionic zinc was the most toxic form, exerting a significant impairment in the hatching rate at all the concentrations tested. This impairment was attributable to mortality except in the case of 10 mg/L-exposed embryos. In this group, 50 % of the surviving embryos did not hatch. Surprisingly, bulk ZnO produced a significant reduction of hatching only in embryos exposed to 0.1 mg Zn/L (Fig. 4b). Exposure to the three tested metal forms caused hatching delay at the highest concentrations (5 and 10 mg Zn/L). Moreover, bulk ZnO also increased the time required to hatch at 0.1 mg Zn/L (Fig. 4c). The malformation prevalence increased significantly only at the highest tested concentration of ionic zinc. Yolk sac oedema and tail flexures were the predominant abnormalities. Malformations were rare under bulk ZnO or ZnO NPs exposures (Fig. 4d).

## Embryo toxicity of titanium dioxide

A LC50 value of 84.095 mg Ti/L was calculated for $TiO_2$-60-DSLS NPs, while for the rest of treatments, LC50 values were either estimated above the highest tested concentration or could not be calculated (Table 2). $TiO_2$-60-DSLS NPs exerted a significant effect on survival and hatching rates at the highest tested concentration (100 mg Ti/L), when compared to their control groups (Fig. 5a, b). However, the same effect was observed when the surfactant was tested alone. Neither $TiO_2$-RUAN-poly NPs (55 % rutile and 45 % anatase) nor $TiO_2$-RUAN-P25 NPs (10 % rutile, 70 % anatase, 20 % unidentifiable material) produced

**Table 2** $LC_{50}$ values for tested substances

| Metal | Tested Forms | Exposure Concentration | LC 50 (mg/L) |
|---|---|---|---|
| Copper | CuO-poly NPs | 0.01–10 mg | >10 |
| | Bulk CuO | Cu/L | >10 |
| | Ionic Cu ($CuCl_2$) | | 3.083 |
| Zinc | ZnO NPs | 0.01–10 mg | >10 |
| | Bulk ZnO | Zn/L | >10 |
| | Ionic Zn ($ZnCl_2$) | | 3.004 |
| Titanium dioxide | $TiO_2$-60-DSLS NPs | 0.1–100 mg Ti/L | 84.095 |
| | $TiO_2$–RUAN-poly NPs | | >100 |
| | $TiO_2$-RUAN-P25 NPs | | >100 |
| | $TiO_2$-BAN | | >100 |
| | $TiO_2$-BRU | | >100 |
| | $TiO_2$-MIX | | >100 |
| | DSLS | 0.0083–8.3 mg/L (*) | 4.825 (58.135) |

(*)Surfactant concentration present in the TiO2-60-DSLS NPs suspension. For DSLS, LC50 value is given in mg/L and in the equivalent metal concentration of the hypothetical NP suspension containing that DSLS concentration (in brackets)

any toxic effect (data not shown). Similarly, no mortality was observed after exposure to any of the bulk $TiO_2$-tested forms and concentrations. None of the $TiO_2$ compounds produced a significant increase in the malformation prevalence. Only in the case of embryos exposed to $TiO_2$-BRU, some malformations such as pericardial oedema and tail curvature were recorded, but the effect was not statistically significant.

### Autometallography

The results for the semi-quantitative analysis of the presence of BSDs are shown in Table 3. BSDs were detected in several organs, such as brain, gill, liver, yolk sac and tail (Fig. 6). Control zebrafish embryos exhibited scarce small uniformly distributed BSDs in their tissues. Embryos exposed to bulk CuO, bulk ZnO, ZnO NPs and ionic zinc showed almost the same amount of homogenously disseminated small BSDs throughout the tissues studied. Sections of organisms exposed to CuO-poly NPs and ionic copper exhibited the highest amount of BSDs among the experimental groups.

## Discussion

The increasing production, use and consequent release of metal oxide nanoparticles into the environment make it necessary to assess the environmental and health hazards that these compounds could exert (Lee et al. 2010; Wang et al. 2013). In the present study, we evaluated the toxic effects of different metal oxide nanoparticles (CuO-poly, ZnO, $TiO_2$-60-DSLS, $TiO_2$-RUAN-poly and $TiO_2$-RUAN-P25) compared to their bulk and ionic (for copper and zinc) counterparts using the zebrafish embryo test.

### Differential toxicity of metal oxide nanoparticles

Except in the case of $TiO_2$-60-DSLS NPs, whose effect on survival could be attributable to the surfactant present in the NP suspension, for the other NPs studied in this work, the LC50 values were estimated above the highest tested concentration or they could not be calculated (Table 2). In agreement with these results, Lin et al. (2012) did not observe a significant increase in embryo mortality after 72 h of exposure to 50 mg/L CuO, ZnO or $TiO_2$. Zhu et al. (2008) found no toxicity for $TiO_2$ NPs after exposing embryos up to 500 mg/L, but they established a LC50 value of 1.793 mg/L (96 h) for ZnO NPs-exposed embryos. The higher toxicity observed by Zhu et al. (2008) for ZnO NPs could be due to the differences in the employed particle size. According to the DLS analysis, NPs employed by Zhu et al. (2008) were smaller (50–360 nm) than the ZnO NPs assayed here (150–1,000 nm).

A toxicity ranking for the studied NPs could be established based on the sublethal parameters analysed in this study; CuO-poly were the most toxic NPs exerting significant effects on embryos (hatching delay, increased malformation prevalence) at concentrations of 1 mg Cu/L, followed by ZnO NPs that caused deleterious effects at concentrations of 5 mg Zn/L (hatching delay). $TiO_2$ NPs were the least toxic. From the three different $TiO_2$ NPs assayed (Table 1), only in the case of $TiO_2$-60-DSLS NPs, effects (reduced survival and hatching rates) were observed at 100 mg Ti/L, but as mentioned, these effects can be

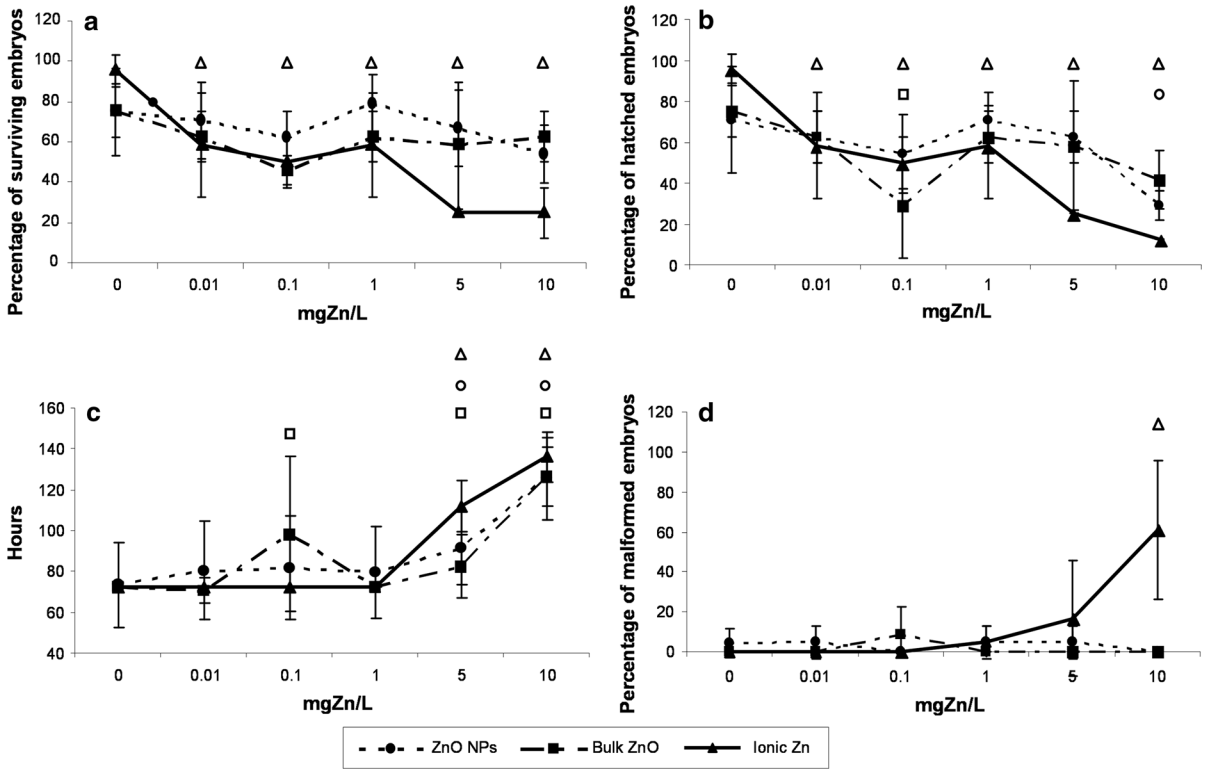


**Fig. 4** Results obtained for embryos exposed to ZnO NPs (*circles*), ionic zinc (*triangles*) and bulk ZnO (*squares*). **a** Percentage of surviving embryos after 120 h of exposure. **b** Percentage of hatched embryos after 120 h. **c** Embryo hatching time. **d** Percentage of malformed over surviving embryos after 120 h. Significant effects towards the unexposed controls are identified for each tested compound by empty forms

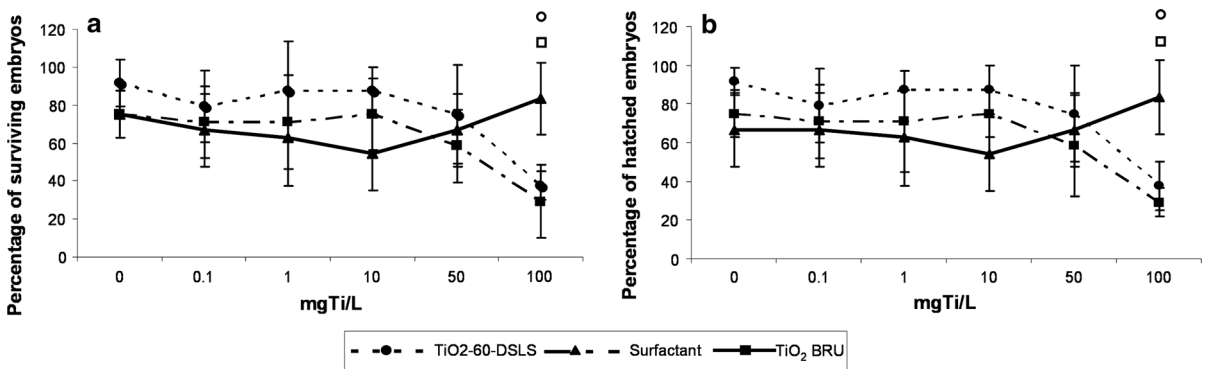


**Fig. 5** Results obtained for embryos exposed to $TiO_2$-60-DSLS NPs (*circles*), the surfactant DSLS (*squares*) and bulk $TiO_2$ (*triangles*). **a** Percentage of surviving embryos after 120 h of exposure. **b** Percentage of hatched embryos after 120 h. Significant effects towards the controls are identified for each tested compound by empty forms

attributed to the surfactant present in the NPs suspension that exerted the same effect when assessed alone (Fig. 5).

Solubility is a fundamental parameter to be considered when NPs toxicity is assessed as it can lead to the delivery of highly toxic ions (Auffan et al. 2009;

**Table 3** Semi-quantitative evaluation of the presence of black silver deposits (BSDs) in whole embryo sections of organisms exposed to different forms of Cu and Zn

| Organism | Control | CuO-poly NPs | Bulk CuO | Ionic Copper | ZnO NPs | Bulk ZnO | Ionic Zinc |
|---|---|---|---|---|---|---|---|
| A | – | + | ++ | ++ | + | – | + |
| B | – | ++ | + | ++ | – | + | + |
| C | + | ++ | + | +++ | + | – | + |
| D | + | +++ | ++ | ++ | + | ++ | ++ |
| E | – | ++ | + | +++ | + | + | + |
| Total number of + | 2 | 10 | 7 | 13 | 4 | 4 | 6 |
| Average number of + | 0.4 | 2 | 1.4 | 2.6 | 0.8 | 0.8 | 1.20 |

(–) Tissues without presence of BSDs, (+) presence of homogeneously distributed small BSDs, (++) presence of homogeneously distributed small BSDs plus the presence of agglomerations of BSDs of larger size and (+++) tissues with a greater presence of homogeneously distributed BSDs plus the presence of abundant large deposits

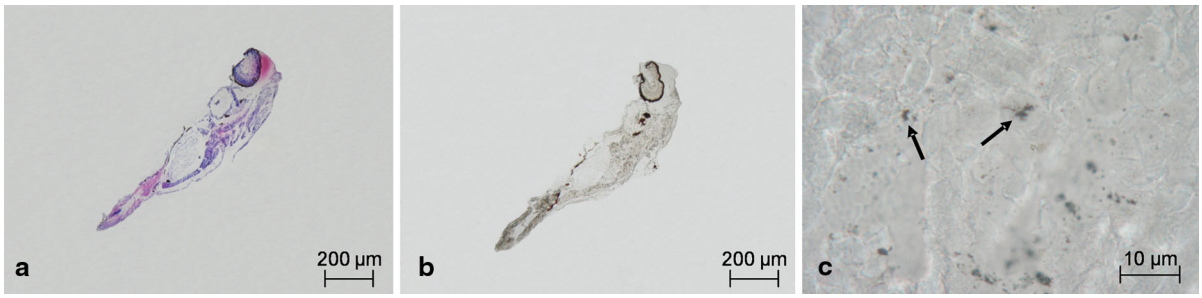


**Fig. 6** Micrographs of a 120 hpf zebrafish embryo exposed to 10 mg Cu/L of CuO-poly NPs. **a** H/E-stained section. **b** Section of the same embryo shown in **a** subjected to autometallographical detection of metals. **c** BSDs (*arrows*) in the liver tissue seen at higher magnification

Misra et al. 2012; Shaw and Handy 2011). Accordingly, CuO-poly and ZnO NPs, which were the most toxic, have been shown to be more soluble than $TiO_2$ NPs (Johnston et al. 2010; Shaw and Handy 2011). Hatching impairment was the main effect shared by CuO-poly NPs- and ZnO NPs-exposed embryos. Both CuO-poly and ZnO NPs reduced the hatching rate in embryos exposed to 10 mg/L and delayed hatching at lower concentrations (Fig. 3b, 4b). It has been demonstrated that due to their chemical characteristics, metal ions released from relatively soluble metal oxide NPs such as CuO and ZnO can fit the active site of the zebrafish hatching enzyme (metalloprotease ZHE1) responsible for the degradation of the chorionic membrane and, consequently, inhibit zebrafish embryo hatching, while exposure to the sparingly soluble $TiO_2$ does not interfere (Lin et al., 2012). Our results are in good agreement with those obtained by Lin et al. (2012), who observed reduced hatching rate at 72 h in embryos exposed to concentrations over 0.5 mg/L and 5 mg/L of CuO and ZnO NPs, respectively. In our study, effects on hatching were registered as delayed hatching (Figs. 3c, 4c) as far as hatching rate was calculated at the end of the test (120 h). These results indicate that even if the soluble fraction arising from the exposure to CuO and ZnO NPs can produce delayed hatching by inhibiting the hatching enzyme at concentrations over 0.5 and 5 mg/L, respectively, this inhibition is not sustained after 120 h and embryos are able to hatch at concentrations below 10 mg/L (Figs. 3b, 4b).

Aggregation is another NP property to be considered when NPs toxicity is assessed, since it leads to changes in NPs size distribution and to differential presence of NPs along the water column (Bai et al. 2010; Johnston et al. 2010). In the present study, even if deionized water was used to run the exposures, NPs aggregated and precipitated as exposure time increased. This effect has previously been reported in other studies with similar NPs (Griffitt et al. 2007;

Hund-Rinke and Simon 2006; Yu et al. 2011). There is evidence that the presence of NPs attached to the chorion surface resulting from NP precipitation could interfere with the regular transport through the chorion pores (Lee et al. 2007). This blockage would result in reduced oxygen supply to the embryo, and consequently, the produced hypoxia would be another factor impairing normal hatching (Bai et al. 2010). The zeta potential values obtained for the tested NPs (Table 1) were all moderate (positive or negative) indicating that they are all likely to have a similar tendency to aggregate, and therefore, aggregation and precipitation should not be parameters explaining the differential toxicity of studied NPs. Thus, even if aggregation is a parameter affecting distinct metal oxide NPs toxicity, the differential toxicity observed for the metal oxide NPs tested in this study depends on their chemical composition and solubility.

The induction of malformations in developing embryos is another common effect observed after zebrafish embryo exposure to metal NPs (Asharani et al. 2008; King-Heiden et al. 2009; Zhao et al. 2013). In our study, only CuO-poly NPs produced increased malformation prevalence at concentrations equal or above 1 mg Cu/L. Nevertheless, it must be considered that the low hatching rate of embryos exposed to ZnO NPs may have acted as a confounding factor when the malformation prevalence was calculated due to the difficulties to register slight anomalies in non-hatched embryos.

For NPs of the same chemical composition, crystal structure has also been considered a parameter affecting NPs toxicity (Warheit et al. 2008). In this study, different forms of $TiO_2$ NPs, containing different proportions of anatase and rutile $TiO_2$, were tested and only $TiO_2$-60-DSLS NPs (100 % rutile) produced effects at 100 mg Ti/L. It has been reported that anatase is more cytotoxic than rutile (Auffan et al. 2009; Katsumiti et al. 2014), but it must be considered that toxicity of $TiO_2$-60-DSLS NPs appeared to be caused by the surfactant agent present in the suspension, indicating that special attention should be paid to the chemicals that are used as additives for nanoparticle formulations. In fact, when the toxicity of $TiO_2$-60-DSLS NPs was tested in mussel haemocytes and gill cells, the surfactant was suggested to be responsible of the $TiO_2$-60-DSLS NPs cytotoxicity (Katsumiti et al. 2014).

Overall, our results indicate that in the concentration range tested in this study and for the particular $TiO_2$ NPs and experimental conditions used, no significant toxicity is observed by any rutile/anatase combination. Indeed, a major conclusion from this study is that the chemical composition of the NPs, along with additives used to disperse them, is the key factor affecting NPs toxicity.

## Nanoparticles toxicity compared to their ionic and bulk counterparts

Several works have studied and compared the relative toxicity of metallic NPs and their ionic counterparts, in terms of equivalent soluble fraction, reaching the conclusion that NPs are more toxic than their soluble ionic counterpart (Bai et al. 2010; Kasemets et al. 2009; Yu et al. 2011; Zhu et al. 2008). In order to provide straight comparable data in terms of the toxicity arising from equal metal quantities, in this work, we exposed zebrafish embryos to identical nominal concentrations of each metal form. When NPs toxicity is considered in comparison with their ionic counterparts in these terms, the ionic forms of copper and zinc resulted significantly more toxic than the CuO-poly and ZnO NPs, respectively (Figs. 3, 4). The ionic counterpart for titanium was not tested in this study since, as mentioned previously, $TiO_2$ is a sparingly soluble compound. Ionic copper caused mortality, reduced the percentage of hatched embryos, delayed hatching, and increased malformation prevalence always at lower concentrations than NPs (Fig. 3). The lower toxicity of copper-bearing NPs when compared to their ionic form has previously been described in different zebrafish developmental stages. Chen et al. (2011b) exposed for 5 days post-fertilization zebrafish larvae to $Cu_2O$ and $CuCl_2$, obtaining a lower LC50 value in the groups of larvae exposed to $CuCl_2$ (0.24 and 0.085 mg/L, respectively). The higher toxicity of soluble copper has also been described by Griffitt et al. (2007, 2008), who after exposing adult zebrafish to both ionic and Cu NPs (0.1 mg Cu/L) registered a higher mortality in the case of fish exposed to the former. Regarding zinc-exposed embryos, no significant effect on survival was exerted neither by the ZnO NPs nor by the bulk form, while significantly increased mortality was observed at all ionic zinc concentrations. The lower toxicity to zebrafish embryos of ZnO NPs in comparison with ionic zinc

(regarding nominal concentrations) has also been described by Bai et al. (2010), who reported delayed hatching at lower doses in embryos exposed to ionic zinc at concentrations higher than 2.152 mg Zn/L.

When NPs toxicity was considered in comparison with their bulk counterparts, different results were obtained for each metal. CuO-poly NPs exerted sublethal toxicity at lower concentrations than bulk CuO, while ZnO NPs produced similar effects to bulk ZnO on the studied endpoints.

CuO NPs have already shown to be more toxic than their bulk counterparts in human epithelial cells (Wang et al. 2012), bacteria and crustaceans (Heinlaan et al. 2008). In the case of ZnO, different results have been reported. Yu et al. (2011) showed that the bulk form of the metal resulted more toxic to adult zebrafish than ZnO NPs and argued that this was a direct consequence of reduced availability of NPs, as a result of their tendency to precipitate. However, in the case of zebrafish embryo exposures, NPs precipitation resulted in an increased concentration around the embryo and consequent increased contact between the embryo and the NPs, which could therefore explain the toxicity recorded for CuO-poly and ZnO NPs. In fact, Zhu et al. (2008) reported a similar toxicity for bulk ZnO and ZnO NPs both producing delayed embryo development and decreased survival and hatching rates. After 96 h, they recorded lower $LC_{50}$ values for both nano ZnO (1.793 mg Zn/L) and bulk ZnO (1.55 mg Zn/L) compared to our study. These differences could be due to the different methodologies employed during exposures, since the study by Zhu et al. (2008) involved shaking of the incubation plates every 12 h.

Assuming that the toxic effects observed in this study for $TiO_2$-60-DSLS NPs were produced by the surfactant present in the NPs suspension, none of the assayed $TiO_2$ NPs and bulk compounds produced any toxic effect to zebrafish embryos at the concentrations tested. $TiO_2$ NPs have been considered innocuous and used as non-toxic control NPs for zebrafish (Griffitt et al. 2008). Our results are in agreement with previous works in which the toxicity of $TiO_2$ has been assessed using zebrafish embryos. Zhu et al. (2008) did not find any toxic effect on zebrafish embryos exposed to concentrations of up to 500 mg/L (96 h) of $TiO_2$ NP and bulk forms. However, toxic effects have been reported after longer exposures of adult zebrafish (Chen et al. 2011a; Xiong et al. 2011), and thus, attention should be paid to the possible effects of $TiO_2$ NPs after longer exposures or different exposure conditions, such as exposure under UV radiation, that could increase their toxicity (Clemente et al. 2013), as well as to other developmental stages which could be affected through different modes of action.

Overall, our results indicate the lower toxicity of NPs when compared to the ionic forms when nominal concentrations are compared, while the relative toxicity of the NPs compared to the bulk forms is dependent on the metal oxide employed. These results are consistent with those obtained in other model organisms, such as yeast (*Saccharomyces cerevisiae*; Kasemets et al. 2009), bacteria (*Vibrio fischeri*; Heinlaan et al. 2008) and mussel cells (Katsumiti et al. 2014). However, in the case of crustaceans (*Daphnia magna* and *Thamnocephalus platyurus*), CuO NPs were also less toxic than the ionic form, while ionic zinc resulted less toxic than the NPs (Heinlaan et al. 2008).

## Metal detection in tissue sections

In order to assess the toxicity of a compound, it is important to establish the final destination that it will have in the organism. To date, the body distribution of nanometals is poorly understood and hampered by a lack of methods for measuring NPs in tissues (Shaw and Handy 2011). Determining whether NPs exposure produces increased metal uptake, and the consequent increased bioaccumulation, is an initial step to allow a better understanding of toxicity mechanisms (Johnston et al. 2010). Autometallography allows to localizing metallic deposits in tissue sections of organisms (Soto et al. 1996). This technique has previously been successfully used to determine the existence of metallic deposits in the gills of turbot (*Scophthalmus maximus*) (Alvarado et al. 2006) or to describe subcellular distribution of metals in molluscs (Marigómez et al. 2002). In adult zebrafish, autometallography has been used to determine methylmercury distribution in the retina after trophic exposure (Mela et al. 2010).

The semi-quantitative results obtained for metal accumulation in embryos exposed to the different metal forms of copper and zinc (Table 3) match the toxicity pattern reported in previous sections. Embryos exposed to the ionic form of copper and

zinc showed the highest presence of BSDs, reinforcing the idea that solubility is a key parameter determining NPs toxicity. Autometallography results showed lower accumulation of metal in embryos exposed to zinc than in those exposed to copper.

The variety of organs and tissues in which BSDs were detected (brain, gills, liver, yolk sac, tail) and the existing literature about NPs fate (Lee et al. 2007; Handy et al. 2008), in which gills, gut, liver and brain have been referenced as targets for nanocompounds, indicate that the distribution pattern inside the organisms is wide and, that to some extent, the fate of metallic compounds in fish is related to the developmental stage and the uptake pathway metals follow. Results suggest that autometallography can be successfully utilized to localize metallic compounds (which may have entered the organism either as dissolved ions or as suspended NPs) in zebrafish embryo tissue sections. The application of this technique could therefore fulfil the lack of simple, routine methods for the direct measurement of metals in tissues. Thus, autometallography could allow a better knowledge of body distribution or serve as a complement for other techniques such as the labour-intensive electron microscopy of dissected tissues.

### Environmental relevance

Even though little information is available about the current environmental concentrations of NPs, some estimations have been reported. The environmental concentration for Cu NPs in major Taiwanese rivers has been estimated to be 0.06 mg/L (Chio et al. 2012). This concentration is within the range of concentrations tested for CuO NPs (Fig. 3). We did not observe any effect at concentrations lower than 1 mg/L suggesting that pollutant levels estimated by Chio and co-workers should not be a cause for concern. The estimated average concentrations for ZnO and $TiO_2$ NPs in European surface and waste waters are 10 and 430 ng/L for ZnO NPs and 15 ng/L and 3.47 μg/L for $TiO_2$ NPs, respectively (Gottschalk et al. 2009). As in the case of CuO NPs, considering that toxic effects were only observed at higher concentrations (Figs. 4, 5), we could conclude that the current environmental concentrations of ZnO and $TiO_2$ NPs should not pose a risk to the aquatic environment if each metal is considered separately. Nevertheless, usually metals appear in the environment as complex mixtures, and therefore, the total toxicity risk should be taken into account. Furthermore, other organisms may display higher sensitivities than zebrafish.

Moreover, it must be considered that the results presented in this study correspond to acute exposures, and therefore, further investigations are needed before discarding long-term effects as consequence of chronic exposures. It must also be taken into account that an increased production and release of NPs are expected to occur in future years. In fact, it has been estimated that the production of engineered NMs will increase to 58,000 tons before 2020 (Maynard et al. 2006). Therefore, even if the concentrations tested in this work are over the currently estimated environmental concentrations, our results indicate that a future increase in CuO, ZnO or $TiO_2$ NPs environmental concentrations may turn into an environmental issue.

## Conclusions

The present study shows that the toxic effect of NPs on developing zebrafish embryos is a function of their chemical composition; CuO NPs are the most toxic of the NPs tested in this study and $TiO_2$ the least toxic. Of all $TiO_2$ NPs tested, toxic effects were only produced by $TiO_2$-60-DSLS NPs (100 mg Ti/L). However, in this case, toxicity was linked with the presence of DSLS surfactant in NPs suspensions. Thus, special attention should be paid to the chemicals used in NP formulations. Finally, when assessing NPs toxicity using the zebrafish embryo model, it is important to consider not only mortality, but the sublethal effects produced by the exposures; otherwise, NPs toxicity could be underestimated.

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