Elevated CO₂ levels increase the toxicity of ZnO nanoparticles to goldfish (*Carassius auratus*) in a water-sediment ecosystem

Ying Yin,[†] Zhengxue Hu,[†] Wenchao Du,^{*,†} Fuxun Ai,[†] Rong Ji,[†] Jorge L. Gardea-Torresdey,^{‡,£,€} and Hongyan Guo,^{*,†}

[†]State Key Laboratory of Pollution Control and Resource Reuse, School of Environment, Nanjing University, Nanjing 210046, China

[‡]Department of Chemistry, The University of Texas at El Paso, El Paso, Texas 79968, United States

[£]Environmental Science and Engineering PhD program, The University of Texas at El Paso, El Paso, Texas 79968, United States

[€]University of California Center for Environmental Implications of Nanotechnology (UC CEIN), The University of Texas at El Paso, El Paso, Texas 79968, United States

*Corresponding author. Tel.: +86-25-89680263; Fax: +86-25-89680263.

E-mail address: hyguo@nju.edu.cn (Guo H Y), du@nju.edu.cn (Du W C).

ABSTRACT

Concerns about the environmental safety of metal-based nanoparticles (MNPs) in aquatic ecosystems are increasing. Simultaneously, elevated atmospheric CO₂ levels are a serious problem worldwide, making it possible for the combined exposure of MNPs and elevated CO₂ to the ecosystem. Here we studied the toxicity of *n*ZnO to goldfish in a water-sediment ecosystem using open-top chambers flushed with ambient ($400 \pm 10 \mu\text{L/L}$) or elevated ($600 \pm 10 \mu\text{L/L}$) CO₂ for 30 days. We measured the content of Zn in suspension and fish, and analyzed physiological and biochemical changes in fish tissues. Results showed that elevated CO₂ increased the Zn content in suspension by reducing the pH value of water and consequently enhanced the bioavailability and toxicity of *n*ZnO. Elevated CO₂ led to higher accumulation of Zn in fish tissues (increased by 43.3%, 86.4% and 22.5% in liver, brain and muscle, respectively) when compared to ambient. Elevated CO₂ also intensified the oxidative damage to fish induced by *n*ZnO, resulting in higher ROS intensity, greater contents of MDA and MT and lower GSH content in liver and brain. Our results suggest that more studies in natural ecosystems are needed to better understand the fate and toxicity of nanoparticles in future CO₂ levels.

Keywords: ZnO nanoparticles, water-sediment, CO₂, fish, toxicology

1. Introduction

The increasing applications of nanotechnology is moving the industry to produce large quantities of nanoparticles (NPs). It is inevitable that NPs and respective by-products will be released directly and indirectly into the aquatic environment.¹ The unique properties of NPs, such as small size and chemical properties, have raised great concerns about their toxicity. Since the Bayer Corporation first provided ZnO nanoparticles (*n*ZnO) to the market, various countries have started large-scale industrial production of *n*ZnO.² Accordingly, *n*ZnO are widely used in consumer products such as cosmetics, medical materials, rubber manufacture, coating and paints.³⁻⁵ It is expected that *n*ZnO will find their way into aquatic environments, raising concerns regarding their conversion, accumulation, and toxicity in aquatic organisms.⁵

Toxicity of metal-based nanoparticles (MNPs) is partly attributed to their specific properties related to the small size and consequent high surface activity, while effects may be further enhanced by the release of free metal ions.⁶ It has been reported that the toxicity of MNPs would be affected by the aquatic chemistry.⁵ Once released to the aquatic system, most MNPs aggregate, resulting in a significant effect on their sedimentation rates. Furthermore, environmental factors such as ionic strength, pH levels, natural organic matter, and other characteristics of the aqueous media affect nanoparticle aggregation, stabilization, and bioavailability.⁷ Thus, once MNPs are in a water-sediment environment, their distribution, form, and fate will be

highly complex and will depend on a variety of physical influences.⁸ For example, $n\text{CeO}_2$ aggregate once they enter aquarium water, and on the third day ceria concentration in water decreases to a relatively constant level of about 1.3% of the initial amount.⁹ Unmodified MNPs, in the absence of natural organic macromolecules, are likely to have a low bioavailability in high-cationic environments.¹⁰ The ecotoxicological hazard of MNPs needs to be considered against the context of a general lack of knowledge of their fate, behavior, and bioavailability in natural systems. Levard et al.¹¹ noted that sulfidation decreases $n\text{Ag}$ toxicity to zebrafish and killifish, and chloride in the exposure medium strongly affects the toxicity by affecting Ag speciation. Specific organic ligands available in the receiving waters can differentially surface modify $n\text{Ag}$ and alter their environmental persistence (changing dissolution dynamics) and subsequently their toxicity.¹² It is therefore imperative to focus on the behavior, fate, and toxicology of MNPs in the natural aquatic environments, yet most studies to date are limited to simple water systems, and available reports on the toxicology of MNPs in natural aquatic ecosystems are very scarce.

According to the Intergovernmental Panel on Climate Change (IPCC), the atmospheric CO_2 concentration has increased from 280 $\mu\text{L/L}$ to more than 380 $\mu\text{L/L}$ over the past two centuries.¹³ It is estimated that 40% of the total CO_2 emissions remain in the atmosphere, while approximately 30% of the total emissions are accumulated in the ocean.¹⁴ The oceans are absorbing CO_2 from the atmosphere, causing chemical changes by making themselves more

acidic. Calculations show that the uptake of CO₂ has led to a 0.1 unit reduction of the pH of surface seawater, and the average pH of oceans could fall by 0.5 units by the year 2100 if global CO₂ emissions continue to rise on current trends.¹⁵ Also, dissolved CO₂ can decrease the pH value and induce the release of metal from sediment in shallow groundwater systems.¹⁶

Elevated CO₂ levels result in high concentrations of dissolved CO₂ and dissolved inorganic carbon, lower pH and low concentrations of nitrate and soluble reactive phosphorus, which may have an effect on the biomass of the microbe and shift their elemental composition in groundwater.¹⁶ In addition, water acidification can potentially mobilize metals and ions by dissolving minerals found in natural sediments or by desorption/adsorption occurring at water-sediments interfaces.¹⁶ However, it is still unclear whether or how elevated CO₂ affects the toxicity of MNPs.

Open-top chambers (OTCs), an exposure technology commonly used in air-pollution research, suitable for investigating the effects of elevated atmospheric CO₂ on ecosystem processes under conditions similar to those of small experimental field plots.¹⁷ Here, we aimed at determining the toxic effect of *n*ZnO (80 ppm) on goldfish (*Carassius auratus*) in a water-sediment ecosystem in OTCs flushed with ambient or with CO₂ enriched air. We examined the stability of *n*ZnO in suspension, their uptake by *C. auratus*, and the physiological damage caused. To our knowledge, this is the first time this type of experiments are performed.

2. Materials and methods

2.1 ZnO nanoparticles

ZnO nanoparticles were purchased from Sigma Chemical (St. Louis, MO, USA), with a purity > 97%, particle size <100 nm, and surface area 15–25 m² g⁻¹. These nanoparticles were nearly slab-like in transmission electron microscopy (TEM) images, and the diameters varied from 30–100 nm, mainly (90.3%) at 40 ± 10 nm.¹⁸

2.2 Animals

Goldfish (*C. auratus*) were obtained from Fuzimiao aquatic breeding base (aquaculture facility, Nanjing, China) with average body mass at 31.64 ± 1.10 g. The fish were acclimatized for two weeks in dechlorinated municipal water prior to the test and the mortality rate was below 1%.

2.3 Aquatic ecosystem model

The experiment was conducted in two OTCs (area 0.603 m², height 1.8 m). One OTC was flushed with ambient CO₂ (hereafter called A-OTC, CO₂ concentration 400 ± 10 µL/L), and the other OTC was flushed with elevated CO₂ (hereafter called E-OTC, CO₂ concentration 600 ± 10 µL/L). The CO₂ enriched air was pushed (1300 m³/h, 24 h/day) inside the E-OTC chambers through perforated polyvinyl tubes. The average CO₂ concentration was determined by CO₂ analyzer. Each OTC held six glass aquaria of 30 cm × 30 cm × 50 cm (L×W×H). Sediment was collected from Tianlai Lake, Nanjing,

China (118°57'E, 32°07'N), and was smoothly spread on the bottom of each aquarium at a thickness of 10 cm. To each aquarium, 27 L water was added. After stabilizing for 7 days, *n*ZnO were added to three aquaria of each OTC as following: 2.16 g nanoparticles were dispersed by ultrasonication (50 w, 40 kHz) for 30 min in 200 mL water taken from the aquarium, and the suspensions were poured into the aquarium and mixed by stirring with a glass rod. The other three aquaria of each OTC served as controls lacking *n*ZnO. Six fish were put into each aquarium 3 days after *n*ZnO addition and exposed for 30 days without any artificial feeding.

2.4 Zn content in the aquatic system

The fraction distribution of Zn in the sediment was analyzed according to BCR sequential extraction procedure.¹⁹ The sediment background contained 134.94 ± 14.71 mg total Zn per kg dry wt. in the following proportions: weak acid soluble fraction, 6.99 ± 1.01%; reducible fraction, 5.24 ± 1.10%; oxidizable fraction, 4.01 ± 0.98%; and residual fraction, 83.75 ± 12.81%.

To measure total Zn concentration in the suspension, 1 mL HNO₃ was added to 100 mL of each water sample, filtered the samples through 0.45 µm membranes, and diluted to a constant volume. To measure the concentration of ionic Zn (Zn²⁺) in the suspension, water sampled from the aquaria was centrifuged at 22,000 g for 5 min.²⁰ The supernatants were used for the concentration assay by atomic absorption spectrophotometer (Hitachi, Z-8100, Japan).

To measure the Zn content in fish tissues, 5 ml HNO₃ was added to 0.1 g (fresh weight) liver, brain, and muscle tissues, respectively. After incubation overnight, the mixtures were heated on a hotplate at 95 °C for 120 min, added with 1 ml 30% H₂O₂, and held at 80 °C for 35 min. After cooling, the total digestions were made up to 25 ml and analyzed on an atomic absorption spectrophotometer (Hitachi, Z-8100, Japan).

2.5 Physiological changes assay

After exposure for 30 days, the fish were euthanized, and the liver and brain tissues were carefully dissected and weighed. A fraction for 0.1 g was removed and homogenized quickly in 1.0 mL 50 mM *a*-phenyl-N-tert-butyl nitrone (PBN, dissolved in DMSO) using a Teflon pestle in a Potter homogenizer for electron paramagnetic resonance (EPR). The rest of the tissues were homogenized at 4 °C for other experiments. Reactive oxygen species (ROS) were detected according to the method of Shi et al.²¹ using Bruker EMX 10/12 X-band spectrometer (Bruker, Germany). Malondialdehyde (MDA) was measured by thiobarbituric acid assay as shown by the formation of thiobarbituric acid reactive substances (TBARS) with some modifications.²² Glutathione (GSH) was assayed according to the method of Hissin and Hilf.²³ Metallothionein (MT) protein amount was evaluated using the cadmium-binding assay according to the method of Onosaka and Cherian²⁴ with minor modifications.

2.6 Statistical analysis

The data are expressed as mean $\pm$ standard deviation of triplicates. Statistical analysis was conducted using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). Difference among treatments was determined using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons at a significance level of 0.05.

3. Results

3.1 Zn release to suspension

Figure 1 shows contents of Zn^{2+} and total Zn in suspension of the aquaria exposed to *nZnO*. It shows that elevated CO_2 enhanced the release of Zn to suspension. In aquaria added with *nZnO*, both Zn^{2+} and total Zn concentrations in suspension from E-OTC (0.21 ± 0.04 and 4.16 ± 0.43 mg/L, respectively) were significantly higher than those from A-OTC (0.11 ± 0.01 and 3.03 ± 0.20 mg/L, respectively) (Figure 1). And Zn^{2+} contents in control (i.e., without *nZnO* exposure) from both OTCs were too low to be detected. Additionally, elevated CO_2 also modified the pH value of the suspension (Figure 2). After 10 days exposure to elevated CO_2 (3 days exposure to *nZnO*), the pH value in E-OTC decreased from initial 8.10 ± 0.11 to 7.12 ± 0.15 , and it was significantly lower than that from A-OTC (7.64 ± 0.17). After 20 days (13 days exposure to *nZnO*), it was stable in both OTCs (8.01 ± 0.13 and 7.73 ± 0.12 for A-OTC and E-OTC, respectively).

3.2 Zn accumulation in fish tissues

Figure 3 shows Zn contents in liver, brain and muscle of *C. auratus* after 30-day exposure in A-OTC and E-OTC. Results show that fish accumulated more Zn in liver (0.06–0.11 mg/g) and brain (0.04–0.15 mg/g), less in muscle (0.006–0.01 mg/g). When exposed to elevated CO₂ alone, fish accumulated more Zn in all tissues when compared to those from A-OTC (increased by 43.3%, 86.4% and 22.5% in liver, brain and muscle, respectively). In fish exposed to *n*ZnO, Zn contents were significantly higher in liver and brain than those from A-OTC (increased by 46.7% and 39.4% for liver and brain respectively, Figure 3A and B). However, Zn content in the muscle exposed to *n*ZnO did not significantly differ between A-OTC (0.011 ± 0.001 mg/g) and E-OTC (0.012 ± 0.001 mg/g, Figure 3C).

3.3 Physiological changes in fish tissues

A typical six-line (triplet of two lines in each, $a^N=13.88\pm0.11G$, $a^H=1.48\pm0.10G$) EPR spectrum of ROS was observed (Figure 4A–C), showing that hydroxyl radical ($\cdot OH$) generated in vivo in fish liver and brain after exposure to *n*ZnO. The difference between the signal intensity of the second hyperfine splitting peak was taken to represent the concentration of the PBN-radical adduct.²⁵ Figure 4D and E show that the signal intensity of ROS in liver was relatively higher than that in brain. *n*ZnO alone enhanced generation of ROS in liver compared to control in A-OTC. Compared with control in A-OTC, ROS intensity exposed to *n*ZnO at 80 mg/L in E-OTC was also higher. But, there was no significant difference between A-OTC and E-OTC at the same

exposure concentration of *n*ZnO. However, either *n*ZnO or elevated CO₂ alone stimulated ROS generated in brain; there was almost twice more ROS formed the in the fish brain exposed to *n*ZnO in E-OTC when compared to that exposed to *n*ZnO in A-OTC.

Changes in MDA content are shown in Figure 5 A and B. *n*ZnO alone caused no significant change in MDA contents either in liver or brain. But, elevated CO₂ alone led to significantly higher MDA contents in those tissues. In fish liver, elevated CO₂ increased MDA content induced by *n*ZnO. MDA content in liver with combined exposure (exposed to *n*ZnO in E-OTC) was over double (221.28%) of that exposed to *n*ZnO in A-OTC (Figure 5A). However, elevated CO₂ did not cause significant difference in brain MDA content induced by *n*ZnO (Figure 5B).

Changes in GSH content are shown in Figure 5 C and D. In A-OTC, *n*ZnO alone did not affect the GSH content in brain but significantly increased GSH content in fish liver compared to the control. Elevated CO₂ alone did not affect the GSH content either in liver or brain. However, combined exposure of *n*ZnO and elevated CO₂ led to obviously lower GSH content in both liver and brain.

Neither *n*ZnO nor elevated CO₂ alone caused obvious change in MT content in liver and brain (Figure 5E and F). However, combined exposure significantly increased MT content in those tissues. In E-OTC, the MT content in fish liver exposed to *n*ZnO was 172.8 ± 24.8 µg/mg protein, which was almost 1.7-fold higher than that exposed to *n*ZnO in A-OTC and also significantly higher than

that of the control. Similarly, the MT content in brain exposed to *n*ZnO in E-OTC was also significantly increased when compare with that exposed to *n*ZnO in A-OTC.

4. Discussion

The agglomeration and stability of particle dispersions are determined by the sum of the attractive and repulsive forces between individual particles. The attraction between particles is due to van der Waals forces, and the electrostatic repulsive force is the interaction of the electrical double layer surrounding each particle.²⁶ Two important properties of the electrical double layer are the thickness and the zeta potential, and an increase in either will result in an increase in the electrostatic repulsive interaction.²⁷ Zeta potential and consequently the hydrodynamic size can be altered by pH. Jiang et al. reported the zeta potential and average diameter of *n*TiO₂ as a function of pH with the ionic strength held constant, when the pH approaches the isoelectric point, the repulsive force is weakened due to low surface charge and the hydrodynamic size increases.²⁸ When pH > 7, *n*ZnO showed a lower solubility at higher pH values, with 3.67% and 4.93% of total Zn shown as Zn²⁺ in suspension from A-OTC and E-OTC, respectively (Figure 1). This decreased solubility could result from the hydroxide layer formed on the surface of *n*ZnO at high pH conditions, inhibiting further dissolution under alkaline environments.²⁹

When NPs enter natural waters, their physicochemical properties are affected by water chemistry (pH, ionic components, dissolved organic matter, etc.). Thus, the dispersion, mobility, bioavailability, and toxicity of NPs in the natural environment will be very different from laboratory measurements.³⁰ Once the NPs are in the water/sediment environment, their form, distribution between media, and ultimate transport will depend on a variety of physical influences.⁸ NPs in aquatic systems could potentially dissolve, agglomerate, sediment, associate, react, or decompose.⁹ Bradford et al.³¹ reported that once released into the coastal environment, Ag NPs are likely to precipitate onto the surface of sediment. Eventually, aquatic sediments will form an important sink for NPs. In our study, with an initial *n*ZnO concentration in aquarium water of 80 mg/L (i.e., total Zn at 64.27 mg/L), only 4.72% was detected in suspension and 0.17% detected as Zn²⁺ in A-OTC (Figure 1). de Orte et al.³² investigated the effects of simulated CO₂ leakage from geological storage sites on the fate of metals incorporated in highly contaminated sediments, and up to 11.21% of Fe was observed in water. In this work, elevated CO₂ led to higher Zn observed in water, with 6.47% detected in suspension and 0.32% as Zn²⁺ in E-OTC (Figure 1).

It is estimated that atmospheric CO₂ levels could approach 600 µL/L by the end of this century, and accordingly sea acidification could change the physical and chemical properties of the water environment.¹³ Thus, the dispersion, mobility, bioavailability, and toxicity of *n*ZnO in water would be affected. The

concentration of dissolved CO₂ in freshwater is approximately 15 µmol/L after the CO₂ partial pressure balanced between air and water. It has been reported that atmospheric CO₂ level at 700 µL/L would result in a pH drop of approximately 0.28.¹⁵ Similarly, in our experiment, the elevated CO₂ levels led to a pH drop at about 0.28, consequently increasing the content of Zn observed in suspension.

With changes in water properties under future CO₂ levels, the release and solubility of *n*ZnO in water will be affected, leading to expected changes in toxicity of *n*ZnO to aquatic organisms. In our study, we observed a significant increase of Zn content in liver, brain, and muscle tissues of fish after 30-day exposure to *n*ZnO (Figure 3). Previous studies proved that NPs can be taken up and translocated to other organs via the blood.³³ Ramsden et al.³⁴ reported that Ti accumulation occurred in the gill, gut, liver, brain, and spleen of rainbow trout during dietary exposure. Hao et al.⁶ also noted that liver and brain might be the target tissues of *n*ZnO in juvenile carp. Similarly, in our study, the target tissues of *n*ZnO to *C. auratus* were shown to be liver and brain, and elevated CO₂ increased the accumulation (Figure 3A and B), indicating greater ecological effect of *n*ZnO in future CO₂ levels. Muscle tissue is shown to be less affected by *n*ZnO, and the accumulation of Zn in muscle tissue was only about 11.5% of that in liver tissue (Figure 3C). No statistically significant difference of Zn concentration was found in fish muscle when comparing E-OTC with A-OTC. It seems to correlate with low blood perfusion rates and

the very low metabolic demand in muscle.^{35,36} Zn in fish tissues may consist of three parts: soluble Zn ions (Zn^{2+} , $\text{Zn}(\text{OH})^+$, ZnCl^+ , etc), nano-sized ZnO that directly enter the cell in water and soluble Zn ions released in the cell from *n*ZnO that enter the cell. Soluble Zn ions that enter with water are influenced by pH, anions (Cl^- , SO_4^{2-}), and redox potential.³⁷ Elevated CO_2 could reduce the water pH, affecting the redox potential and surface charge of NPs by reducing the water pH.³⁸⁻⁴⁰ Tortiglione et al.³⁹ noted that only particles with positive surface charge are actively internalized by tentacle and body ectodermal cells, while negatively charged nanoparticles are not uptaken. Fabrega et al.⁴⁰ also noted a higher uptake of NPs at lower pH in *Pseudomonas putida*. Nevertheless, the mechanisms of *n*ZnO internalization, transportation, and deposition in fish remain unclear and deserve more research.

The accumulation of Zn in fish liver and brain observed in this study indicated that continuous exposure to *n*ZnO would be toxic to fish. It is widely accepted that the toxicity of *n*ZnO is usually ROS mediated.⁴¹ There are some reports that nano- C_{60} , *n*CeO₂, and other NPs may alter ROS production, and thereby, cause interference in biological antioxidant defense responses.⁴²⁻⁴⁴ In our study, elevated CO_2 induced ROS production in the liver and brain, which is consistent with the high concentration of Zn found in the fish organs from the E-OTC group. Antioxidant defense systems include a series of antioxidant enzymes and low-molecular non-enzymatic antioxidants, playing a role in free

radicals scavenging and serving as an important biological defense against oxidative stress at the cellular level.⁴⁵ Under normal physiological conditions, antioxidant defense systems can remove ROS. However, a severe oxidative stress may suppress the activities of these enzymes and lead to oxidative damage to DNA, lipid, protein, and other key molecules.²⁵ MDA is the final product of lipid peroxidation showing damage as a consequence of ROS, and it has been used extensively as a biomarker of oxidative stress in fish.⁴⁶ An increase in MDA was found in fish liver exposed to *n*ZnO in E-OTC due to increased accumulation of Zn (Figure 5A). Similar increase in MDA contents, as a result of *n*ZnO exposure was also recorded in zebrafish by Zhao et al.¹ In addition, a significantly higher level of MDA found in E-OTC compared to A-OTC suggested that elevated CO₂ enhanced the oxidative damage to fish caused by *n*ZnO.

GSH is one of the most important antioxidants and plays a number of key roles in many biochemical pathways. The peptide is highly reactive and forms conjugates with other molecules via its sulfhydryl moiety and complexes of GSH with metal ions as a new biochemical marker of aquatic environment pollution.⁴⁷ In our study, due to the synthesis of a new GSH triggered by *n*ZnO, the GSH content in liver of fish in A-OTC increased significantly. The lower GSH content in brain under combined exposure was considered to be related to more severe oxidative stress in E-OTC inhibiting related enzyme activity.⁴⁶ Furthermore, the decrease of GSH would weaken the detoxification capability

of antioxidant defense system and alter the redox status of cell, therefore more ROS accumulated and more MDA would be formed.

The cysteine residues of MT serve to coordinate heavy metal atoms such as zinc, copper, and cadmium via mercaptide linkages.⁴⁸ In addition, they were postulated to detoxify metals, play a role in zinc homeostasis during development, regulate synthesis, assembly, or activity of zinc metalloproteins, and protect against reactive oxygen species.⁴⁸ Thus, MT can be taken as one of the most important indices of *n*ZnO toxicity. Its basal level is low in most tissues, but a variety of stimuli such as metals and oxidant-induced stress can markedly induce the synthesis of MT.⁴⁹ It has been reported that Ag exposure could increase MT content, providing a protective effect against Ag-induced oxidative stress.⁵⁰ In this study, *n*ZnO in E-OTC induced more MT synthesis in both liver and brain compared to A-OTC (Figure 5E and F), similar to the Zn accumulation and ROS generation in such tissues. A GSH depletion and MT induction levels in liver and brain of fish exposed to *n*ZnO in E-OTC was observed. It seems to correlate with the depletion of GSH caused by ROS triggering an increase in MT, thereby, maintaining a constant level of sulfhydryl in cell.⁵¹

5. Conclusions

Further investigations are required to elucidate the toxicity of *n*ZnO to natural ecosystems in future conditions and to characterize the protective role of antioxidant defense systems. Our study was the first attempt at understanding

the toxicity of *n*ZnO to fish in a water-sediment ecosystem exposed to both ambient CO₂ and elevated CO₂ levels. Elevated CO₂ reduced the pH of the water in such ecosystems, thus, increasing the content and bioavailability of Zn in suspension. Under elevated CO₂ levels, with higher levels of Zn accumulated in the liver and brain of fish, more ROS were induced, leading to greater challenge to the antioxidant defense system. We hypothesize that with the elevated CO₂ levels predicted for the latter part of this century, the fate of *n*ZnO in aquatic environments will change, with enhanced toxicity to aquatic organisms.

Financial and competing interest disclosure

The authors declare no competing financial interests.

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Figure captions

Fig. 1. Concentration of (A) ionic Zn and (B) total Zn in water in the aquaria in OTCs with ambient (A-OTC, $400 \pm 10 \mu\text{L/L}$) or elevated CO_2 levels (E-OTC, $600 \pm 10 \mu\text{L/L}$). Data are means of three replicates $\pm$ standard deviation. Different letters among columns indicate statically significant differences at $p \leq 0.05$.

Fig. 2. pH value of water in aquaria exposed to ambient (A-OTC, $400 \pm 10 \mu\text{L/L}$) or elevated CO_2 levels (E-OTC, $600 \pm 10 \mu\text{L/L}$). The initial pH value was measured immediately after the aquaria were set up (day 0). ZnO NPs were added to three of six aquaria of each OTC on day 7. Six fish were placed into each aquarium on day 10. Data are means of three replicates $\pm$ standard deviation.

Fig. 3. Zn concentration in (A) liver, (B) brain and (C) muscle of *C. auratus* exposed to with ambient (A-OTC, $400 \pm 10 \mu\text{L/L}$) or elevated CO_2 levels (E-OTC, $600 \pm 10 \mu\text{L/L}$) for 30 days. Data are means of three replicates $\pm$ standard deviation. Different letters among columns indicate statically significant differences at $p \leq 0.05$.

Fig. 4. EPR detection of the PBN-radical adducts in (A) liver and (B) brain of *C. auratus* after exposure to ZnO NPs. (C) EPR spectrum of 50mM PBN in DMSO. EPR signal intensity of ROS in (D) liver and (E) brain of *C. auratus* exposed to 0 and 80 mg/L *n*ZnO with ambient (A-OTC, $400 \pm 10 \mu\text{L/L}$) or elevated CO_2 levels (E-OTC, $600 \pm 10 \mu\text{L/L}$) for 30 days. Data are means of

three replicates $\pm$ standard deviation. Different letters among columns indicate statically significant differences at $p \leq 0.05$.

Fig. 5. Content of (A, B) MDA, (C, D) GSH and (E, F) MT in (A, C, E) liver and (B, D, F) brain of *C. auratus* exposed to 0 and 80 mg/L *n*ZnO with ambient (A-OTC, 400 ± 10 μ L/L) or elevated CO₂ levels (E-OTC, 600 ± 10 μ L/L) for 30 days. Data are means of three replicates $\pm$ standard deviation. Different letters among columns indicate statically significant differences at $p \leq 0.05$.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.