Journal of Hazardous Materials 192 (2011) 1565–1571

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Metal oxide nanomaterials in seawater: Linking physicochemical characteristics with biological response in sea urchin development

Elise A. Fairbairn[a], Arturo A. Keller[b], Lutz Mädler[c,d], Dongxu Zhou[b], Suman Pokhrel[c,d], Gary N. Cherr[a,e,*]

[a] University of California Davis, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923, USA
[b] Bren School of Environmental Science and Management, 3420 Bren Hall, University of California Santa Barbara, Santa Barbara, CA 93106, USA
[c] IWT, Foundation Institute of Materials Science, Department of Production Engineering, University of Bremen, Badgasteiner Str. 3, 28359 Bremen, Germany
[d] Center for Nanoscience Institute, University of California Los Angeles, Los Angeles, CA 90095, USA
[e] Departments of Environmental Toxicology & Nutrition, University of California Davis, Davis, USA

ARTICLE INFO

Article history:
Received 25 January 2011
Received in revised form 27 May 2011
Accepted 28 June 2011
Available online 2 July 2011

Keywords:
Seawater
Engineered nanomaterial
Sea urchin embryo
Metal oxide nanoparticles
Aggregation

ABSTRACT

The fate and behavior of nanomaterials (NMs) in environmental media has important consequences for toxicity. The majority of aquatic research to date has focused on NM behavior in freshwater systems. However, pH and salinity differences of seawater affect dissolution and aggregation of NMs. In this study, physical characteristics of metal oxide NMs in seawater were linked with their toxicity to developing sea urchins. The metal oxide NMs TiO_2 and CeO_2 up to 10 mg/L were not toxic to the embryos of the white sea urchin (*Lytechinus pictus*). In contrast, ZnO NM was highly toxic to these embryos (EC_{50} = 99.5 μg/L). The toxicity of ZnO NM was not significantly different from bulk ZnO or soluble Zn^{2+} (from $ZnSO_4 \cdot 7H_2O$), suggesting that the toxicity of ZnO NM can be attributed to soluble Zn^{2+}. Furthermore, solubility data indicate that at the concentrations used in our sea urchin embryo experiments, ZnO NM was rapidly and completely solubilized in seawater. The present study also demonstrated that Fe-doped NMs were less soluble in seawater compared to pure ZnO NMs, but there was no concomitant reduction in toxicity.

1. Introduction

The impact of nanomaterials (NMs) on the aquatic environment is an important question in ecotoxicology. Nanomaterial pollution during manufacturing processes, accidents during transport, consumer use and improper disposal can reach the aquatic environment via wastewater and runoff [1]. The coastal marine environment is susceptible to human impacts via rivers, estuaries and wastewater outfalls that discharge into the nearshore environment. Several metal oxide NMs (e.g. TiO_2, CeO_2 and ZnO) are widely used in consumer products such as pigments and catalysts, as well as in personal care products. TiO_2 and ZnO NMs have intrinsic UV-absorbing properties and are commonly used in sunscreens [1], and researchers have estimated that 25% of sunscreen that is applied to skin is washed off within 20 min in water [2]. Although nanomaterial pollution has not been directly measured in marine environments, chemical UV absorbers (like butylparaben) found in some sunscreens have been shown to accumulate and have adverse effects in marine species [1,2]. This highlights the possibility that NMs from consumer products may also impact the marine environment. However, the potential toxicological impacts of metal oxide NMs in the marine environment have received little attention.

The behavior and fate of NMs in the environment, both at the nano–bio interface and in terms of larger scale transport, mobility, persistence and bioavailability, are known to directly influence toxicity [3–6]. Aggregation of NMs in environmental media, as well as the dissolution of ions from NMs, are both influenced by the physical and chemical characteristics of the media [3,4]. Aggregation and dissolution, in turn, are expected to greatly affect bioavailability and toxicity [3,4]. The ionic strength, ionic composition, and the presence of dissolved organic matter can influence the stability of metal oxide NMs in water [7]. The majority of aquatic research to date on the environmental fate, transport and toxicity of NMs has been conducted in freshwater systems and organisms. Expectations and early evidence predict that the behavior of NMs in seawater may be quite different than in freshwater [3,7,8]. For example, increasing ionic strength has been shown to increase aggregation and can reduce solubility [7].

The toxicity of ZnO NMs has been previously attributed to the dissolution of Zn^{2+} from the NM into freshwater [9–11], seawater [8,12], and cell culture media [13]. ZnO NMs have been widely

* Corresponding author at: Bodega Marine Lab, PO Box 247 Bodega Bay, CA 94923, USA. Tel.: +1 707 875 2051; fax: +1 707 875 2009.
E-mail address: gncherr@ucdavis.edu (G.N. Cherr).

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doi:10.1016/j.jhazmat.2011.06.080

studied in freshwater systems, and it has been estimated that at concentrations less than 1 mg/L, an average of 83% of nano and bulk ZnO is ionized to Zn^{2+} [11]. In contrast to ZnO NM, the toxicities of TiO_2 and CeO_2 NMs have been reported to be low [4,12–14], although TiO_2 has been shown to induce oxidative stress [15].

The diversity of NMs in production and use is large and growing, and precludes the ability to evaluate the environmental fate and toxicity of each one. However, there has been an effort to identify the properties of NMs associated with toxicity by concentrating research efforts on a subset of NMs [13]. Given the large number of variables in determining mobility, persistence, bioavailability and toxicity, it is critical that these representative NMs are tested in diverse environmental media and organisms, and at sensitive life stages (e.g. during embryogenesis).

Critical to understanding NM toxicity is the need to identify whether toxicity can be attributed to the bulk properties of the compound in question, or whether there are particle-specific effects. In this study, metal oxide NMs as well as other zinc-containing compounds were dispersed in natural seawater. The toxicities of CeO_2, TiO_2, ZnO NMs, bulk ZnO and soluble Zn^{2+} (from $ZnSO_4{\cdot}7H_2O$) to highly sensitive developing sea urchin embryos [16,17] were investigated. Physical characteristics of the NMs in seawater were investigated and correlated with the toxicity of the NMs to sea urchin embryos. Sea urchin development is a classic model and has been widely used to determine the effects of toxicants in seawater. A previous study has shown that iron doping of ZnO NMs can reduce solubility as well as toxicity to mammalian cells in culture [18], and this was also investigated in this study using sea urchin embryos in seawater. In order to compare the results obtained in the previous study using cells in culture [18] with developing embryos in seawater, the toxicity, dissolution rate and sedimentation characteristics of iron-doped ZnO NM were compared to pure ZnO NM in seawater.

2. Materials and methods

2.1. Synthesis of ZnO NM and Fe-doped ZnO NM

ZnO and Fe-doped ZnO NMs were synthesized by flame spray pyrolysis [19] as described in George et al. [18].

2.2. Chemicals

CeO_2 NM was purchased from Meliorum Technologies (Rochester, NY). TiO_2 NM was purchased from Evonik Degussa (Parsippany. NJ). Bulk ZnO, $ZnSO_4{\cdot}7H_2O$, alginic acid and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). The physicochemical characteristics of CeO_2 and TiO_2 NMs can be found in Keller et al. [7], and reproduced in Table S1. The physicochemical characteristics of ZnO and Fe-doped ZnO NMs are presented in Table 1.

2.3. Metal oxide nanomaterial dispersal in seawater

Stock solutions (1 g/L) of metal oxide nanomaterials and the other Zn-containing compounds were prepared in de-ionized water and then sonicated in a Branson model 2510 sonic bath (Danbury, CT) for 30 min (max. 100 W). Each stock solution (unfiltered) was then diluted into 0.45 μm filtered (to remove particulates) seawater containing 10 mg/L alginic acid as a source of dissolved organic carbon.

2.4. Dissolution and particle aggregation

After dispersion, the ZnO stock solutions were placed for increasing time periods in Amicon Ultra-15 Ultracel 3 centrifuge tubes (3 kDa cutoff, Millipore, Billerica, MA). Three initial concentrations were evaluated: 0.1, 1.0 and 10 mg/L. They were then centrifuged for 30 min in a Sorvall RC5B Plus centrifuge (Thermo Scientific, Asheville, NC) with a swinging bucket rotor at 4000 × *g*. The filtrate was then analyzed via ICP-AES (iCAP 6300, Thermo Scientific, Waltham, MA). All experiments were performed at 22 ± 2 °C.

Particle size at different stages of the aggregation process was determined via dynamic light scattering (DLS) using a Malvern Nanosizer (Malvern Instruments, Worcestershire, UK). Initial particle concentration was 100 mg/L. The sedimentation process was measured using a UV–vis spectrophotometer (BioSpec 1601, Shimadzu, MD) using time-resolved optical absorbency (ZnO at 378 nm). Optical absorbency was measured every 6 min for 360 min. The experiments were run in triplicate, and the results presented are the mean value of each run.

Table 1
Physicochemical characteristics of ZnO and Fe-doped ZnO nanomaterials (NMs).

Sample	Surface area (m^2/g)	d_{BET}[a] (nm)	d_{XRD}[b] (nm)
ZnO NM	52.11	20.2	18.8
10% Fe-doped ZnO NM	129	8.3	5.5

[a] Brunauer–Emmett–Teller analysis (d_{BET}).
[b] X-ray powder diffraction (d_{XRD}).

2.5. Sea urchin embryo development

Sea urchin (*Lytechinus pictus*) embryos were exposed to a range of concentrations of nanomaterials and other Zn-containing compounds diluted into 0.45 μm-filtered seawater (FSW) containing a final concentration of 0.2–0.3 mg/L alginate (Sigma, #A2158). The pH of the FSW was 8.2, and was unaffected by 0.2–0.3 mg/L alginate. The pH of the ZnO, CeO_2 and TiO_2-containing solutions was between 8.0 and 8.2, well within the range known to result in normal development. Embryos were exposed from the 2-cell stage until the pluteus larval stage (approximately 96 h after fertilization) in 12-well polystyrene culture plates, and incubated at 15 °C. When controls (0.2 mg/L alginate in 0.45 μm filtered seawater) reached the pluteus stage, all samples were fixed with 0.1% paraformaldehyde in seawater (final concentration) and assessed for normal development. The research by Kobayashi and Okamura [20] was used as a reference for determining the normal pluteus larval morphology in control and Zn-exposed embryos. Briefly, embryos were considered abnormal if radialized plutei, Apollo-like gastrula, exogastrulation, or inhibited or delayed development were observed. Experiments were conducted at least three times, and for each experiment, treatments were always run in triplicate. For each replicate, 100 embryos were assessed for normal development using a Nikon AZ100 macrozoom stereo microscope at 40× magnification.

Statistical analysis was accomplished by fitting a logistic generalized linear mixed model (GLMM) with logit link, binomial error and "experiment" as a random factor. 95% confidence intervals (CI) for EC_{50} values were estimated using Monte Carlo simulation. The logistic regression equation was solved for 50% abnormality, and a distribution of 10,000 estimates of EC_{50} was generated using coefficient values drawn from the multivariate normal distribution defined by the means and covariances of the regression model coefficients. EC_{50}s with non-overlapping CIs were determined to be significantly different. Logistic GLMM was performed using the lme4 package [21] in R 2.10.0 [22].

3. Results

3.1. Particle size distribution, aggregation kinetics and sedimentation

The initial particle size distribution indicates that the pure ZnO NMs dispersed in 0.45 μm filtered Bodega Marine Laboratory seawater aggregated to approximately 300–350 nm, the Fe-doped ZnO NMs to approximately 450 nm, while the bulk ZnO had an initial size of approximately 615 nm (Fig. 1). However, there was a range of initial aggregate sizes for each sample. All three particles aggregated rapidly to sizes above 1000 nm within 30–40 min, at approximately the same rate of aggregation (Fig. 2). However, due to their initial larger size, the bulk ZnO sedimented rapidly in seawater, decreasing their concentration by 60% within 60 min (Fig. 3). The Fe-doped ZnO NM also sedimented relatively fast, although the concentration decreased only by 40% within the first 60 min. The pure ZnO NMs remained stably dispersed for the first 45 min, but then the two main processes, aggregation and dissolution, rapidly reduced the number of NMs in the water column. The addition of 10 mg/L alginate significantly increased the dispersion of the three metal oxide NMs in seawater (Fig. 4). This helped to maintain the NMs in suspension for a longer time during the toxicity experiments.

3.2. Particle dissolution rates in seawater

Overall, the rate of dissolution was fastest for the pure ZnO NMs, followed by the bulk ZnO and slowest for the 10.2% Fe-doped ZnO NMs. At low [ZnO] (0.1 and 1 mg/L), dissolution was almost complete within 10–20 h, but at higher [ZnO] (10 mg/L) the rate of dissolution slowed considerably after the first 24 h. For [ZnO] = 10 mg/L, the final percent dissolved was 66% for the pure ZnO NMs, 24% for the Fe-doped ZnO NMs and only 9% for the bulk ZnO (Fig. 5). However, at concentrations near those used in the sea urchin embryo toxicity experiments, (for ZnO, 0.1 mg/L) the final percent dissolved was ~100% for the pure ZnO NMs, ~80% for the Fe-doped ZnO NMs and ~100% for bulk ZnO. $ZnSO_4 \cdot 7H_2O$ dissolved completely within the first 30 min (data not shown).

3.3. Embryo toxicity

Sea urchin embryos were exposed to NMs and Zn-containing compounds during a 96 h developmental time course, in which normal/abnormal morphology was the experimental endpoint. CeO_2 and TiO_2 NMs did not induce developmental abnormalities in sea urchin embryos under the conditions of our experiments (Fig. 6). All of the Zn-containing compounds were toxic to developing sea urchin embryos at the low μg/L range (Fig. 7). The morphological abnormalities of pluteus larvae that had been exposed to Zn-containing compounds were diverse (Fig. S1), but resembled those described by Kobayashi and Okamura [20]. Treatments with overlapping 95% confidence intervals were not significantly different from one another. The Fe-doped ZnO NM treatment was significantly different from $ZnSO_4 \cdot 7H_2O$ treatment, but the other treatments were not significantly different from each other. Specifically, the effects of bulk ZnO, $ZnSO_4 \cdot 7H_2O$, and pure ZnO NM were not significantly different from one another (Fig. 8). Furthermore, the effects of pure ZnO NM did not differ significantly from the Fe-doped ZnO NM (Fig. 9).

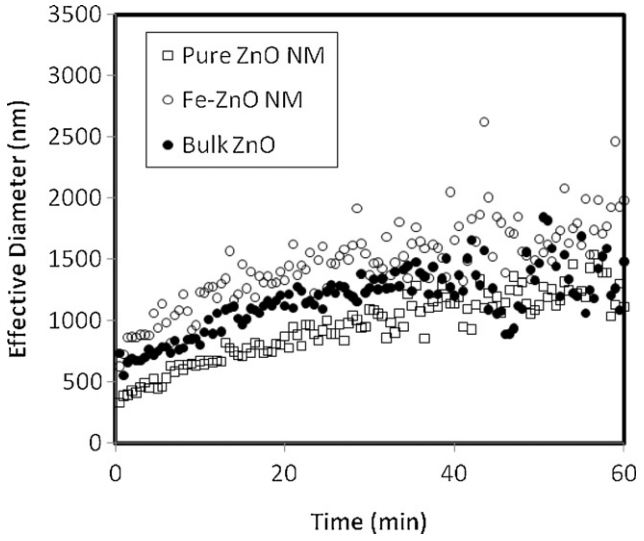

Fig. 2. Aggregation dynamics for ZnO particles, presenting the increase in diameter as a function of time.

4. Discussion

The bioavailability and toxicity of nanomaterials in the environment is a relatively new and pressing concern, and thus far,

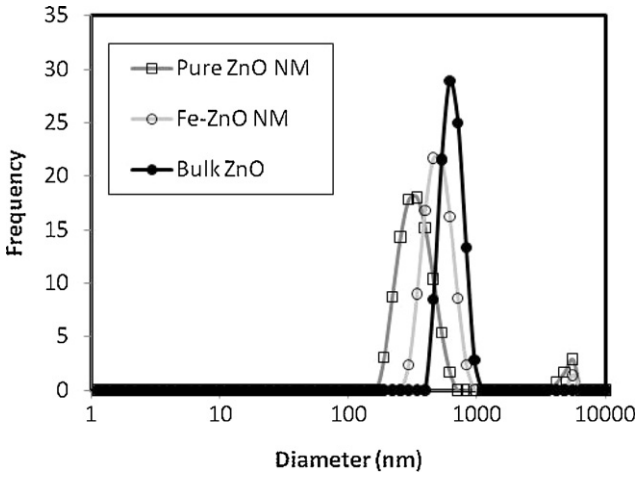

Fig. 1. Initial ZnO particle diameter distribution when dispersed in seawater.

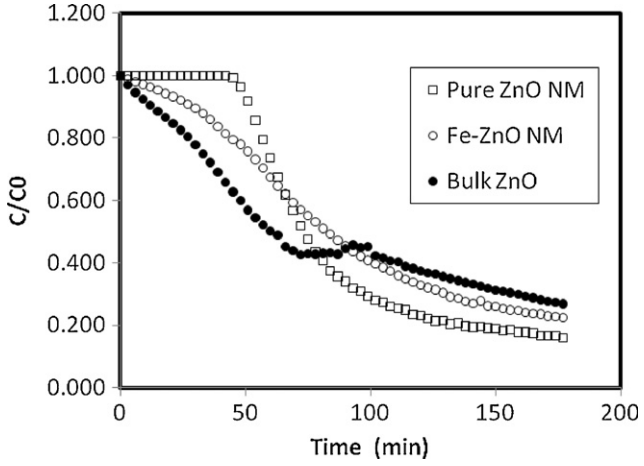

Fig. 3. Sedimentation rate of ZnO particles, presenting the decrease in particles in suspension as a function of time.

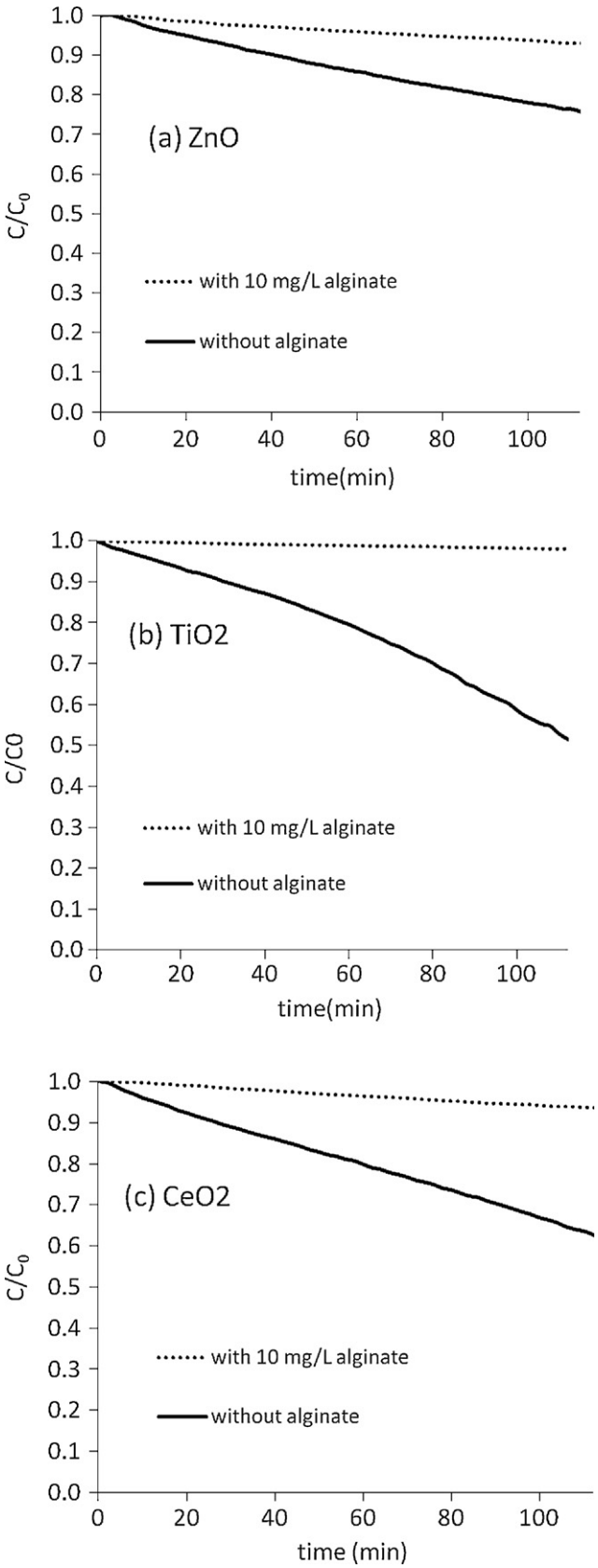

Fig. 4. Stabilization of the NPs in seawater using 10 mg/L alginate: (a) ZnO; (b) TiO_2; and (c) CeO_2.

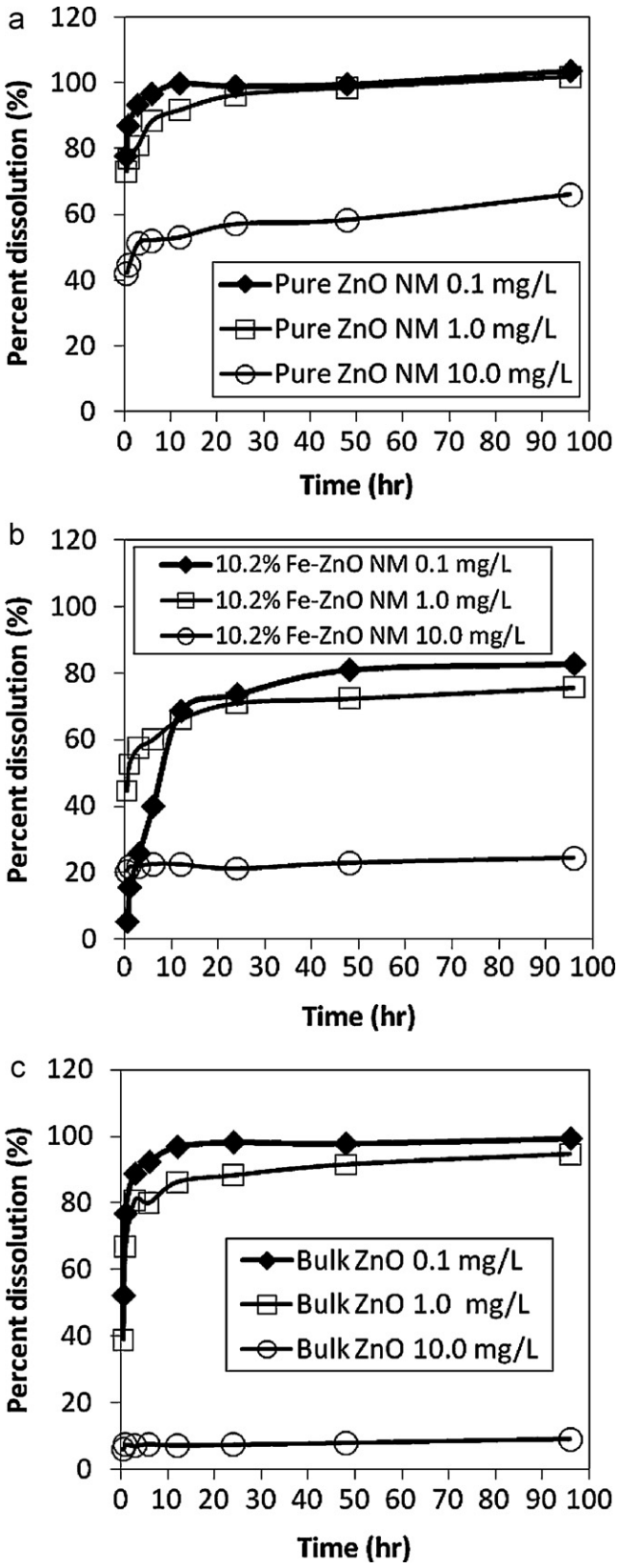

Fig. 5. Dissolution kinetics for the three different ZnO particles, indicating the percent dissolved as a function of time: (a) ZnO nanoparticles; (b) Fe-doped ZnO nanoparticles; and (c) bulk ZnO particles.

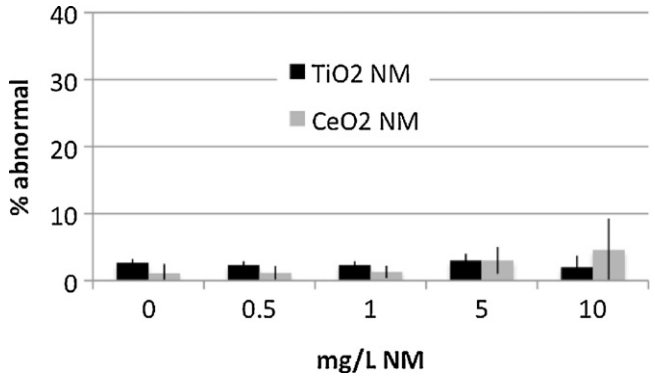

Fig. 6. Effects of TiO_2 NM and CeO_2 NM on sea urchin development in a 96 h developmental assay. Bars represent standard deviation from the mean.

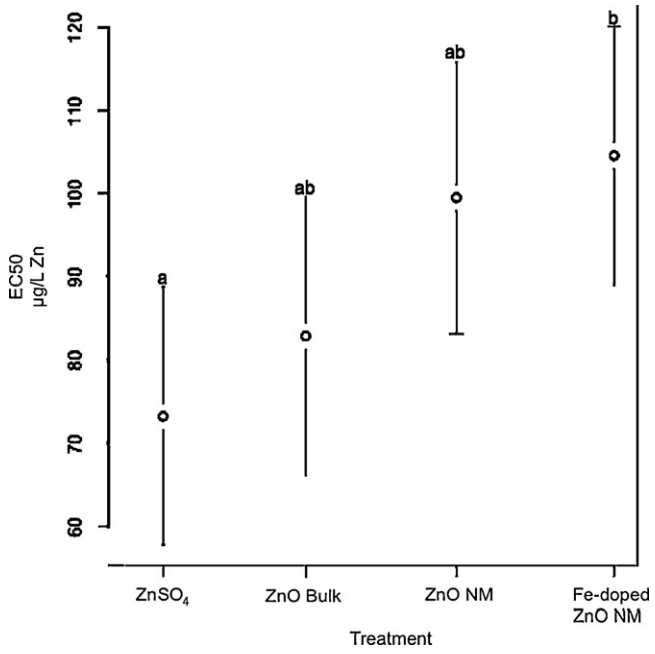

Fig. 7. EC_{50} values (μg/L Zn) for Zn-containing compounds tested in 96 h sea urchin developmental bioassay. Bars represent 95% confidence intervals (CIs). Treatments with overlapping 95% CIs are not significantly different from one another.

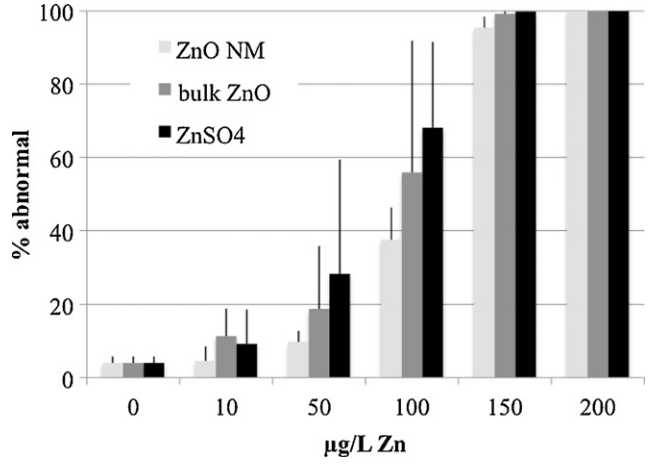

Fig. 8. Effects of ZnO NM, bulk ZnO and $ZnSO_4{\cdot}7H_2O$ on sea urchin development in a 96 h developmental assay. Bars represent standard deviation from the mean.

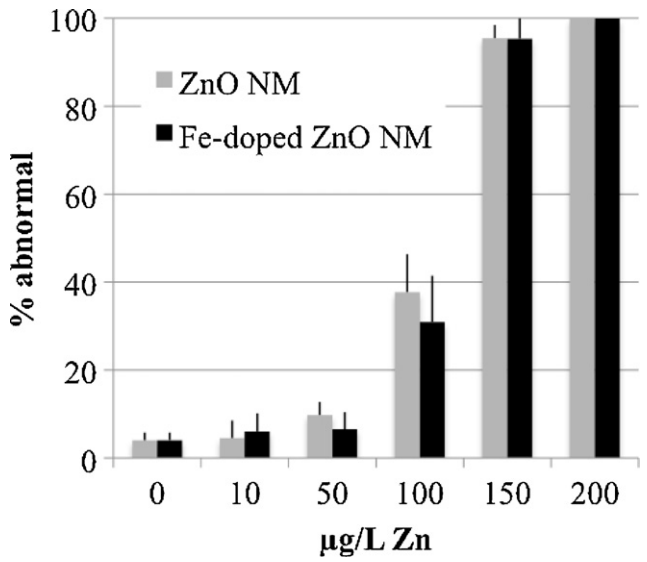

Fig. 9. Effects of pure ZnO NM and Fe-doped ZnO NM on sea urchin development in a 96 h developmental assay. Bars represent standard deviation from the mean.

freshwater species and ecosystems have received the bulk of the attention. This study linked the physicochemical characteristics of ZnO NMs in seawater with their toxicities in developing sea urchin embryos, and showed that the metal oxide NM ZnO is highly toxic, affecting development in the low μg/L range (EC_{50} = 99.5 μg/L). In contrast, neither CeO_2 NM nor TiO_2 NM was toxic to developing sea urchin embryos under the conditions of the bioassay, even at mg/L concentrations. This study demonstrates that some NMs may be non-toxic to highly sensitive developing marine embryos, while others can undergo specific environmental fate processes (e.g. dissolution) that result in toxicity.

The low toxicities of TiO_2 and CeO_2 NMs reported here are consistent with the literature, although TiO_2 NM has been shown to induce oxidative stress in other systems [4,13,14]. Both TiO_2 and CeO_2 NMs have been shown to aggregate and sediment rapidly (within 30–60 min) in natural waters, including seawater, and are insoluble [7].

The dissolution of ZnO NM and bulk ZnO in seawater was highly dependent on the initial concentraion. Wong et al. [8] demonstrated that the solubility of ZnO NM in seawater was 3.7 mg Zn/L. These results are comparable with those reported here. The 10.0 mg/L ZnO NM seawater solution reached approximately 60% dissolution over the course of the assay, corresponding to a solubility of approximately 4.8 mg Zn/L. In contrast, at low initial concentrations (0.1 mg/L), both nano and bulk ZnO were highly soluble. However, the percent dissolution of bulk ZnO was significantly lower than ZnO NM at higher initial concentrations, reaching only 9% dissolution when the starting concentration was 10 mg/L, an effect that is related to size and aggregation. There are two main factors that influence the dissolution rate of the particles, namely the concentration gradient and the surface area available for dissolution. A primary particle dissolving within the water column will have a steeper concentration gradient around it than a particle within an aggregate of the same primary particles, due to the proximity of the other particles. The concentration gradient around an aggregate that has settled out of solution may be even smaller, since there is reduced transport of dissolved ions to the bulk aqueous solution. Furthermore, the points of contact between nanoparticles decrease to some extent the surface area available for dissolution, particularly when there are multiple contacts among particles. Thus, the rapid aggregation and sedimentation serves to

explain the decrease in dissolution rates at higher particle concentrations.

The toxicity of ZnO NM to developing sea urchin embryos appears to be largely attributable to the dissolution of Zn^{2+} into the seawater. At the concentrations used in our bioassay, ZnO is rapidly and completely solubilized. These results suggest that the sea urchin embryos/larvae were exposed primarily to soluble Zn^{2+} throughout the 96 h developmental assay. Additionally, the EC_{50} values for ZnO NM and bulk ZnO were not significantly different than the EC_{50} for $ZnSO_4{\cdot}7H_2O$, a zinc salt that is completely solubilized in less than 30 min. The morphological abnormalities that were observed were varied, but were consistent with previous research that showed that Zn^{2+}, in the μg/L range, could cause a suite of abnormalities in developing sea urchin embryos [20]. The most common abnormalities observed in our experiments were delayed/arrested development (gastrula stage), exogastrulation, skeletal abnormalities and radialized plutei.

The majority of the aquatic research to date supports the hypothesis that the toxicity of ZnO NM and bulk ZnO can be attributed largely to solubilized Zn ions. In seawater, the toxicities of ZnO NM to diatoms, crustaceans and fish were previously shown to be influenced significantly by Zn^{2+}. However, in contrast to the work presented here, differences in toxicity between ZnO NM and bulk ZnO were observed in that study [8]. The toxicity of ZnO NM has been assessed in a large number of freshwater species. Studies on microalgae [9,10,12], bacteria and crustaceans [11], and zebrafish [23] have concluded that solubilized Zn-ions from ZnO NMs play a role in toxicity. It is worth noting that Zn^{2+}, from all of the Zn-containing compounds tested in this study, was highly toxic to developing sea urchin embryos. Effects on development were observed at low μg/L levels, suggesting that sea urchin embryos are particularly sensitive to Zn^{2+}.

George et al. [18] hypothesized that for NMs whose toxicities can largely be attributed to the dissolution of metal ions, toxicities could be attenuated if it were possible to engineer the NMs with reduced solubility. Consistent with this hypothesis, they demonstrated that Fe-doped ZnO NM was less soluble than pure ZnO NM in cell media, and that the Fe-doped ZnO NM was less cytotoxic in two cell culture lines. Using this same NM we evaluated the physical characteristics of Fe-doped ZnO NMs in seawater, as well as toxicity in our *in vivo* sea urchin development experimental system. Consistent with the previous research, Fe-doped ZnO NM was less soluble than pure ZnO NM. However, in contrast to the reduced toxicity observed in the *in vitro* cell culture system, no significant difference between toxicity with Fe-doped ZnO NM and the pure ZnO NM was observed in our sea urchin developmental exposures. It was hypothesized that the 20% reduction in solubility of Fe-doped ZnO NM as compared to the pure ZnO NM would result in a reduction in toxicity. The fact that this difference was not observed suggests that there was a small Fe-doped ZnO NM particulate effect. Alternatively, detection of this subtle toxicological effect may not have been resolved under our experimental conditions.

Despite the reduced solubility and higher aggregation rates of ZnO NM in seawater compared to freshwater, we found that at environmentally relevant concentrations, ZnO NM was highly soluble and induced morphological abnormalities in developing sea urchin embryos. Consistent with previous research, our results indicate that the toxicological effect of ZnO NM is mediated through the dissolution of Zn^{2+}, rather than through particle-specific effects. However, reducing the solubility of ZnO NMs through iron doping did not decrease toxicity. These results highlight the importance of toxicity assessments both *in vitro* and *in vivo*, in numerous species and environmental media, and at various life stages. While the reduced solubility of Fe-doped ZnO NM was protective for *in vitro* cell culture tests [18], the benefits were marginal, at best, for a Zn^{2+}-sensitive marine embryo, under environmentally relevant conditions.

Acknowledgments

This material is based upon work supported by the National Science Foundation and the Environmental Protection Agency under Cooperative Agreement Number DBI-0830117. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation or the Environmental Protection Agency. This work has not been subjected to EPA review and no official endorsement should be inferred. We also thank Will White, Maia Colyar and Casey Leavitt for their help.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.06.080.

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