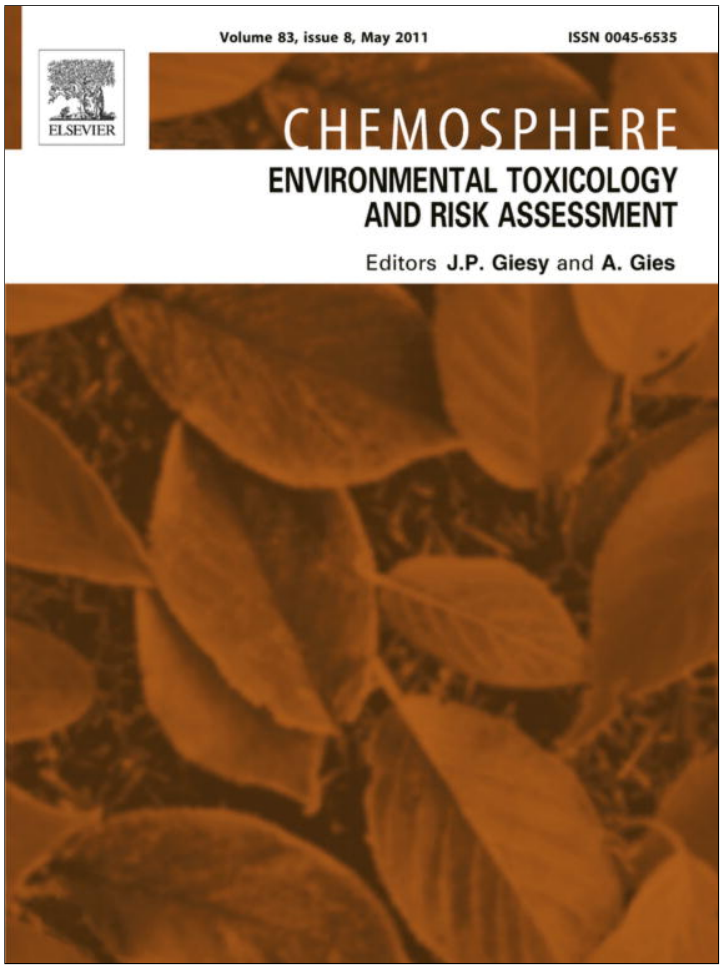

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Acute effects of Fe_2O_3, TiO_2, ZnO and CuO nanomaterials on *Xenopus laevis*

Shawna Nations [a,*,1], Mike Wages [a], Jaclyn E. Cañas [a], Jonathan Maul [a], Chris Theodorakis [b], George P. Cobb [a]

[a] Department of Environmental Toxicology, The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX 79409, USA
[b] Department of Biological Sciences, Southern Illinois University Edwardsville, Edwardsville, IL 62026, USA

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ABSTRACT

Metal oxide nanomaterials have exhibited toxicity to a variety of aquatic organisms, especially microbes and invertebrates. To date, few studies have evaluated the toxicity of metal oxide nanomaterials on aquatic vertebrates. Therefore, this study examined effects of ZnO, TiO_2, Fe_2O_3, and CuO nanomaterials (20–100 nm) on amphibians utilizing the Frog Embryo Teratogenesis Assay Xenopus (FETAX) protocol, a 96 h exposure with daily solution exchanges. Nanomaterials were dispersed in reconstituted moderately hard test medium. These exposures did not increase mortality in static renewal exposures containing up to 1000 mg L^{-1} for TiO_2, Fe_2O_3, CuO, and ZnO, but did induce developmental abnormalities. Gastrointestinal, spinal, and other abnormalities were observed in CuO and ZnO nanomaterial exposures at concentrations as low as 3.16 mg L^{-1} (ZnO). An EC_{50} of 10.3 mg L^{-1} ZnO was observed for total malformations. The minimum concentration to inhibit growth of tadpoles exposed to CuO or ZnO nanomaterials was 10 mg L^{-1}. The results indicate that select nanomaterials can negatively affect amphibians during development. Evaluation of nanomaterial exposure on vertebrate organisms are imperative to responsible production and introduction of nanomaterials in everyday products to ensure human and environmental safety.

1. Introduction

Nanomaterials are components for emerging technologies that have numerous applications in electrical engineering, chemistry, material science, and medicine. Many advances in these industries are accelerating the introduction of nanomaterials into a variety of products such as marine paint and cosmetics. Toxicological testing of nanomaterials is imperative because the size of nanomaterials may create new toxicological issues for compounds that are relatively inert as bulk material. Some fine and ultra fine (<100 nm) particles have exhibited more toxicity than larger particles of the same chemical composition (Borm, 2002). Nanomaterials that contain compounds that are normally considered inert may have increased toxicological effects due to the reduced size and increased surface area of nanomaterials. Surface area and chemical properties influence material interactions with biological systems. For example, TiO_2 nanomaterials have the ability to inhibit cancer cell growth due to increased binding and reactivity (Borm, 2002; Lovern and Klaper, 2006). In addition, polytetrafluoroethylene nanomaterials (PTFE) inhalation exposures have been studied using rats (Service, 2003). After a fifteen minute exposure to 20 nm diameter PTFE nanomaterials, most of the rats died within four hours (Service, 2003). The result was much different when the rats were exposed to 130 nm PTFE particles; there were no negative effects observed (Service, 2003). In other studies, *Daphnia magna* exhibited higher mortality when exposed to TiO_2 nanomaterials with an average diameter of 30 nm than those exposed to 100–500 nm TiO_2 nanomaterials (Lovern and Klaper, 2006). Finally, investigation of differential toxicity for 10–20 nm fullerenes and 20–100 nm fullerenes demonstrated that smaller fullerenes proved to be more toxic with a LC_{50} at 0.46 mg L^{-1} while the larger fullerenes had a LC_{50} of 7.90 mg L^{-1} (Lovern and Klaper, 2006). Based on these studies, it is clear that size determination is important when considering the possible toxic effects of nanomaterials.

Metal oxide nanomaterials have been found to adversely affect mammalian cells and some aquatic organisms. For example, CuO nanomaterials induced death of H4 and SH-SY5Y cells in a dose dependent manner (Chen et al., 2007). Several types of metal oxide nanomaterials affected mitochondrial functions and induced lactate dehydrogenase (LDH) leakage at concentrations as low as 50–100 μg L^{-1} (Hussain et al., 2005; Jeng and Swanson, 2006). Metal oxide nanomaterial exposure caused abnormal cell morphology, such as abnormal size and shrinkage. Aquatic *in vivo* studies have shown that metal oxide nanomaterials can induce significant mortality at low mg L^{-1} exposures, such as 96 h LC_{50} of 1.793 mg L^{-1} ZnO in Zebrafish (*Danio rerio*) and LC_{50} of 5.5 mg L^{-1} TiO_2 in *Daphnia magna* (Lovern and Klaper, 2006; Zhu et al., 2008).

* Corresponding author. Address: Department of Environmental Toxicology, The Institute of Environmental and Human Health, Texas Tech University, PO Box 41163, Lubbock, TX 79409, USA. Tel.: +1 512 779 0061.
E-mail address: shaylobo42@hotmail.com (S. Nations).
[1] Present address: 107 Hague, St. Hutto, TX 78634, USA.

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Amphibian decline has been an issue of concern since the late 1950s. Rapid population declines were observed in the 1950s and have continued to the present (Houlahan et al., 2000; Alford et al., 2001; Sodhi et al., 2008; Wake and Vrendenburg, 2008). The increased use of metal oxide nanomaterials in catalysis, sensors, environmental remediation, and commercial products such as personal care products may adversely affect already impacted amphibian populations (Franklin et al. 2007). Titanium dioxide, Fe_2O_3, ZnO and CuO nanomaterials are utilized in cosmetics and antimicrobial products, so there is a strong possibility that these particular nanomaterials will ultimately enter aquatic ecosystems through waste water discharges and wash off during recreational activities such as swimming and water skiing. Currently, no other studies have been conducted to examine toxicity of metal oxide nanomaterials in amphibians. Thus, aquatic toxicity of 4 metal oxide nanomaterials was evaluated using the Frog Embryo Teratogenesis Assay Xenopus Assay (FETAX). The purpose of this study was to determine the effect of select metal oxide nanomaterials on *Xenopus laevis* (*X. laevis*) during organogenesis.

2. Methods and materials

Nanomaterials were obtained from Alfa Aesar (Ward Hill, MA). Each type of nanomaterial was supplied with average particle size (APS) and surface area data as follows. Characteristics for the respective nanomaterials were TiO_2, 32 nm and 45 $m^2\,g^{-1}$; ZnO, 40–100 nm and 10–25 $m^2\,g^{-1}$; CuO, 23–37 nm and 25–40 $m^2\,g^{-1}$; and Fe_2O_3, 20–40 nm, 30–60 $m^2\,g^{-1}$. All FETAX salts were obtained from VWR (West Chester, PA): NaCl (100% purity), $NaHCO_3$ (99–100% purity), KCl (100% purity), $CaCl_2$ (99–100% purity), $CaSO_4{\cdot}2H_2O$ (98–100% purity), and $MgSO_4$ (99–100% purity). Trace metal grade nitric acid (70%) was obtained from Fisher Scientific (Fisher, Waltham, MA). Human chorionic gonadotropin (HCG) and L-cysteine (⩾98%, from non-animal source, cell culture) were obtained from Sigma–Aldrich (St. Louis, MO).

2.1. Test solution preparation

Tests were conducted in FETAX solution, which contained 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4{\cdot}2H_2O$, and 75 mg $MgSO_4$ per liter of DI water. A metal oxide nanomaterial stock solution of 1000 mg L^{-1} served as the highest concentration tested and was prepared by adding metal oxide nanomaterials to FETAX solution. A FETAX medium control and seven exposure concentrations at log intervals from 0.001 mg L^{-1} to 1000 mg L^{-1} were utilized for range finding studies for all metal oxide nanomaterials. Each solution was prepared by serial dilution of constantly stirred stock. Copper oxide definitive assay dosing solutions were made with the same technique as above and included the following nominal concentrations: 0.1–1000 mg L^{-1} in log dose intervals. For the ZnO definitive assay, a stock solution of 100 mg L^{-1} was prepared by adding ZnO nanomaterials to FETAX solution. The remaining nominal concentrations, half-log dose intervals, of 31.6, 10, 3.16, 1, 0.316 and 0.1 mg L^{-1} were prepared by serial dilutions of stock. All test solutions were constantly stirred with stir bars and stir plates throughout the toxicity test. Test solutions were not stirred in test dishes, as this would have introduced an additional stressor to the test organisms. It was observed that metal oxide nanomaterials precipitated out of solution, and this was deemed expectable; if nanomaterials would precipitate in the lab they may precipitate in the environment as well.

2.2. Determination of metal cations

Metal cations, Zn and Cu, concentrations were quantified for definitive assays with flame atomic absorption spectroscopy (M Series AA Spectrometer, Thermo, Waltham, MA). Aliquots of dosing solutions were acidified with concentrated nitric acid. An equal amount (5 mL) of concentrated nitric acid was added to an aliquot of dosing solution and diluted to an appropriate volume with 3% nitric acid. This technique allowed total digestion of metal oxides so that total cation concentration could be determined. The curve used to analyze for Zn and Cu in dosing solutions consisted of standards ranging from 0.05 mg L^{-1} to 3 mg L^{-1}. Calibration (1 mg L^{-1} solution) and blank checks were analyzed every 10 samples.

2.3. Breeding and embryo collection

Three *X. laevis* mating pairs were used to ensure adequate supply of viable embryos. One male *X. laevis* and one female *X. laevis* were placed in a tank 1/3 full of FETAX solution with a mesh platform to separate adults from eggs. They were then injected with 250 IU HCG into the male dorsal lymph sac and 750 IU HCG into the female dorsal lymph sac. Tests for each type of metal oxide nanomaterial used embryos from a single mating. Eggs were cleaned by gently swirling in an L-cysteine (2% w/v in test water) solution with a pH of 8.1. Embryos were then rinsed six times with FETAX solution. Viable embryos within Nieuwkoop and Faber stage 8 and 11 were selected for testing (Nieuwkoop and Faber, 1975).

2.4. FETAX assay

In general, standard FETAX protocols were used to conduct the assay (ASTM, 1999). The temperature range for the 96 h exposures was 23 ± 3 °C, within temperature ranges accepted by ASTM E1439-98 (ASTM, 1999). Each exposure concentration for the range finding study was tested in duplicate with five embryos per Petri dish containing 10 mL of solution. Each definitive study exposure concentration was tested in triplicate with 10 embryos per replicate. Dishes were randomly placed in a 113 L aquarium with 5 cm of aerated DI water under the dish support. Test solutions were changed daily. At the end of the 96 h exposure, embryos were euthanized with buffered MS-222. All embryos from each exposure were stored in 10% buffered formalin.

Nanomaterial toxicity was evaluated using the following endpoints: mortality, malformations, stage, snout vent length (SVL), and total body length (TBL). Mortality was determined by lack of movement or response from external stimulus. Malformations were determined using Bantle's Atlas of Abnormalities: A Guide for the Performance of FETAX (Bantle et al., 1989). Stage was determined by Nieuwkoop and Faber table of development to track physiological development of the tadpole as the FETAX assay is designed to observe organogenesis (Nieuwkoop and Faber, 1975). Snout vent length was measured from the tip of the nose to the anal vent to the nearest tenth of a mm. Total body length was measured from the tip of the nose to the tip of the tail to the nearest tenth of a mm. Snout vent length and total body length are tools used to evaluate the effects of exposure on growth such as growth inhibition or stimulation.

2.5. Observations

Daily observations included mortality, malformations, stage of development, air temperature, and water temperature. Observations were conducted using a stereoscope (Motic K series), and electron microscopy (vide infra). Observations at the end of the 96 h exposure included mortality, malformations, stage, snout vent

length, and total body length. Water quality (pH and ammonia) was measured at the end of the exposure period for each Petri dish.

2.6. Scanning electron microscopy

Scanning electron microscopy (SEM) was conducted using a Hitachi S4300VP (Pleasanton, CA). SEM was utilized to verify size of nanomaterials as described below. SEM was also employed to visualize any variations in *X. laevis* skin morphology with exposure to metal oxide nanomaterials during organogenesis.

2.6.1. Nanomaterial preparation for SEM

Double-sided conductive tape was placed on a small SEM mount and a small amount of nanomaterial powder was deposited on the tape. The excess nanomaterials were removed with a steam of nitrogen gas, thus creating a monolayer of nanomaterials on the stub. A 5 nm thick coating of gold/palladium alloy was applied over a 30 s time span on the nanomaterials with a Hummer V Sputter Coater. This procedure was followed for each of the nanomaterials.

2.6.2. Tissue preparation for SEM

Biological samples can require more preparation than abiotic materials for SEM. Tadpoles were rinsed three times with phosphate buffer solution, and dehydrated in a series of ethanol exchanges: 10%, 30%, 50%, 70%, 95%, and 100%. Each step of the series lasted 20–30 min. Critical point drying was achieved with a Balzars CP 030 Critical Point Dryer. This removed the ethanol from the tadpole and replaced it with CO_2. Tadpoles were mounted using double-sided conductive tape on a SEM stub. Tadpoles were then sputter coated in the Hummer V Sputter Coater for one minute applying approximately 10 nm coat of gold/palladium alloy.

2.7. Statistics

Snout vent length, TBL, and malformation incidence were compared among concentrations utilizing nested one-way analysis of variance (ANOVA) followed by Tukey Honestly Significant Differences test when significance was determined with ANOVA ($\alpha = 0.05$). Nested ANOVA was used to eliminate "tank effect" from significance evaluation and determine that concentration was the cause of the observed effects. Effect concentrations for malformations were determined utilizing probit analysis and include Effective Concentration affecting 50% of the population (EC_{50}) as well as the Lowest Observable Effect and No Observable Effect Concentrations (LOEC and NOEC, respectively.

3. Results

3.1. FETAX assay results

Water quality parameters were within ASTM guidelines established to prevent mortality or malformation incidence (Tietge et al., 2000). The pH for the exposures ranged from 6.94 ± 0.03 to 7.68 ± 0.02 (mean ± SE), and total ammonia for all exposures was below 0.55 mg L^{-1}. Minimum inhibition of growth is a common endpoint with the FETAX assay; it is equivalent to the lowest observable effect concentration in regards to growth. Nanomaterials concentrations referenced within the following results are nominal concentrations unless otherwise stated.

3.1.1. TiO_2 and Fe_2O_3 range finding FETAX assay

Titanium dioxide and Fe_2O_3 exposure caused no mortality or significant malformation. The average stage at the study conclusion of for embryos exposed to either TiO_2 or Fe_2O_3 nanomaterials was 48. There was no significant difference between dose groups for developmental stages ($p > 0.05$). Titanium dioxide and Fe_2O_3 nanomaterial exposure decreased tadpole snout vent length from control to 0.001 mg L^{-1}, then produced an increase to 1 mg L^{-1}, and finally steadily decreased through the higher concentrations (10, 100, and 1000 mg L^{-1}). Tadpoles exposed to 1000 mg L^{-1} TiO_2 or 1000 mg L^{-1} Fe_2O_3 had significantly reduced SVL ($p \leqslant 0.003$) and TBL ($p \leqslant 0.033$) than control tadpoles. No further testing was conducted with TiO_2 and Fe_2O_3 nanomaterials due to the lack of acute toxicity with the range finding test.

3.1.2. Definitive ZnO FETAX assay

Zinc oxide nanomaterials did not affect mortality at any concentration tested. The percent malformation for all dose groups ranged from 0% to 81%. The control group exhibited 3% malformation, within acceptability standards (<10% malformation) for the FETAX assay (ASTM, 1999). Dose groups containing ZnO at 3.16 and 10 mg L^{-1} had similar malformation rates, 40% and 42% respectively, and tadpoles in the 31.6 mg L^{-1} ZnO solution had the highest malformation rate with 81%, all of which were significantly higher than control tadpoles. The EC_{50} for ZnO was 10.3 mg L^{-1} (95% Confidence Interval: 6.5–17.0 mg L^{-1}). Several types of malformations were observed in organisms receiving ZnO treatment. Axial/tail, gut, head, eye abnormalities, and blistering were the typical malformations observed in organisms receiving ZnO treatment. The majority (89%) of abnormalities were gut malformations (Fig. 1).

After 96 h, the average developmental stage across all doses was 49. Dose groups showed no significant difference in developmental stage. Average SVL for all tadpoles in this exposure was 3.84 ± 0.01 mm. There was no significant difference in SVL among exposure groups (Fig. 2A). There was a significant difference in TBL following exposure to 10 and 31.6 mg L^{-1} ZnO nanomaterials ($p < 0.001$) compared to control TBL (Fig. 2B). Tadpoles exposed to 10 and 31.6 mg L^{-1} ZnO also had significantly shorter TBL than tadpoles exposed to 0.1, 0.316, and 1 mg L^{-1} ZnO ($p \leqslant 0.003$). The minimum concentration of ZnO to inhibit growth of *X. laevis* tadpoles was 10 mg L^{-1}.

3.1.3. Definitive CuO FETAX assay

Copper oxide nanomaterial exposure did not induce significant mortality for any of the tested concentrations. The highest tested concentration (1000 mg L^{-1}) generated the highest incidence of malformation (33%) which was greater than controls ($p \leqslant 0.05$). Similar abnormalities (axial/tail, gut, head, etc.) were seen with CuO treatment as observed with ZnO treatment. As observed with ZnO, the majority (61%) of abnormalities were gut malformations (Fig. 1). Tail or axial abnormalities comprised 19% of total abnormalities observed with CuO exposure. There was no significant difference between the dose groups for developmental stages due to all tadpoles reaching the same developmental stage of 48.

Copper oxide nanomaterials induced a decrease in SVL starting exposure to 0.1 mg L^{-1} through the 1000 mg L^{-1} treatment (Fig. 2C). Snout vent length was significantly reduced in the three highest concentrations compared to controls ($p \leqslant 0.05$). In contrast, TBL did not vary among exposure concentrations below 100 mg L^{-1}. However, TBL was lower for organisms experiencing 1000 mg L^{-1} of CuO (9.80 ± 0.13 mm) compared to controls (10.72 ± 0.05 mm) ($p < 0.001$) (Fig. 2D). The minimum concentration of CuO to inhibit growth of *X. laevis* tadpoles was 10 mg L^{-1}.

3.2. Metal cation concentrations

Zinc cation concentrations were lower than nominal predictions. Zinc cation concentration was verified in dosing solutions as well as the stock solution (100 mg L^{-1}). Stock solution Zn concentration had a percent of nominal over 90%. Concentrations ranging from 3.16 to 31.6 mg L^{-1} ZnO had over 54% of the nominal

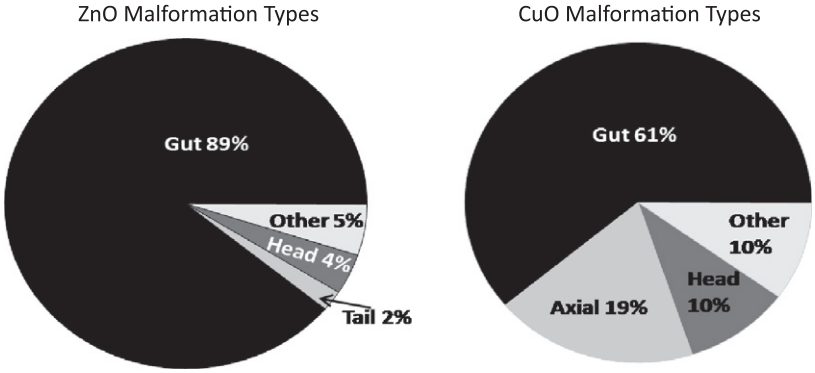

Fig. 1. Percent of malformation types induced by exposure to ZnO or CuO nanomaterials overall for all samples.

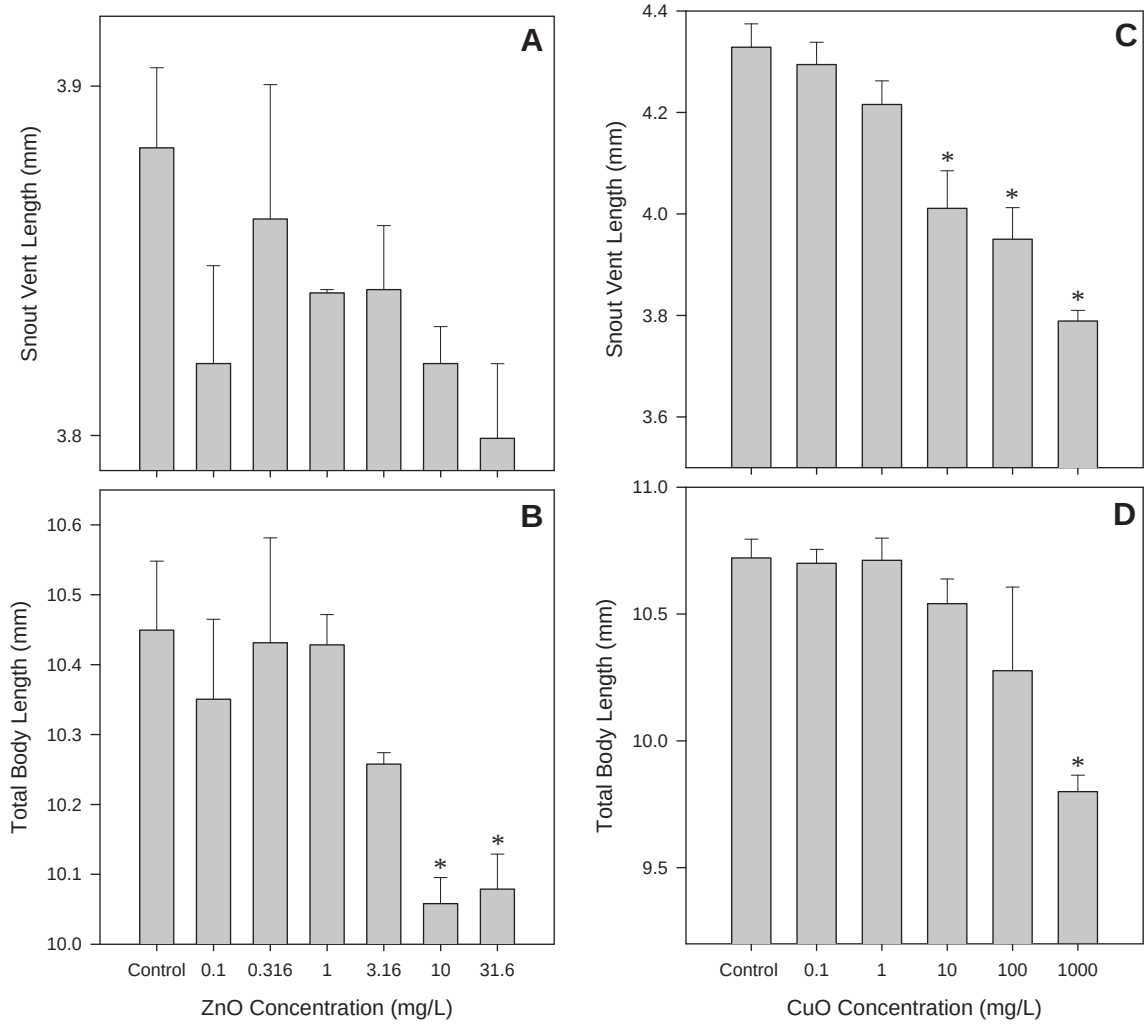

Fig. 2. Snout vent length (A) and total body length (B) (mean ± standard error) of *X. laevis* tadpoles exposed to ZnO nanomaterials. Snout vent length (C) and total body length (D) (mean ± standard error) of *X. laevis* tadpoles exposed to CuO nanomaterials. * = $p \leqslant 0.05$ with ANOVA and TukeyHSD compared to control.

Zn cation determined in solution and increased in percent up to 83% with increasing ZnO concentration. Two ZnO dosing solutions, 0.316 and 1 mg L^{-1}, contained approximately 20% of the calculated Zn concentration, and the dosing concentration of 0.1 mg L^{-1} ZnO had 42% of the calculated Zn cation in solution.

Copper from toxicant concentrations were higher than nominal predictions. The stock solution contained 108% of the nominal Cu 1000 mg L^{-1} concentration, while the remaining dosing solutions contained anywhere from 89% to 163% of calculated Cu concentration in solution. The lower CuO nanomaterial concentrations, 0.1 and 1 mg L^{-1}, contained higher percentage (126% and 163% respectively) of the respective nominal concentration compared to other dosing solutions; 10 mg L^{-1} CuO with 103% and 100 mg L^{-1} CuO with 89%.

3.3. Scanning electron microscopy: nanomaterial characterization

At least five images were taken for each nanomaterial (Fe_2O_3, TiO_2, CuO, and ZnO) to obtain a variety of locations to determine particle size. A minimum of 20 measurements were taken to obtain

an average individual particle size (APS) and four measurements to obtain an average aggregate size. Iron oxide had an APS of 44 nm and ranged in size from 17 to 98 nm. Titanium dioxide had an APS of 56 nm with a range from 21 to 135 nm. Zinc oxide nanomaterials had an average APS of 45 nm and included sizes from 7 nm to 94 nm. Copper oxide nanomaterial size ranged from 18 to 285 nm with an APS of 57 nm. Overall all average nanomaterial sizes were marginally larger than the reported APS by a minimum of 12%. Aggregate size was evaluated in addition to individual particle size. Iron oxide, TiO_2, and ZnO had average aggregate sizes greater than 5 μm. Copper oxide nanomaterials formed smaller aggregates, <1 μm, than the other metal oxide nanomaterials.

3.4. Scanning electron microscopy: X. laevis

Three tadpoles, one from each replicate, were taken from each concentration to represent each dosing group. At least two images were produced per tadpole to compare skin cell morphology. It appeared that nanomaterials had an affinity for ciliated and secretion ectoderm cells, as this is where nanomaterials were adhering most to the tadpoles.

As ZnO concentration increased, there was evidence of nanomaterials adhering to the skin of the tadpole. Zinc oxide nanomaterial adhesion was observed at concentrations as low as 1 mg L^{-1}. The ZnO nanomaterials sparsely adhered to both ciliated cells and secretion cells. The number of cells affected by exposure increased with increasing concentration of nanomaterials. Ectoderm cell morphology was not affected by the toxicant concentrations evaluated. Micrographs of range-finding nominal concentrations including 100 and 1000 mg L^{-1} were evaluated in addition to the definitive assay. Skin cell vaulting (Fig. 3 F) was induced with 1000 mg L^{-1} ZnO nanomaterial exposure.

Copper oxide nanomaterial exposure induced similar adhesion results as ZnO nanomaterial exposure. The first evidence of nanomaterial adhesion was observed at 10 mg L^{-1}. Nanomaterial aggregate adhesion did not uniformly cover the tadpoles. Copper oxide nanomaterial adhesion to skin cells increased with an increase in concentration. Skin cells became vaulted in some areas when exposed to 1000 mg L^{-1} CuO, but this effect was also not uniform for the entire tadpole (Fig. 4).

Two tadpoles, one from each replicate, were examined from each concentration of $TiO_2$and Fe_2O_3. Trends seen for both TiO_2 and Fe_2O_3 were similar to those observed with CuO and ZnO (data not shown). Some nanomaterial adhesion was visible at concentrations as low as 1 mg L^{-1} and no visible abnormalities were observed until 1000 mg L^{-1}. Abnormal vaulting and cilia reduction on ciliated cells was present, but not uniform over the entire tadpole.

4. Discussion

All four metal oxide nanomaterials (TiO_2, Fe_2O_3, CuO, and ZnO) affected SVL and TBL at the highest tested concentrations, 1000 mg L^{-1} (TiO_2, Fe_2O_3, CuO) and 31.6 mg L^{-1} (ZnO). Snout vent length responses were more variable with changes in metal oxide nanomaterials concentration than were TBL responses, indicating that SVL could be a more sensitive endpoint for metal oxide nanomaterial exposure. There is no precedent of using SVL as an endpoint in FETAX assays, but it is an endpoint utilized in *X. laevis* chronic exposures (OECD, 2007). If so, this would allow more reliable comparison of acute and chronic growth data. Titanium dioxide and Fe_2O_3 nanomaterials did not significantly affect mortality or malformation frequencies. These results indicate that TiO_2 and Fe_2O_3 have relatively little developmental or teratogenic effects on *X. laevis* during the first 96 h of life. The Ecotox Database (http://cfpub.epa.gov/ecotox/) contained no toxicity data for TiO_2 nor Fe_2O_3.

In previously conducted range finding studies (data not reported), tadpoles exposed to ZnO nanomaterials exhibited increased SVL and TBL compared to controls exposed to ZnO at concentrations less than 10 mg L^{-1}. The increase in SVL at doses below 10 mg L^{-1}, followed by a reduction in SVL at higher concentrations, suggests that ZnO nanomaterials may have a hormetic trend in both SVL and TBL. True hormesis has not been unequivocally shown in our study as we have not evaluated the compensatory mechanisms that are required to complete the definition of hormesis. The essential nature of Zn in vertebrate development has been noted as a reason for Zn hormesis in aquatic organisms (Calabrese and Blain, 2004; Bodar et al., 2005), which supports the probability of a hormetic effect for ZnO nanomaterials. Dietary Zn does support normal growth and development, which is consis-

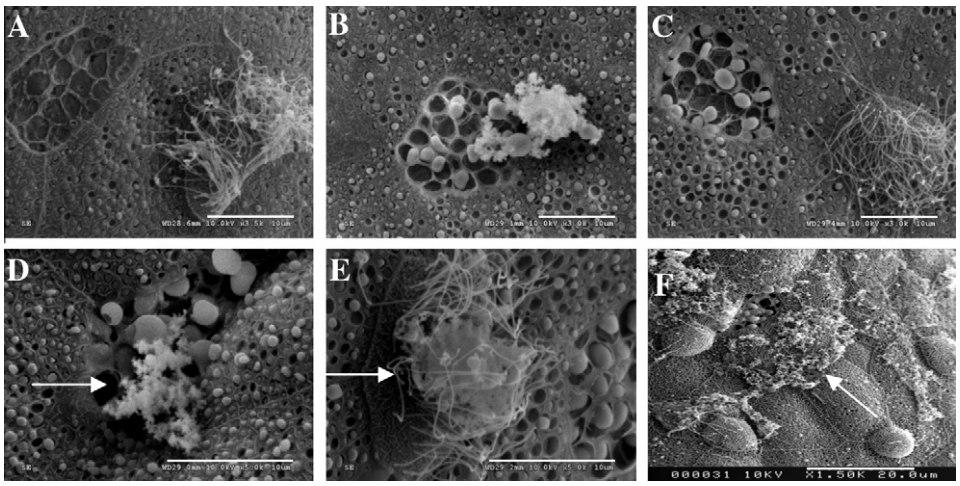

Fig. 3. Micrographs of tadpole exposed to ZnO nanomaterials, including control (A), 10 mg L^{-1} (B), 31.6 mg L^{-1} (C, D, E) and 1000 mg L^{-1} (F). The white scale line for mircrograph A through E is 10 μm and 20 μm for micrograph F. Micrograph A is an image of a control ciliated cell. Micrograph B is an example of nanomaterial adhesion to a secretion cell at 10 mg L^{-1}. Micrographs C, D, and E illustrate the variety of skin cells observed at 31.6 mg L^{-1}; which includes cells with nanomaterial adhesion (D and E) and normal skin cells (C). Arrows in micrograph D and E are highlighting nanomaterials adhesion to different cell types; secretion cells in micrograph D and ciliated cells in micrograph E. Micrograph F was from an earlier study (identical procedures) including the dose 1000 mg L^{-1} ZnO. Micrograph F reveals vaulting of skin cells and irregular morphology of ciliated cells.

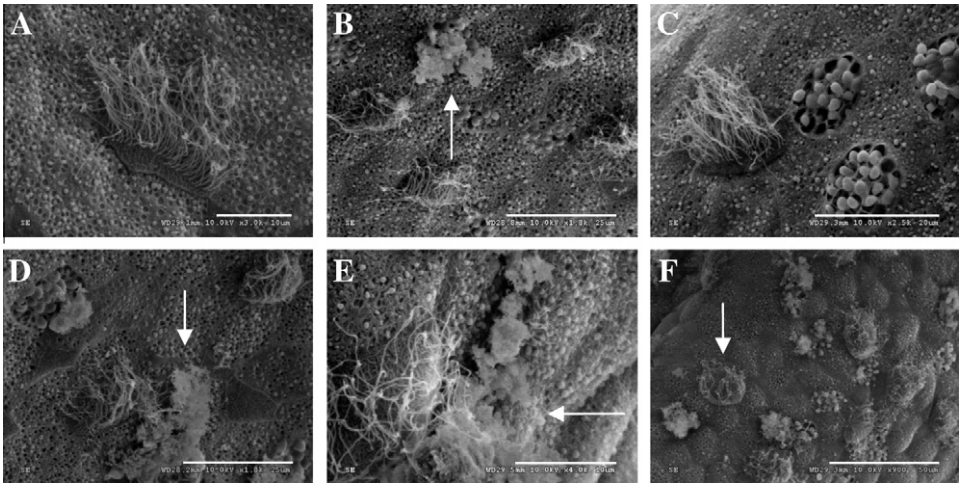

Fig. 4. Micrographs of tadpole exposed to CuO nanomaterials, including control (A), 10 mg L^{-1} (B and C), 100 mg L^{-1} (D) and 1000 mg L^{-1} (E and F). The white scale line for mircrograph A and E is 10 μm, 20 μm for micrograph C, 25 μm for micrograph B and D, 50 μm for micrograph F. Micrograph A is an image of a control ciliated cell. Micrographs B and C is an example different areas of tadpole skin, with nanomaterial adhesion to a secretion cell highlighted by the arrow in mircograph B and D and normal cells at 10 mg L^{-1} in micrograph C. Micrograph D illustrate the affinity of nanoparticles, see arrow, to both secretion and ciliated cells at 100 mg L^{-1}. Micrograph E and F reveal vaulting of skin cells and irregular morphology of ciliated cells indicated by the arrow.

tent with the stimulatory effects on growth seen in the present study (Zinc and Human Health, 2008).

Zinc oxide nanomaterials did affect tadpole length, but at much higher concentrations than dissolved Zn. Ecotox Database (http://cfpub.epa.gov/ecotox/) LOEC values for Zn on *X. laevis* length ranged between 0.75 and 2.0 mg L^{-1}. The lowest concentration of ZnO nanomaterials that affected length was 10 mg L^{-1} (6.12 mg Zn L^{-1}). Zinc oxide nanomaterial exposure did increase malformations at concentrations higher than 3.16 mg L^{-1} and a malformation EC_{50} was determined to be 10.26 mg L^{-1} (~6.6 mg Zn L^{-1}). Our EC_{50} is approximately three times higher than reported conventional Zn EC_{50}s (2.65–2.83 mg L^{-1}) for *X. laevis* (http://cfpub.epa.gov/ecotox/). Zinc exposure also enhanced mortality with reported LC_{50} values ranging from 20 to 34.5 mg L^{-1} (http://cfpub.epa.gov/ecotox/), whereas ZnO nanomaterial exposure produced no mortality. Zinc oxide nanomaterials were relatively less toxic than dissolved Zn to *X. laevis* as indicated by higher malformation effect concentrations, effects on length, and lack of lethal effects with ZnO nanomaterials.

Copper oxide nanomaterial exposure did not affect mortality, and effects on malformations and growth did not manifest until concentrations exceeded reported Cu effect concentrations for these endpoints, indicating that CuO nanomaterials may not be as toxic as conventional Cu. Copper has a reported LOEC of 0.75 mg L^{-1} on TBL of *X. laevis* (http://cfpub.epa.gov/ecotox/). The lowest concentration of CuO nanomaterials to affect TBL was 1000 mg L^{-1} (862.81 mg Cu L^{-1}). Copper has a reported malformation EC_{50} of 0.740–0.880 mg L^{-1} (http://cfpub.epa.gov/ecotox/). An EC_{50} could not be calculated from this study for CuO nanomaterial exposure as none of the concentrations evaluated within this study caused over 33% of the organisms to develop malformations. Copper LC_{50}s range from 0.150 to 1.080 mg L^{-1} (http://cfpub.epa.gov/ecotox/). Copper oxide nanomaterial exposure did not induce any significant mortality indicating that CuO nanomaterials are less toxic than Cu cation. Several studies attribute metal oxide nanomaterial toxicity to dissolution of metal oxide with the subsequent increase in metal cation concentration in solution (Franklin et al., 2007; Heinlaan et al., 2008; Zhu et al., 2008; Aruoja et al., 2009,). Our study determined exposure to ZnO and CuO nanomaterials induced less mortality and negative effects on growth when compared to extensive historical results of their respective metal cation from soluble sources. Bioavailability of CuO and ZnO nanomaterials were investigated in some aquatic studies, and in one study it was concluded that ZnO and CuO nanomaterials were not as bioavailable as soluble compounds (Heinlaan et al., 2008). A lower concentration of bioavailable Cu and Zn could be a factor in the reduced toxicity of ZnO and CuO nanomaterials observed within this study.

Zinc oxide and CuO nanomaterials appear to increase malformations and possibly reduce growth. Malformations were not observed until day four of nanomaterial exposure. There were little to no visible abnormalities observed on day 1 through day 3 in the highest exposure concentrations. This may indicate that nanomaterials do not enter tadpoles until later stages of development. *X. laevis* tadpoles feed from their yolk until between stages 41 and 47 (days 3–4) (Mitchell et al., 2005). On day 3, or stage 40/41, the *X. laevis* larvae mouth begins to open (Mitchell et al., 2005). *X. laevis* tadpoles are filter feeders so it is possible on day 3 tadpoles begin to ingest nanomaterials. Metal oxide nanomaterials could be visualized within tadpole gut coils with the use of a stereoscope on day 4, suggesting that nanomaterials were being ingested (Fig. 5). Ingestion of metal oxide nanomaterials increases contact with cells in the intestinal tract, which possibly contributes to gut malformations such as edema (Heinlaan et al., 2008; Zhu et al., 2008). Gut malformations were the most prominent type of malformation observed with ZnO and CuO nanomaterial exposure. However, the proportions of malformation types were different between the two nanomaterials, as ZnO exposure produced more gut malformations than exposure to CuO. Zinc is a more effective inducer of metallothionein compared to Cu, therefore Cu would have a greater probability to be absorbed through the gastrointestinal tract and transported to other areas of a tadpole body. This increase in Cu to other portions of the organism could lead to an increased variety of malformations.

Ectoderm cells did not appear to be adversely affected by ZnO and CuO nanomaterials with many of the exposures. As the concentration of nanomaterials increased, there was evidence of nanomaterials adhering to ectoderm cells. Ciliated cells appeared atypical in shape and had less cilium at 1000 mg L^{-1} than compared to control tadpoles. Similar cell morphology abnormalities have been observed with other metals with low level exposure; such as 0.5 mg L^{-1} Cd exposure and in a 2 mg L^{-1} Cd exposure all cilia were gone (Herkovits et al., 1997). Exposure to 2 mg L^{-1} Cd also induced gross malformations (axial incurvations, stunted size,

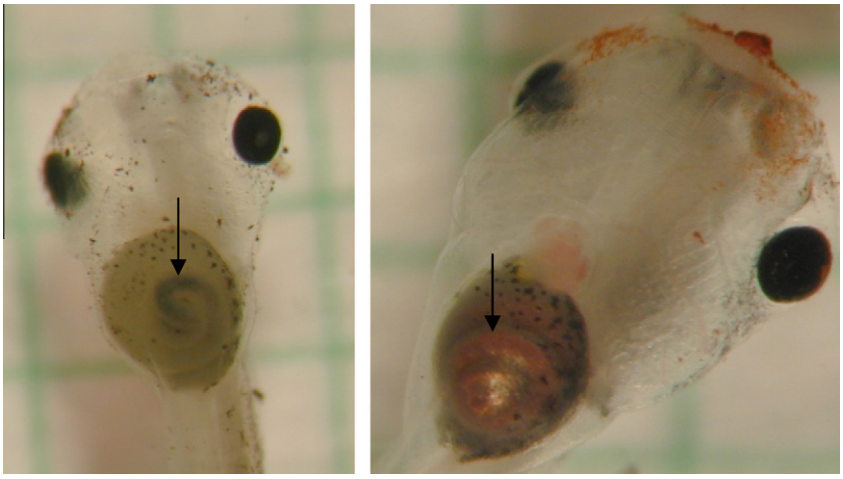

Fig. 5. Evidence of metal oxide nanomaterials within gut coils. Left picture is a tadpole exposed to 1000 mg L^{-1} of CuO nanomaterials and the picture on the right is a tadpole exposed to 1000 mg L^{-1} of Fe_2O_3 nanomaterials with the arrows pointing to visual confirmation of nanomaterials within gut coils indicating ingestion.

abnormal tail, fin, and eyes) and mortality (Herkovits et al., 1997). Cilia, especially those around the external nares (nostrils or nasal passage), are believed to have chemosensory roles, such as identifying predator odor (Nokhbatolfoghahai et al., 2005, 2006). Heavy metals, such as Zn and Cu, can affect an amphibian's ability to sense predator odor, therefore the additional adhesion of nanomaterials to cilia and/or reduction of cilia due to metal oxide nanomaterial exposure could make tadpoles more susceptible to predation (Lefcort et al., 1998, 1999). Exposure to fullerenes significantly affected *Daphnia magna* by inducing physiological changes that are linked to increased predation (Lovern et al., 2007). Nanomaterial exposure can induce alterations to an organism that may negatively affect them in an ecosystem not necessarily seen in lab exposures. For example, size can affect survivability; as larger organisms have a better probability of reaching adulthood and smaller organisms are more likely to become prey to the larger organisms (Lefcort et al., 1998, 1999). Therefore if chemical exposure inhibits growth, it could cause a decrease or delay in young organisms reaching sexually maturity to procreate and replenish the population within an ecosystem.

There were relatively large differences observed between measured and nominal Zn concentrations. The largest differences occurred with the lower concentrations (0.1–1 mg L^{-1}). This variation could be attributed to bioconcentration by tadpoles. Tadpoles could bioconcentrate Zn in the lower solutions resulting in much lower solution concentrations because they have not reached their biological threshold for Zn; where as tadpoles exposed to the higher concentrations could only utilize so much Zn before reaching a threshold and potentially resulting in higher percentage of Zn remaining in solution. Adhesion of nanomaterials to ectoderm cells of tadpoles could also affect absorption of Zn. Tadpoles exposed to higher concentrations of ZnO nanomaterials experienced adhesion of nanomaterials which could reduce absorption by tadpoles. Reduced absorption of Zn by the tadpoles can results in relatively higher Zn concentrations in solution. Tissue concentration could determine how much Zn tadpoles are concentrating during the exposure. Dilution errors during preparation of the serial dilutions could have caused some of the variation; however this seems unlikely as the lowest concentration (1 mg L^{-1}) contained 42% of the nominal concentration while 0.316 and 1 mg L^{-1} had approximately 20% of the nominal concentrations. The digestion process could also have affected recovery from solution if the digestion process was not vigorous enough to thoroughly breakdown ZnO nanomaterials into elemental Zn. It is not believed to be the sole cause as the stock solution recovery was over 90%. The combination of the aforementioned possible causes of reduced measured concentrations compared to nominal concentrations is also likely.

In conclusion, this study found TiO_2 and Fe_2O_3 nanomaterials of the size and shape tested to be relatively non-toxic at the tested concentrations and average particle size of 56 nm and 44 nm, respectively. Therefore, we do not consider TiO_2 and Fe_2O_3 nanomaterials alone to pose risks to amphibian populations. However, it is important to remember that nanomaterial size can affect toxicity. Several types of nanomaterials did not cause significant mortality, malformations, or decrease growth in a variety of study organisms until the size of the nanomaterials was less than 20 nm (Service, 2003; Lovern and Klaper, 2006). Our findings support data collected from a variety of studies evaluating toxicity of metal oxide nanomaterials on aquatic organisms (Table 1). None of these studies evaluated Fe_2O_3 nanomaterial toxicity, indicating that our study is one of the first to evaluate effects caused by exposure to Fe_2O_3 nanomaterials. Several studies (Table 1) have determined that TiO_2 nanomaterials did not negatively affect their respective organism(s) tested, whereas CuO and ZnO did negatively affect growth and/or survival of many aquatic species. When comparing mortality, malformation, and growth effects from exposure to CuO and ZnO nanomaterials and their respective metal cations, Cu and Zn, nanomaterials were less toxic than their corresponding metal cations.

Chronic studies have also been performed whereby with *X. laevis* were exposed to CuO and ZnO nanomaterials (Nations et al., 2011). Chronic study results indicated that chronic exposure to metal oxide nanomaterials was significantly more toxic than acute exposures. This was especially true of CuO, which produced 60% mortality after 5 d of exposure to concentrations that produced EC_{15} response for malformation at day 4. Using both acute and chronic results suggests that the scientific community should not yet conclude that nano-TiO_2 is non-toxic in aquatic systems. In addition to known chronic effects, there are photochemical interactions with nano-TiO_2 that could pose risks to unicellular and higher order organisms (Zhang, 2010). Caution should be used with release of CuO and ZnO nanomaterials as increasing concentrations of these nanomaterials may increase the incidence of malformations and reduce growth as seen in our study. Combining acute and chronic toxicological responses will provide much needed data to better assess the risks associated with metal oxide nanomaterials. These results will be instrumental in assessing ecological risk

Table 1
Acute toxicity of metal oxide nanomaterials (ZnO, CuO, TiO_2, and Fe_2O_3) on aquatic organisms.

Organism	Effect	Endpoint	ZnO (mg L^{-1})	CuO (mg L^{-1})	TiO_2 (mg L^{-1})	Fe_2O_3 (mg L^{-1})
Vibrio fischeri[a]	Inhibition of luminescence	30 min EC_{50} (Cuvette)	4.8	68.1	nt[h]	nt[h]
Vibrio fischeri[a]	Inhibition of luminescence	30 min EC_{50} (Microplate)	3.8	204	nt[h]	nt[h]
Vibrio fischeri[b]	Inhibition of luminescence	30 min EC_{50}	1.9	79	>20 000	nt[h]
Vibrio fischeri[b]	Growth	MIC[f]	100	200	>20 000	nt[h]
Pseudokirchneriella subcapitata[c]	Growth	72 h IC_{50}	0.042	0.71	5.83	nt[h]
Daphnia magna[b]	Mortality	48 h LC_{50}	3.2	3.2	~20 000	nt[h]
Thamnocephalus platyurus[b]	Mortality	48 h LC_{50}	0.18	2.1	>20 000	nt[h]
Danio rerio[d]	Mortality	96 h LC_{50}	1.793	nt[h]	no effect	nt[h]
Danio rerio[d]	Hatching	84 h EC_{50}	2.065	nt[h]	no effect	nt[h]
Xenopus laevis[e]	Malformation	96 h EC_{50}	10.3	>1000	>1000	>1000
Xenopus laevis[e]	Malformation	96 h EC_{10}	1.3	2.1	>1000	>1000
Xenopus laevis[e]	Growth	96 h MCIG[g]	10	10	1000	1000

[a] Mortimer et al. (2008)
[b] Heinlaan et al.
[c] Aruoja et al.
[d] Zhu et al.
[e] *X. laevis* results from this study.
[f] Minimum Inhibitory Concentration (lowest tested concentration of compound that totally inhibits growth).
[g] Minimum Concentration to Inhibit Growth (lowest tested concentration of compound that significantly inhibits growth).
[h] Not tested.

to aquatic organisms, especially in regards to declining amphibian populations.

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