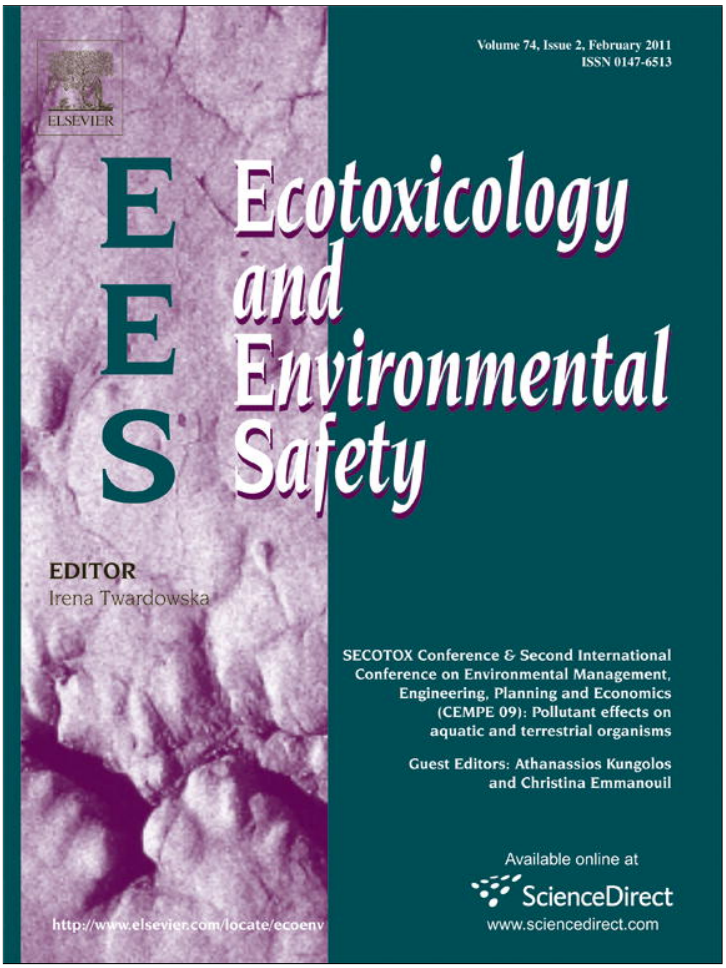

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Effects of ZnO nanomaterials on *Xenopus laevis* growth and development

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ABSTRACT

The objectives of this study were to quantify uptake and developmental effects of zinc oxide nanomaterials (nano-ZnO) on *Xenopus laevis* throughout the metomormosis process. To accomplish this, *X. laevis* were exposed to aqueous suspensions of 40–100 nm nano-ZnO beginning in-ovo and proceeding through metamorphosis. Nanomaterials were dispersed via sonication methods into reconstituted moderately hard water test solutions. A flow-through system was utilized to decrease the likelihood of depletion in ZnO concentration. Exposure to 2 mg/L nano-ZnO significantly increased mortality incidence to 40% and negatively affected metamorphosis of *X. laevis*. Tadpoles exposed to 2 mg/L nano-ZnO developed slower as indicated by tadpoles with an average stage of 56 at the conclusion of the study which was significantly lower than the control tadpole stages. No tadpoles exposed to 2 mg/L of nano-ZnO completed metamorphosis by the conclusion of the study. Tadpoles exposed to 0.125 mg/L nano-ZnO experienced faster development along with larger body measurements indicating that low dose exposure to nano-ZnO can stimulate growth and metamorphosis of *X. laevis*.

1. Introduction

The possible uses and advancements of nanomaterials and the resulting technologies have increased due to the significantly different electrical, optical, thermal, and surface area properties of materials with sizes less than 100 nm (Borm, 2002). As surface area increases, reactivity increases due to surface atoms control of the particles properties. Therefore, nanomaterials behave more like quantum particles (Kumar, 2006). For example, TiO_2 stability varies with size; bulk TiO_2 is thermodynamically most stable in rutile form while nano-sized TiO_2 is most thermodynamically stable in anatase form (Kumar, 2006). In addition, nano-ZnO are more efficient at reflecting or scattering ultraviolet light than bulk ZnO. Nano-ZnO also increased antibacterial properties with reduced size (Mortimer et al., 2008).

Current metal oxide production volumes are among the highest among nanomaterials (Kumar, 2006). One of the reasons metal oxide nanomaterials are in such high demand is that these materials can treat a wide variety of hazardous materials such as chlorinated solvents, microbes, pesticides, and mustards (Kumar, 2006). Zinc oxide nanomaterials have been shown to efficiently transform hydrogen sulfide, H_2S to ZnS, and to dehalogenate chlorinated solvents (Kumar, 2006). Zinc oxide nanomaterials have antimicrobial properties and are used in sunscreens, cosmetics, coatings, caulks, and adhesives (Australian Academy of Science, 2008). Antimicrobial wallpaper has been created by scientists from National Tsing Hua University in Taiwan by coating paper with nano-ZnO (Richards, 2006). Zinc oxide nanomaterial does not appear to penetrate human skin past the upper stratum corneum (Cross et al., 2007). Zinc oxide nanomaterials have a high probability of entering the aquatic environment with the multitude of commercial and consumer applications. This makes it imperative to investigate possible ecological effects due to ZnO nanomaterial exposure.

Metal oxide nanomaterials toxicity has been documented in the following aquatic organisms: freshwater microalga (*Pseudokirchneriella subcapita), Daphnia magna,* and zebrafish (*Danio rerio).* Freshwater microalga IC50 for growth inhibition at 72 h exposure to ZnO was 49 μg/L (Franklin et al., 2007). Titanium dioxide nanomaterial exposure exhibited a dose response mortality effect in *Daphnia magna* resulting in a LC_{50} of 5.5 mg/L, but behavioral or physiological changes at the lowest observable effect concentration for mortality (2 mg/L) were not observed (Lovern and Klaper, 2006; Lovern et al., 2007). Zinc oxide nanomaterial exposure significantly increased mortality and decreased hatchability of zebrafish at 1 mg/L (Zhu et al., 2008). Zebrafish also had an increased incidence of tissue ulceration beginning 72 h post fertilization (hpf) and reaching 100% tissue ulceration by 108 hpf (Zhu et al., 2008). Our study of acute ZnO exposures in the Frog Embryo Teratogenesis Assay Xenopus (FETAX) suggested no lethality at the concentrations

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tested; however significant malformations were observed at 3.16 mg/L (Nations, 2009).

The aforementioned studies have reported acute effects and to date there have been no published studies that describe the chronic toxicity of nano-ZnO. Chronic studies are important tools to evaluate the effects that prolonged exposures to sublethal concentrations of a contaminant cause in organism growth and maturation. *Xenopus laevis* is an excellent test species for a metamorphosis-based bioassay, as morphological changes that this species undergoes is well documented from hatching until the completion of metamorphosis (OECD, 2007). Several chronic studies have been conducted utilizing *X. laevis* from egg or young larval stages to completion of metamorphosis. Ammonium perchlorate was found to inhibit metamorphosis by significantly reduced number of tadpoles completing tail resorption at 16 μg/L and a complete lack of tadpoles completing metamorphosis at concentrations as low as 147 μg/L (Goleman et al., 2002). Two studies revealed atrazine, which has a reported 96-h lowest observable adverse effect concentration of 1.1 mg/L in *X. laevis,* did not affect metamorphosis or growth at 25 μg/L in .*X laevis* (Carr et al., 2003; Coady et al., 2005). An acute test (96-h) of depleted uranium (U) produced no significant mortality, malformation, or growth effect with exposure to the following range of depleted U concentrations 4.78–77.72 mg/L (Mitchell et al., 2005). Chronic exposure (64 d) to depleted U (13.1–54.3 mg/L) delayed metamorphosis and was attributed to increased tissue concentrations (0.98–2.82 mg/L) (Mitchell et al., 2005). Carbon nanotubes also cause blockage of gills and the GI tract, which produced mortality at 10 mg/L and above (Mouchet et al., 2008).

The purpose of this study was to determine effects of nano-ZnO on *X. laevis* larvae through metamorphosis (Nieuwkoop and Faber stage 66). Results of this study, in combination with previously conducted acute studies, may demonstrate ecological implications of nano-ZnO release in aquatic ecosystems. Our results also provide critical information to regulatory agencies and industry to determine the need for monitoring and regulation regarding nano-ZnO.

2. Methods and materials

Zinc oxide nanomaterials were obtained from Alfa Aesar (Ward Hill, MA). Alfa Aesar reported average particle size (APS) and surface area of NanoTek® nano-ZnO used in this project to be 40–100 nm and 10–25 m^2/g respectively. 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). For chemical analysis of water and tissue, trace metal grade nitric acid (70%) and hydrogen peroxide (30%) were obtained from Fisher Scientific (Waltham, MA). Human chorionic gonadotropin (HCG) and L-cysteine ($\geq$98%, from non-animal source, cell culture) were obtained from Sigma-Aldrich (St. Louis, MO). The use of live organisms was conducted in accordance to protocols submitted and approved by Texas Tech University Animal Care and Use Committee which insures that experimental procedures adhere to guidelines from the AAALAC.

2.1. Nanomaterial solution preparation

Nanomaterial solutions were prepared in FETAX solution, referred to hereafter as FETAX. FETAX is a media used to culture *X. laevis* larvae containing 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4.2\ H_2O$, and 75 mg $MgSO_4$ per liter of deionized or distilled water. Dosing solutions were sonicated with Fisher Scientific Model 500 Sonic Dismembrator assembled with a disruptor horn (Fisher, Waltham, MA). A pulsed sonication mode was utilized (1 s on, 0.5 s off, 30 s total time) with 40% amplitude, approximately 160 watts. The pulsed sonication was repeated until all particles appeared suspended and no longer precipitating from solution in the beaker. Dissolution did not require more than five minutes of total sonication time. Pulsed sonication was utilized to minimize temperature increases and excessive solution turbulence that can occur with horn sonication and to reduce the need for chemical solvents. Each ZnO dosing solution was prepared by creating a concentrated solution in a 1 L beaker and diluting into the appropriate 208 L drum. Acute 96-h studies were conducted to establish doses for the developmental study. A calculated total malformation EC_{15}, 1.9 mg/L (Nations, 2009), was used to establish the highest nominal dose. Doses for the developmental test were as follows: control, 0.125, 0.250, 0.500, 1, and 2 mg/L.

2.2. Breeding

Four mating pairs of *X. laevis* adults were bred to increase the possibility that at least one pair of frogs would produce an adequate supply of viable embryos. Embryos from a single mating pair were used for the exposure. Males were injected with 250 IU of human chorionic gonadotropin (HCG) in the dorsal lymph sac, and females were injected with 750 IU HCG in the dorsal lymph sac to induce reproduction. Each mating pair was housed in a 37.9 L tank containing aerated FETAX and a coated mesh platform one inch above the bottom of the tank. The platform protected the eggs from the adults until time of collection.

2.3. Embryo collection

Embryo collection began approximately 24 h after HCG injection. Preparation of embryos for use in FETAX assay began with dejellying the eggs that contained embryos. The embryos eggs were dejellied with a 2% L-cysteine solution. A plastic transfer pipette was used to collect and transfer embryos to a Petri dish. Embryos were inspected with a stereoscope to identify viable embryos. Viable embryos have been fertilized and are between stages 8 and 11 according to Nieuwkoop and Faber stages (Nieuwkoop and Faber, 1975). Unviable embryos were removed from the Petri dish with a 1 mL syringe.

2.4. Dosing

Each concentration was tested in triplicate with 15 tadpoles in each replicate. Small 9.5 L tanks were used as exposure chambers (see below). Each tank was labeled with concentration, chemical, replicate identifier, and date of test. Nanomaterial solutions were pumped from 208 L drums to each tank. To begin the exposure, 6 L of dosing solution was placed in every tank. After the solution was added to each tank, at least 20 tadpoles were added. Tadpoles were transferred with plastic transfer pipettes. Tadpoles were thinned to 15 after hatching was completed.

2.5. Test chamber and preparation

The test preparation included 18 exposure tanks, three temperature control tanks, six 208 L drums, aeration (for each exposure tank and dosing drum), 2–3 tank heaters, 2–3 thermometers, and a living stream reservoir used as a water bath. The living stream reservoir had a stand pipe keeping the level of water within the reservoir the same throughout the exposure period. Tap water was used to initially fill the reservoir 2/3 full. This amount allowed the dosing tanks and temperature tanks to be submersed enough to maintain relatively constant temperature for the duration of exposure. The water in the reservoir was heated to $23 \pm 2\ ^{\circ}C$. Dosing tanks were randomly arranged within the reservoir.

One drum was designated for each nano-Zn exposure concentration. Each drum was aerated to aid water quality and mix solutions. A flow through system was used to replenish water. Every tank had a line running from a dose drum through a peristaltic pump, (Cole Parmer Masterflex L/S Economy Variable-Speed Digital Drive). Each tank had an overflow spout at 6 L. The flow rate into each tank was 12.5 mL/min; which allowed for approximately 18 L a day to be pumped through each tank.

Tadpoles were fed a diet specifically developed for culturing *X. laevis* tadpoles. The "tadpole cocktail" composed of 25% Nutrafin Max, 25% Aquamax Trout Chow, and 50% Nasco frog brittle pellets dry volume (Koss and Wakeford, 2000). Each of the above ingredients were ground to a fine powder and thoroughly mixed with water, 50% by volume. This mixture was fed daily to tadpoles beginning on day 5.

2.6. Observations

Water quality parameters were monitored every other day and included : ammonia, temperature, conductivity, salinity, dissolved oxygen, and pH. Mortality and tank temperature were recorded daily. Mortality was determined by lack of movement or response from external stimulus. Every five days body measurements were conducted to the nearest mm; these measurements included snout-vent length (SVL), total body length (TBL), hind-limb length (HLL), and stage. Snout vent length is measured from the tip of the nose to the anal vent. Total body length is measured from the tip of the nose to the tip of the tail. Hind limb length is measured from the apex of the leg to the end of the toes. Stage was determined by Nieuwkoop and Faber table of development (Nieuwkoop and Faber, 1975). Measurements (stage, TBL, SVL, and HLL) from day 40, 45, and 46 include measurements from stage 66 juveniles euthanized before the aforementioned measure days. Time to metamorphosis was also evaluated as a growth endpoint and was defined as the percentage of tadpoles that completed metamorphosis

(NF stage 66). Time to metamorphosis was tracked from the first day tadpoles completed metamorphosis to the completion of the study. Malformations were also noted with body measurements and were determined using Bantle's Atlas of Abnormalities: A Guide for the Performance of FETAX (Bantle et al., 1989). Malformations and staging were identified using a Motic K series stereoscope.

2.7. Euthanization and Storage

Dead tadpoles were removed and stored in 10% buffered formalin. When tadpoles reached Nieuwkoop and Faber stage 66, they were removed from the tank, euthanized with MS-222 and stored in 10% buffered formalin. Stage 66 juveniles had TBL and HLL measured at time of euthanization. Removing tadpoles when they reach stage 66 ensured survival of smaller, less developed tadpoles since *X. laevis* can be cannibalistic. The study was concluded when 90% of the control tadpoles reached stage 66. It is standard practice to conclude a FETAX Assay when 90% of tadpoles reach stage 46 or at 96 h (ASTM, 1999). At the end of the study, all remaining tadpoles and frogs were euthanized with MS-222 and stored in 10% formalin.

2.8. Metal cation concentration

Metal cation, Zn, concentration was determined in dosing solutions with flame atomic absorption spectroscopy (M Series AA Spectrometer, Thermo, Waltham, MA). Dosing solutions were acidified, to pH of 2, by adding 150 μL concentrated nitric acid to 30 mL of sample. Concentration was determined before new solution was added and 24 h after the new solution was added. The analysis was conducted within 48 h of sample collection.

2.9. Tissue concentration

Individual tadpoles were weighed to the nearest mg after freeze drying (FreeZone 2.5, Labconco, Kansas City, MO) to obtain dry weight for analytical techniques. Tadpoles were composited in groups of 15 samples, five from each replicate. In addition to tadpole samples, one standard reference material (DOLT-4, NRC-CNRC, Ottawa, Ontario, Canada) and sample blank were processed with every 24 samples. Samples were acid digested using a modified EPA method 3050B. Tadpoles were pre-digested overnight using a 1:1 nitric acid solution. Concentrated nitric acid (5 mL per g dry weight) was added to the pre-digest solution and heated to 95 °C for 1 h or until digest was liquid. An additional aliquot of nitric acid, half the initial amount of concentrated nitric, was added to the cooled digests. If brown fumes were generated an additional aliquot of nitric acid was added and digests were heated to 95 °C without boiling for 2 h. Hydrogen peroxide was added after digests were cooled in an ice bath. Digests were returned to heat at 95 °C for an additional 2 h to complete digestion processes. Tissue digests were diluted to 25 mL (control through 0.5 mg/L samples) or 50 mL (1 and 2 mg/L samples) with 3% nitric acid. Flame atomic absorption spectroscopy was utilized to determine Zn tissue concentration (M Series AA Spectrometer, Thermo, Waltham, MA).

2.10. Statistical analyses

All exposure concentrations are reported as measured zinc in solution, and these measured concentrations are use in statistical evaluations. Measured concentrations can be translated to target exposures of ZnO using data reported in the results section. Growth measurements were compared among concentrations using a nested one-way analysis of variance (ANOVA) followed by Tukey Honestly Significant Differences test upon significant ANOVAs ($p \leq 0.05$). Growth measurements were analyzed at every measure day and the conclusion of the study. Total body length and HLL of stage 66 juveniles were also compared among concentrations. Mortality was analyzed using ANOVA and Tukey HSD multiple comparison test at the conclusion of the study. Zinc concentration in solution and Zn tissue concentration were analyzed with nested ANOVA and Tukey test as well to determine differences in Zn concentration between doses.

3. Results

3.1. Water quality

Temperature and pH were within acceptable ranges, according to ASTM E1439-98 (24 ± 2 °C and pH=6.5–9.0), throughout the study with averages of 23.6 °C and 7.47 respectively (Table 1). Conductivity, ammonia, and dissolved oxygen were within ranges previously reported by other similar studies (Carr et al., 2003; Coady et al., 2005; Mitchell et al., 2005; Tietge et al., 2005).

Table 1
Water quality of dosing solutions characterized throughout the exposure.

Water Quality[a]	
Temperature (°C)	23.6 ± 0.05
pH	7.47 ± 0.02
Conductivity (μs/cm)	1740 ± 13
Salinity (ppt)	0.97 ± 0.01
NH (mg/L)	1.19 ± 0.08
Un-ionized NH_3 (mg/L)	0.02 ± 0.00
Dissolved Oxygen (mg/L)	5.46 ± 1.89

[a] Reported as mean ± SE.

3.2. Zn concentrations

Zinc concentrations were lower than nominal predictions (amount of Zn in solution resulting from 100% dissolution of ZnO) and decreased over time in the following ZnO nanomaterial solutions: 0.125, 0.25, and 2 mg/L (Table 2). Both 0.25 and 0.5 mg/L had the highest nominal percent determined over 75%, while 0.125 and 1 mg/L were over 63% of nominal Zn concentration. The 2 mg/L exposure solutions had the lowest nominal percentage with just below 50% of the calculated nominal Zn concentration. Due to these departures from nominal concentrations, X. laevis response to ZnO exposures will be described in relation to measured Zn concentrations, rather than nominal ZnO concentration for the remainder of this article. Measured Zn concentrations among exposure groups were different ($p \leq 0.001$). FETAX has been reported to contain less than 0.01 mg/L Zn as determined by atomic absorption analysis when made with deionized distilled water (Cross et al., 2007) and our FETAX control contained no quantifiable Zn.

The decrease in Zn concentration over time could be attributed to the nature of nano-ZnO. Zinc oxide nanomaterials had a tendency to precipitate from solution. There was visible precipitation in tubing that ran from the drum through the pump to the tank and in the tanks themselves. It is also possible that nano-ZnO precipitated in the drum; this was not visibly seen as the drum was a translucent white.

3.3. Concentration of zinc in tissue

Average dry weight for all tissue samples was 0.174 g ranging from as small as 0.012–0.321 g. A total of four DOLT-4 (a standard reference material) samples were utilized for this test. DOLT-4 is reported to contain 116 ± 6 μg/g of Zn, and the average Zn concentration recovered from DOLT-4 in this study was 125.66 ± 4.20 μg/g. Zinc concentration in tissue increased in a dose-dependent manner (Table 3). Significant increases in Zn concentration in tissue were observed beginning at 0.305 mg/L Zn when compared to control Zn tissue concentration ($p \leq 0.001$). Zinc concentration in tissue of tadpoles exposed to 0.159 mg/L Zn was approaching a significant increase with a p-value of 0.086. The highest dose also had a significantly higher Zn concentration in tissue when compared to all other ZnO nanomaterial tested concentrations ($p \leq 0.001$).

3.4. Mortality

Three doses (0.067, 0.159, and 0.305 mg/L) induced an average mortality equal to or less than 10% (Fig. 1). Control and 0.513 mg/L exposed tadpoles had mortality of 12 and 11% respectively, each tank within these doses lost a maximum of 1 or 2 tadpoles. Control mortality of $12 \pm 0.4\%$ was comparable to other chronic *X. laevis* studies and verified that our toxicity test was performed under well controlled conditions. (Carr et al., 2003; Coady et al.,

Table 2
Concentration of Zn in ZnO nanomaterial dosing solutions.

ZnO Dose (mg/L)	Calculated Zn (mg/L)	Actual Zn (mg/L)[a] (% Nominal)	Range of Concentration (mg/L)
Control	> 0.01	0.000 ± 0.001	0–0.009
0.125	0.1	0.067 ± 0.004* (67.0)	0.016–0.092
0.25	0.201	0.159 ± 0.007* (79.1)	0.084–0.214
0.5	0.402	0.305 ± 0.007 (75.9)	0.249–0.363
1	0.804	0.513 ± 0.010 (63.8)	0.428–0.609
2	1.607	0.799 ± 0.020* (49.7)	0.489–1.018

[a] Reported as mean ± SE.
* Linear regression (time vs concentration) $p \leq 0.05$.

Table 3
Concentration of Zn in *Xenopus laevis* whole body samples from ZnO nanomaterial exposure.

ZnO Dose	Tissue Zn (μg/g)[a] Dry Weight	Tissue Zn (μg/g)[a] Wet Weight
Control	120.58 ± 16.11	22.08 ± 1.17
0.125	259.92 ± 26.88	50.86 ± 2.18
0.25	417.82 ± 37.05	78.05 ± 2.62
0.5	583.98 ± 47.33*	111.84 ± 2.93*
1	732.72 ± 102.17*	124.96 ± 7.22*
2	1910.85 ± 532.54*	172.56 ± 17.97*

[a] Reported as mean ± SE.
* $p \leq 0.05$ ANOVA and TukeyHSD.

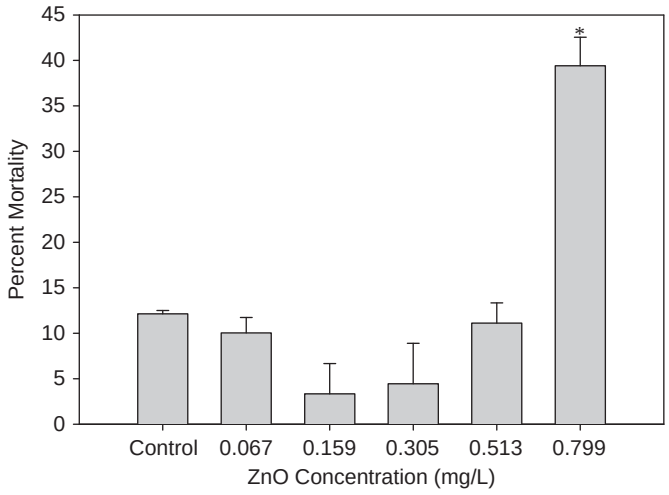

Fig. 1. Mortality of *Xenopus laevis* tadpoles induced by exposure of ZnO nanomaterial. Measured concentrations of Zn reported as mean percent mortality ± standard deviation. *: $p \leq 0.05$ ANOVA and TukeyHSD.

2005). A significant increase in mortality was observed at the highest tested concentration (0.799 mg/L) compared to all treatments including the controls, with a mortality incidence of 40% ($p=0.002$). *X. laevis* mortality after day 4 constituted 97% of all mortality. Three exposures produced tadpole mortality before day 5, control, 0.067 mg/L, and 0.799 mg/L Zn. In the remaining dosing concentrations, all tadpole mortality was observed after day 5.

3.5. Growth

3.5.1. Nieuwkoop and faber stage

In general, all tadpoles increased in developmental stage over time, but there was variation in stage progression (Fig. 2A). On the first measure day (day 5), all tadpoles were at the same average stage of 50. By day 10 there was a difference in staging. Tadpoles in 0.067 mg/L Zn reached significantly higher stages on the following measure days: 10, 15, 20, 30, and 35 ($p \leq 0.002$). Tadpoles exposed to 0.159 mg/L reached more advanced stages compared to control tadpoles on some measure days; day 10, 30, and 35 ($p \leq 0.014$). On days 10 through 20, tadpoles exposed to 0.305 mg/L Zn reached significantly lower stages than control tadpoles ($p < 0.001$). Beginning on day 25, stages for tadpoles exposed to 0.305 mg/L Zn were similar to control tadpoles for the remainder of the study. Tadpoles exposed to high doses (0.513 and 0.799 mg/L Zn) had reached significantly lower average stages ($p \leq 0.034$) for the majority of the study, which indicated slower development. At the conclusion of the study, tadpoles exposed to doses below 0.305 mg/L Zn nanomaterials had average stages of 66, while 0.513 mg/L Zn dosed tadpoles had an average stage of 65 ranging from stage 60 to 66. The highest dose, 0.799 mg/L, had the lowest average staged tadpoles at the conclusion of the study with an average stage of 56 ranging from stage 52 to 64.

3.5.2. Snout Vent Length (SVL)

SVL generally increased steadily to approximately stage 58 and then decreased for a short time before platueaing (Fig. 2B). This pattern was evident for all concentrations except 0.799 mg/L, as tadpoles in this dose experienced an increase in SVL for the entire study, and did not exhibit a peak SVL as observed in other doses.

On the first measure day (day 5), there were no statistical differences in SVL measurements. For many timepoints after day 5, there were significant differerences in SVL, especially for organisms exposed to 0.799 mg/L Zn. On days 10–46, 0.799 mg/L tadpoles were significantly shorter with respect to SVL than control tadpoles ($p \leq 0.05$). Two other doses contained tadpoles with significantly reduced SVLs during the exposure ($p \leq 0.004$), 0.305 mg/L (day 10 and 15) and 0.513 mg/L (day 10 through day 35). On day 10 and 20, 0.067 mg/L had significantly longer SVL than control ($p < 0.001$). Exposure to 0.159 and 0.305 mg/L Zn produced tadpoles with similar SVLs that were similar to control tadpole SVL for a majority of the study. The maximum average SVL for the 0.513 mg/L exposure group was 23.5 ± 0.5 mm, which is 5 mm shorter than average SVL control tadpoles achieved (27.5 ± 0.9 mm).

An additional method to evaluate the effect of ZnO nanomaterial on growth was to examine the relationship between SVL and stage (Fig. 3). These data suggested that at later stages tadpoles exposed to 0.067 mg/L appear to be larger than controls, while tadpoles exposed to 0.513 and 0.799 mg/L Zn nanomaterials appear to be smaller than controls (Fig. 4).

3.5.3. Total body length (TBL)

TBL followed a similar trend in length as SVL in regard to achieving a peak length and then decreasing in TBL (Fig. 2D). TBL begins a steep decline after stage 59/60. The decline visually appears to be steeper in TBL than SVL. TBL at stage 59/60 is on average 69 mm and drops to 22 mm by stage 66, whereas SVL is 27 mm at

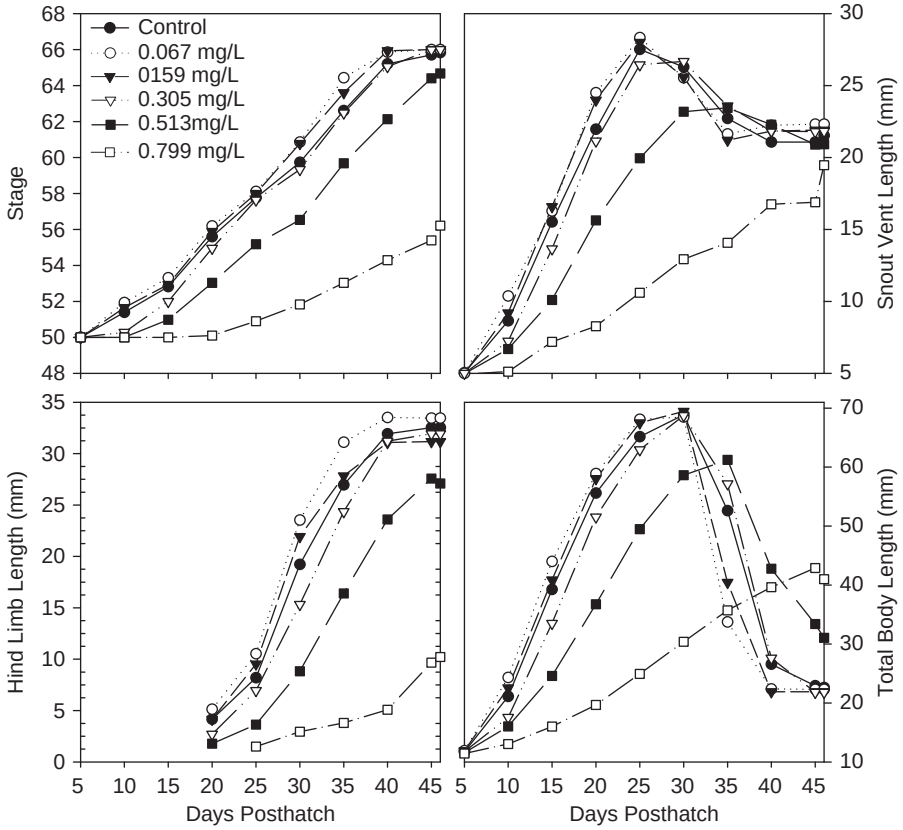

Fig. 2. Trends of *Xenopus laevis* growth endpoints throughout the 46 d exposure to ZnO nanomaterials: A-Stage, B-Snout Vent Length, C-Hind Limb Length, and D-Total Body Length. Data is reported as mean of each respective measurement from tadpoles for each measured Zn concentration per measure day.

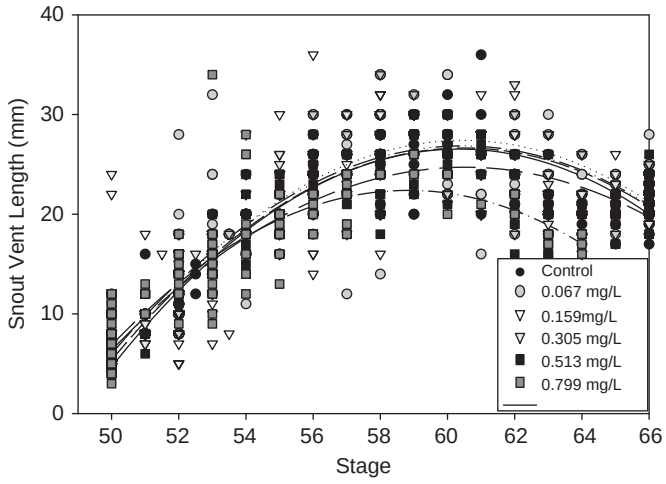

Fig. 3. The relationship between stage and snout vent length of *Xenopus laevis* with exposure to ZnO nanomaterials. Regression lines in order from top to bottom: 0.067(dotted line), 0.305 (dash-dot-dot line), 0.159 (short dash line), control (solid line), 0.513, (long dash), and 0.799 mg/L (dash-dot line) of Zn as measured in test waters.

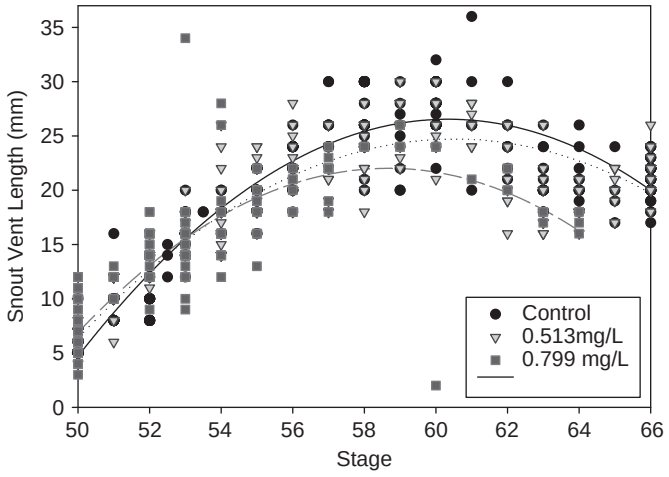

Fig. 4. The relationship between stage and snout vent length of control *Xenopus laevis* tadpoles (solid line) as well as those exposed to ZnO nanomaterial that produced 0.513 mg/L (dotted line) and 0.799 mg/L (dashed line) Zn.

stage 58 and drops to 22 mm by stage 66. From day 10 to day 46, TBL of tadpoles exposed to 0.799 mg/L Zn was significantly shorter than control TBL ($p \leq 0.001$). Two other doses had tadpoles with significantly smaller TBL for a short period of time; 0.305 mg/L on days 10 through 20 and 0.513 mg/L from days 10 to day 46 ($p \leq 0.023$). On days 10, 15, and 35, TBL for tadpoles exposed to 0.067 mg/L Zn were significantly longer than controls TBLs ($p \leq 0.001$). As seen with SVL, exposures to 0.159 and 0.305 mg/L Zn resulted in tadpoles with similar TBL measurements as control TBL for a majority of the study. The maximum average TBL for control tadpoles was 68.9 ± 1.0 mm, while tadpoles exposed to 0.513 mg/L Zn achieved a maximum average of 61.2 ± 1.4 mm and 0.799 mg/L exposed tadpoles reached 41.0 ± 2.4 mm.

3.5.4. Hind Limb Length (HLL)

HLL increased from initial appearance throughout the study (Fig. 2C). HLL could not be measured until at least day 20 as the hind limb was easier to visualize after stage 51/52. Tadpoles exposed to 0.799 mg/L Zn did not have measurable hind limbs

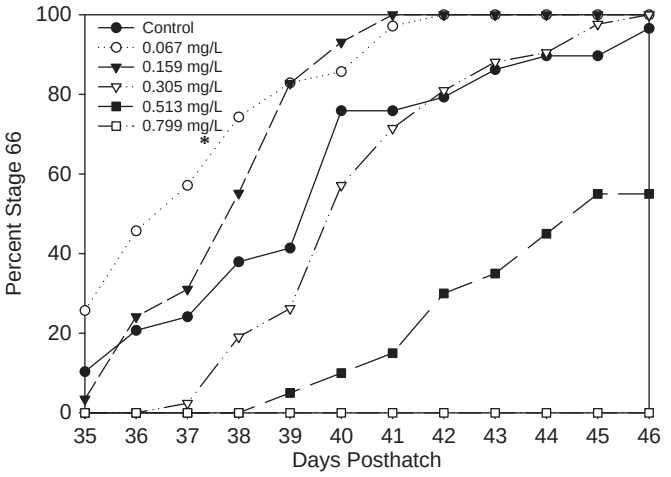

Fig. 5. Percent of *Xenopus laevis* tadpoles to complete metamorphosis in the presence of measured Zn concentrations resulting from ZnO nanomaterial addition over exposure times from day 35 to completion of study, day 46. *No tadpoles completed metamorphosis at 0.799 mg/L.

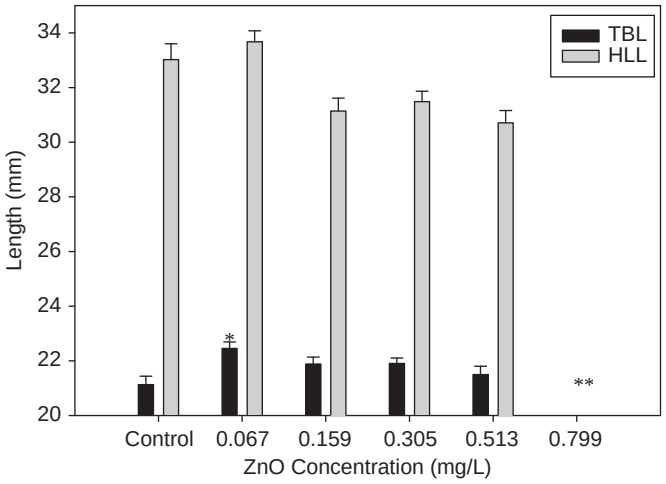

Fig. 6. Body measurements (mean ± standard error), total body length (TBL) and hind limb length (HLL), of stage 66 *Xenopus laevis* juveniles continuously exposed to ZnO nanomaterials to produce each measured Zn concentration. *: $p \leq 0.05$ ANOVA and TukeyHSD. **: no data (zero tadpoles completed metamorphosis).

until the fifth measure day (day 25). Tadpoles exposed to 0.799 mg/L Zn had significantly shorter hind limbs throughout the entire study in relation to control tadpoles ($p < 0.001$). From day 20 to day 46, tadpoles experiencing 0.513 mg/L Zn had significantly shorter hind limbs than control tadpoles ($p < 0.001$). On days 20 through 30, tadpoles in 0.305 mg/L Zn had HLLs significantly shorter than control tadpole HLL ($p \leq 0.049$). On days 20 through 35, tadpoles exposed to 0.067 mg/L had significantly longer HLL than control tadpoles ($p \leq 0.002$). Tadpoles in 0.159 mg/L Zn experienced similar HLL as control tadpoles for all measure days except day 25 where HLL of 0.159 mg/L exposed tadpoles had longer HLL than control tadpoles ($p = 0.034$). On days 40 through 46, tadpoles exposed to low doses of nano-ZnO had similar HLL as control tadpoles.

3.5.5. Time to metamorphosis

The first tadpoles to complete metamorphosis (stage 66) occurred on day 35 in the following concentrations: control (10%), 0.067 mg/L (25%) and 0.159 mg/L (4%) (Fig. 5). On day 37 and 39, at least one tadpole completed metamorphosis in the 0.305 and 0.513 mg/L Zn concentration respectively. By the time 90% of control tadpoles completed metamorphosis, all tadpoles in the 0.067, 0.159, and 0.305 mg/L groups had completed metamorphosis. On day 42, tadpoles exposed to 0.305 mg/L Zn surpassed the percent of control tadpoles that completed metamorphosis. Both 0.067 and 0.159 mg/L had 100% complete metamorphosis a minimum of 5 days before controls reached 90% completion. Tadpoles exposed to 0.513 mg/L only had 58% metamorphic completion, and no tadpoles achieved stage 66 in the 0.799 mg/L treatment group.

3.5.6. Stage 66 Juveniles

Stage 66 juveniles in the 0.067 mg/L Zn nanomaterial treatment group had significantly longer TBL than stage 66 control juveniles (Fig. 6). Control juveniles had an average TBL of 21 mm and 0.067 mg/L juveniles averaged 23 mm for TBL ($p = 0.001$). Stage 66 juveniles from 0.159 and 0.305 mg/L had TBL approaching significance with p-values of 0.081 and 0.091 respectively. Control and 0.067 mg/L juveniles had slightly longer hind limbs, over 33 mm, than 0.159, 0.305, and 0.513 mg/L juveniles with HLL of 31 mm. The HLL of juveniles exposed to 0.067 mg/L Zn had significantly longer HLL than 0.159, 0.305 and 0.513 mg/L juveniles ($p \leq 0.05$).

4. Discussion

Previous studies have demonstrated that nano-ZnO increase mortality in crustaceans and zebrafish (Zhu et al., 2008; Heinlaan et al., 2008). *Daphnia magna* experienced a 48-h LC_{50} of 3.2 mg ZnO/L while *Thamnocephalus platyurus* had a 24-h LC_{50} of 0.18 mg ZnO/L (Heinlaan et al., 2008). Exposure of zebrafish (*Danio rerio*) to nano-ZnO induced a 96-h LC_{50} of 1.793 mg/L (Zhu et al., 2008). *X. laevis* exposed to ZnO nanomaterial concentrations in this study did not exhibit a dose-response relationship for mortality. Mortality was only observed at the highest tested concentration (0.799 mg/L Zn).

Chronic exposure of nano-ZnO affected mortality more significantly than would be predicted from results of previous acute nano-ZnO exposures. No significant mortality occurred during acute studies, even at concentrations as high as 31.6 mg/L ZnO (Nations, 2009); whereas the chronic exposure demonstrated a significant mortality effect (40%) at 0.799 mg/L Zn nanomaterial exposure. An increase in mortality with chronic exposure could be due to delayed feeding or impaired digestion of tadpole larvae. Tadpole larvae obtain nutrition by yolk resorption for the first 3 to 4 days post-hatch (Bernardini, 1999; Raising Tadpoles: Tadpole Care, 2007); thereafter, nutrition is actively acquired by filter feeding. The feeding habits of xenopous larvae increases the possibility of nano-ZnO ingestion thereby providing lethal Zn concentrations with longer exposures. A similar trend of longer exposure time increasing mortality was confirmed in a 7 day study evaluating the use of *X. laevis* as a model for testing pollution of Zn (Haywood et al., 2004). Mortality incidence increased on or after day 4 for all tested concentrations, including 5, 10, 15, and 20 mg/L (Haywood et al., 2004). From day 3 to the conclusion of the study, 20 mg/L exposure had no tadpoles survive, 15 mg/L had a mean less than 1 surviving tadpole, 10 mg/L had a mean of approximately 5 tadpoles surviving, and even 5 mg/L dropped from a mean of 12 tadpoles to a mean of approximately 9 tadpoles (Haywood et al., 2004). The majority of all mortality occurred after day 4 (~70%) similarly to the results of this exposure (~80%).

Growth endpoints revealed nano-ZnO affected metamorphosis of *X. laevis*. Exposure to higher nano-ZnO concentrations inhibited stage progression and growth throughout metamorphosis. Alternatively, exposure to lower concentrations of nano-ZnO increased stage progression and growth throughout metamorphosis.

This type of dose response can be deemed hormetic, but that term implies a changing temporal effect of the stressor and some compensatory response (Calabrese, 2008). Given the known essential nature of Zn for proper growth, the term hormesis may not be entirely accurate for the observed dose response profile.

Our growth and development results complement previous studies that evaluated effects of Zn compounds on growth and/or metamorphosis. Exposure to Zn, $ZnCl_2$ and $ZnSO_4$, for 4 to 7 days inhibited growth of *X. laevis* tadpoles (Haywood et al., 2004; Dawson et al., 1988; Luo et al., 1993). Zn delayed metamorphic completion for Columbia spotted frog (*Rana luteiventris*) tadpoles as compared to controls (Lefcort et al., 1998, 1999). None of these studies indicated that exposure to relatively low concentrations of Zn or nano-ZnO increased metamorphic rate or positively influence growth as observed in our study.

Amphibians can experience accelerated metamorphic development due to stress and the principle of energy allocation (Newman and Clements, 2008). TBL of stage 66 juveniles does not support this reasoning as all nano-ZnO doses with juveniles tended to have longer average TBL than control juveniles. Total body length measurements indicate that nano-ZnO improved growth. When an organism is forced to allocate resources to development to alleviate stress, the organism would tend to be smaller in size (Steyermark, 2002). As Zn is an essential nutrient that facilitates growth, it was expected that Zn would have a beneficial effect on TBL (Zinc and Human Health, 2008). For example, 1500–2000 mg Zn/kg dietary ZnO improved post-weaning pig growth and feed response (Hill et al., 2001). There could also be a stress relief to the developing Xenopus from the antimicrobial properties of nano-ZnO (Zhang et al., 2010).

Zinc concentrations in whole-body samples from our control were similar compared to other tissues from several studies. Whole body Zn concentrations in rainbow trout were 32.57 ± 1.94 μg/g w.w. (Alsop et al., 1999). *X. laevis* whole body Zn concentration, in our study was 22.08 ± 1.17 μg/g w.w. Several fish species from the Yellowstone River Basin contained between 61.9 and 882 μg/g d.w. of Zn in liver samples (Peterson and Boughton, 2000), and control tadpoles in our study contained 120.58 ± 16.11 μg Zn/g dry weight total body samples. Zinc concentrations in tissue were significantly higher in 0.799 mg/L tadpoles exposed to nano-ZnO as compared to all other doses. Negative effects on growth and survivability were also noted in this exposure group throughout the study. This would indicate Zn concentrations in tissue exceeding 1300 μg Zn/g dry weight are detrimental to growth and metamorphosis. Limited resources precluded Zn quantification in different organs. Given the high concentrations, we would expect that elevated concentrations would be found in the GI tract, blood, liver and kidney. There is evidence that exposure to high concentrations of Zn has a protective effect with respect to lead toxicosis by providing a significant excess of Zn for metal binding sites in ALAD, thereby overwhelming the competition that lead demonstrates for these binding sites (Wetmur et al., 1991). Accumulation in liver and kidney would likely be accompanied by increased metallothioneine induction and presence in these tissues (Heier et al., 2007).

Zinc concentration in *X. laevis* could increase over time with continuous exposure to nano-ZnO in solution. Uptake of Zn was evaluated in guppy fish (*Poecilia reticulate*) by exposing fish to 10 mg/L Zn for 6 days, and there was an increase in Zn concentration in tissue from a background of 91 ± 122 μg/g to a tissue concentration of 1500 μg/g (Widianarko et al., 2001). The uptake of nano-ZnO could be evaluated in future studies by sacrificing tadpoles at various time points to determine accumulation of Zn in various tissues throughout the study. By studying the accumulation of Zn in *X. laevis*, it may be possible to determine how concentrations in tissue affect metamorphosis and explain how 0.305 mg/L Zn exposed tadpoles began metamorphosis at a relatively slower rate than controls and surpassed control tadpoles around day 30 in respect to stage progression, SVL, and TBL.

Addition of nano-ZnO could produce Zn concentrations that are hazardous to aquatic organisms. Background concentrations for Zn in water bodies range from 1 to 40 μg/L (Bodar et al., 2005), and the maximum acceptable toxicant concentration (MATC) for fathead minnows in hard water is 30–180 μg/L (USEPA, 1995). Addition of 0.305 mg/L nano-ZnO to FETAX solution, increased the aqueous Zn to 305 ± 7 μg/L, which is almost twice the fathead minnow MATC (180 μg/L). With the addition of 2 mg/L nano-ZnO, aqueous Zn reached 4 times the MATC.

Zinc oxide nanomaterials negatively affected survival, growth, and/or malformation incidence of several aquatic species, zebra fish, African clawed frog, microalgae, and bacteria (Franklin et al., 2007; Zhu et al., 2008; Lovern and Klaper, 2006; Lovern et al., 2007; Tollefsen et al., 2008). So it is important to realize that nano-ZnO could ultimately enter water bodies through industrial products and wastes, as well as personal care product release via wastewater effluent or direct dissolution into surface water. A number of studies evaluated effects of nano-ZnO exposure also evaluated effects of $ZnSO_4$. This allowed comparison of nano-ZnO and ionic Zn toxicity. In general, the toxicity caused by exposure to nano-ZnO was similar to toxicity observed with exposure to $ZnSO_4$ when comparing nominal concentrations of the toxicants. Many studies concluded that nano-ZnO toxicity was due to soluble Zn (Mortimer et al., 2008; Franklin et al., 2007; Heinlaan et al., 2008; Aruoja et al., 2009). Nano-ZnO in water produces higher soluble Zn concentrations in the water column, and Zn is highly toxic to aquatic organisms at elevated concentrations (USEPA, 2005). Exposure to high Zn concentrations can cause mortality, while lower exposure concentrations cause malformation, affect olfactory senses, and affect growth and development, which in turn can decrease survival (Haywood et al., 2004; Lefcort et al., 1999; Aruoja et al., 2009).

Exposure to nano-ZnO could also reduce food resources such as algae. Exposure of nano-ZnO inhibited growth of freshwater microalga (*Pseudokirchneriella subcapitata*) with 72 h EC_{50}s ranging from 0.042 to 0.069 mg Zn/L (Franklin et al., 2007; Aruoja et al., 2009). Nano-ZnO exposure to aquatic ecosystems could negatively affect development of many organisms by inhibiting growth as well as reducing food sources such as microalgae. A reduction in size can increase predation in aquatic ecosystems (Haywood et al., 2004). Therefore decreased food sources in conjunction with reduced size can adversely effect ecosystems in a complex fashion.

5. Conclusions

X. laevis exposed to high nano-ZnO concentrations (i.e. those producing 0.513 and 0.799 mg/L Zn) died or developed slower, which produced fewer and smaller organisms in these treatment groups. Release of nano-ZnO into aquatic ecosystems could also reduce food resources such as algae. Conversely, exposure to lower concentrations of nano-ZnO (those producing 0.067 mg/L Zn) increased tadpole development and body size. Therefore, we conclude nano-ZnO released into aquatic ecosystems in high concentrations could have detrimental effects on aquatic organisms such as amphibians. One possible conclusion of this research is that control of nano-ZnO release would have beneficial effects on aquatic vertebrates. The possibility of beneficial effects should be viewed with care and evaluations of nano-ZnO effects on the base of aquatic food webs must be seriously considered, before

any assertion of overall benefits from nano-ZnO to an aquatic ecosystem can be made.

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