**Enhanced toxicity of environmentally transformed ZnO nanoparticles relative to Zn ions in the epibenthic amphipod *Hyalella azteca***

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<td>Poynton, Helen; University of Massachusetts Boston School for the Environment, Chen, Chun; University of Kentucky College of Agriculture Food and Environment, Department of Plant and Soil Sciences; Northwestern A&amp;F University, College of Life Sciences, State Key Laboratory of Crop Stress Biology in Arid Areas, Alexander, Shaun; University of Massachusetts Boston School for the Environment, Major, Kaley; University of Massachusetts Boston School for the Environment, Blalock, Bonnie; University of Massachusetts Boston School for the Environment, Unrine, Jason; University of Kentucky,</td>
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Enhanced toxicity of environmentally transformed ZnO nanoparticles relative to Zn ions in the epibenthic amphipod *Hyalella azteca*

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Environmental Significance statement:
Sediment dwelling organisms, including the amphipod *Hyalella azteca*, are at particular risk from engineered nanomaterials. Zinc oxide nanoparticles undergo complex transformations in the environment, including pH dependent changes in speciation and solubility and transformations into phosphates, sulfides and carbonates. This is one of the first studies to compare and demonstrate differences in the toxicity of environmentally transformed ZnO nanoparticles. *Hyalella azteca* are particularly sensitive to phosphate-transformed ZnO nanoparticles, which form in wastewater treatment plants and aquatic environments with enriched phosphate. However, both phosphate and sulfide-transformed ZnO nanoparticles accumulate in *H. azteca*, thus providing a route for particles to enter the food web.

Abstract:
After release into the aquatic environment, engineered nanomaterials (ENMs) undergo complex chemical and physical transformations that alter their environmental fate and toxicity to aquatic organisms. *Hyalella azteca* are sediment-dwelling amphipods predicted to have a high exposure level to ENMs and have previously shown to be highly sensitive to ZnO nanoparticles (NPs). To investigate the impacts of environmentally transformed ZnO NPs and determine the route of uptake for these particles, we exposed *H. azteca* to ZnSO₄, ZnO NPs, and environmental aged ZnO NPs which resulted in three types of particles: 30 nm ZnO-Zn₃(PO₄)₂ core-shell structures (p8-ZnO NPs), micron scale hopeite-like phase Zn₃(PO₄)₂*4H₂O (p6-ZnO NPs), and ZnS nano-clusters (s-ZnO NPs). Treatments included freshwater, saltwater (3 ppt), and the presence of sediment, with a final treatment where animals were contained within mesh baskets to prevent burrowing in the sediment. Dissolution was close to 100% for the pristine ZnO NPs and phosphate transformed NPs, while s-ZnO NPs resulted in only 20% dissolution in the water only exposures. In the freshwater exposure, the pristine and phosphate transformed ZnO NPs were more toxic (LC₅₀ values 0.11-0.18 mg L⁻¹) than ZnSO₄ (LC₅₀ = 0.26 mg L⁻¹) and the s-ZnO NPs (LC₅₀ = 0.29 mg L⁻¹). Saltwater treatments reduced the toxicity of ZnSO₄ and all the ZnO NPs. In the presence of sediment, water column concentrations of Zn were reduced to 10% nominal concentrations and toxicity in the sediment with basket treatment was similarly reduced by a factor of 10. Toxicity was further reduced in the sediment only treatments where...
the sediments appeared to provide a refuge for *H. azteca*. In addition, particle specific differences in toxicity were less apparent in the presence of sediment. Bioaccumulation was similar across the different Zn exposures, but decreased with reduced toxicity in the saltwater and sediment treatments. Overall, the results suggest that *H. azteca* is exposed to ZnO NPs through the water column and NP transformations in the presence of phosphate do not reduce their toxicity. Sulfidized ZnO NPs have reduced toxicity, but their similar level of bioaccumulation in *H. azteca* suggests that trophic transfer of these particles will occur.

**Introduction:**

Zinc oxide nanomaterials (ZnO NMs) are a class of nanomaterials utilized to provide UV protection in personal healthcare products,¹ for cancer therapies in medicine,² and they are attractive materials for photovoltaic cells and electrical sensors.³ Modeling studies suggested that the highest environmental concentrations of ZnO nanoparticles (NPs) occurs in wastewater treatment facility (WWTF) effluent with current concentrations estimated to be 0.3- 0.4 µg L⁻¹⁴ while measured values were slightly higher.⁵ These values are expected to climb, especially within sediments,⁶ as world-wide production of ZnO NPs increases and they are further incorporated into novel products.

As ZnO NPs make their way into the aquatic environment, water chemistry influences their fate and transport,⁷,⁸ and the extent of their dissolution.⁹,¹⁰ For example, high ionic strength and alkaline pH cause ZnO NPs to aggregate and settle out of suspension,⁹ but dissolved organic matter helps to stabilize NP suspensions.¹¹,¹² Dissolution of ZnO NPs has been documented as a major determinant of their toxicity;³,¹³ however, other studies have found that the NPs themselves cause toxicity.¹⁴-¹⁷ Although the fate and transport of these particles is complex, sedimentation of ZnO NPs is expected in most surface water environments, which will put benthic organisms in these areas at particular risk.¹⁸

Environmental transformations of engineered nanomaterials have been shown to have a profound effect on their fate, bioavailability, and effects.¹⁹-²³ The impact of these transformations often results in a reduction in bioavailability and toxicity; however, in some cases increases in bioaccumulation²⁴ and toxicity have been observed.¹⁹,²⁵ Recent studies have demonstrated that ZnO NPs also undergo a complex set of transformations in the environment, including pH dependent changes in speciation and solubility, changes in surface chemistry from binding inorganic and organic ligands, and transformations into phosphates, sulfides, and carbonates.²⁶ For example, during wastewater treatment, it has been demonstrated that ZnO NPs were completely transformed to ZnS, Zn₃(PO₄)₂ and Zn²⁺ ions were
adsorbed to mineral surfaces such as iron oxohydroxides (FeOOH). It is often assumed that such transformation products have similar bioavailability and toxicity as corresponding bulk materials, and therefore do not exhibit nano-specific effects. However, there is evidence that some of these transformation products may themselves be nanoscale materials. For example, Ma et al. demonstrated that the product of sulfidation of 30 nm ZnO NPs was 30-40 nm clusters of < 5 nm ZnS nanoparticles under a variety of conditions. In our studies of ZnO NP aging, ~30 nm ZnO-Zn₃(PO₄)₂ core-shell structures were formed when ZnO was aged in phosphate at slightly alkaline pH. The same particles dissolved and precipitated as a micron scale hopeite-like phase (Zn₃(PO₄)₂·4H₂O) at pH 6. Further, increased bioavailability and toxicity of Zn from the ZnO-Zn₃(PO₄)₂ core-shell structures relative to the micron scale hopeite-like phases was observed in wheat (Triticum aestivum) seedlings. The ZnO-Zn₃(PO₄)₂ core-shell structures had similar solubility as Zn₃(PO₄)₂, indicating that the Zn₃(PO₄)₂ shell protected the ZnO core from dissolution.

Sediments are predicted to be an important site for the accumulation of environmentally transformed ZnO NPs; therefore, investigations into the impacts of NPs on sediment dwelling organisms are essential for understanding their environmental risk. Several studies have begun to examine the effects of ZnO NPs to sediment-dwelling and soil organisms, including our study with the epibenthic amphipod Hyalella azteca. However, the number of these studies is limited in comparison with aquatic organisms (highlighted in recent reviews). The range in sensitivity of different benthic organisms spans orders of magnitude, while we still have little understanding of the factors that influence the large differences in toxicity across taxa. In addition, very few studies have investigated the impacts of environmentally relevant exposure scenarios, including exposures to transformed ZnO NPs.

The sediment-dwelling amphipod H. azteca is a model species for sediment toxicity studies and was the subject of a recent genome sequencing project that focused on pollution related gene families including ENM responsive genes. H. azteca’s epibenthic ecology, living and scavenging at the sediment surface, puts them at a high risk of exposure. Indeed, it has been found to be one of the most sensitive invertebrate species to ENMs including ZnO NPs. In addition, it is one of only a few species tested to date to have a higher sensitivity toward ZnO NPs compared with Zn²⁺. Understanding the mechanism for increased NP sensitivity to H. azteca would enable us to predict which organisms in the aquatic environment are at greatest risk for NP exposure and toxicity.

The objective of the present study was to investigate the toxicity of environmentally transformed ZnO NPs on H. azteca within four different exposure scenarios designed to
uncover the mechanisms responsible for NP uptake and toxicity. Based on previous results, we hypothesized that *H. azteca* are exposed to higher concentrations of ZnO NPs compared with Zn$^{2+}$ from ZnSO$_4$ due to their close proximity to the sediment surface and their scavenging behavior. Therefore, we exposed *H. azteca* to different ZnO NPs in the presence and absence of sediment. In a separate exposure we prevented them from scavenging by placing them in a mesh basket suspended above the sediments. Finally, we exposed them to the ZnO NPs at a higher salinity to increase aggregation of the particles and their sedimentation onto the beaker surface. We also measured dissolution of the particles and bioaccumulation in the amphipods. Comparing the toxicity and bioaccumulation of the different ZnO NPs under these exposure scenarios allowed us to begin to tease apart the importance of aggregation, sedimentation, and the scavenging behavior of *H. azteca* on the toxicity of environmentally transformed ZnO NPs.

**Materials and Methods:**

**Nanoparticle synthesis, transformation, and initial characterization**

Pristine and uncoated ZnO NPs were synthesized by wet chemical method using ZnCl$_2$ and NaOH as precursors. The procedures are described by Becheri et al. with minor modification. Briefly, the solutions of ZnCl$_2$ (0.2 M) were stirred at 85 - 90 °C for 15 min, followed by dropwise addition of 5M NaOH (5 M). The precipitate was washed three times with 18 MΩ deionized (DI) water and separated by centrifugation. The pellet was then lyophilized for storage in a dessicator.

Phosphate-aging procedures are based on those we have described previously, and sulfide-aging procedures are those used by Ma et al. Phosphate-aged ZnO NPs (p6-ZnO NPs and p8-ZnO NPs) were generated by weighing as-synthesized pristine ZnO NPs (approximately 5 mg Zn ) into 50 mL polypropylene centrifuge tube and dispersing in a 1.6 mM solution of Na$_2$HPO$_4$ (pH adjusted to 6 and 8, respectively, with HNO$_3$ or NaOH). Sulfidized ZnO NPs (s-ZnO NPs) were generated by weighing as-synthesized pristine ZnO dispersion (approximately 60 mg Zn) into 50 mL centrifuge tube and dispersing in 0.044 M solution of Na$_2$S in He-sparged water. The resulting ratio of S/Zn was approximately 2, which was shown to cause complete transformation of ZnO NPs. The particles were re-dispersed in aqueous media by sonicating in a cup horn sonicator for 30 min (Misonix, Newtown CT, USA). The tubes were filled to maximum capacity to minimize the headspace in the tube, then were capped and sealed with parafilm and placed horizontally on a reciprocating shaker for 5 days. Following incubation, tubes were centrifuged at 3320 g for 2-h. Supernatants were decanted and pellet was re-
suspended in DI water. This process was repeated three times to ensure that all Na$_2$HPO$_4$ or Na$_2$S was removed. After the final washing and centrifugation, pelleted NPs were lyophilized and stored at room temperature in a dessicator until used in toxicity assays.

The primary size and shape of the as-synthesized and transformed ZnO NPs were characterized through transmission electron microscopy (TEM, Jeol 2010F, Tokyo, Japan) at an accelerating voltage of 200 keV. The size distribution of pristine and aged ZnO NPs was determined from more than 100 particles viewed from multiple TEM images across the samples and the diameters of particles were quantified with ImageJ software (http://rsb.info.nih.gov/ij/). The composition and elemental distribution were further mapped using energy dispersive x-ray spectrometry (EDS). The crystal structure of the transformation products was confirmed using powder X-ray diffraction (X’Pert Pro, PANalytical, Malvern, United Kingdom) by comparing to authentic standards.

Zinc concentrations of all stock solutions used to prepare the exposures were determined using inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500cx, Santa Clara, CA, USA) after digestion of the particles with trace-metal grade HNO$_3$ following EPA methods 3005A$^{42}$ and 200.8.$^{43}$ The percent Zn of the pristine and aged samples was also determined by carefully weighing dried particle samples, dissolving in ultra-high purity HNO$_3$, diluting and analyzing zinc content of the sample by ICP-MS. Stoichiometric calculations were used to calculate the percent Zn.

**Experimental animals and culturing**

*Hyalella azteca* (US Lab Strain$^{44}$) were originally obtained from the U.S. Environmental Protection Agency (US EPA), Office of Research and Development Laboratory in Cincinnati, OH, USA, and have been maintained in culture at the University of Massachusetts Boston, USA since 2010. *H. azteca* were cultured according to the US EPA$^{45}$ in reconstituted “Duluth-100” water supplemented with 0.05 mg/L NaBr and fed three times weekly: desalted *Thalassiosira weissflogii* (Reed Mariculture, Inc., Campbell, CA) and Tetramin slurry (Tetra Holding, Blacksburg, VA). *H. azteca* used in all exposure tests were obtained from breeding cultures containing approximately 30 adult *H. azteca* in 1 L of media. Offspring were collected three times weekly and cultured separately until test initiation. Juvenile *H. azteca* used in the tests were 7-9 days old.

**Toxicity and bioaccumulation tests**
The overall study design followed US EPA methods for sediment toxicity testing\textsuperscript{45} utilizing a 96-h test. However, to determine how exposure conditions affected the toxicity of the NPs, we set up four different exposure scenarios (Fig. 1). These exposure scenarios were designed to determine how different environmental conditions affect the toxicity of ZnO NPs and differentiate between exposure through sediment and sediment surface exposure versus water exposure. Freshwater exposures provide controlled conditions to measure toxicity and uptake, but because \textit{H. azteca} are epibenthic organisms, these exposures do not adequately represent environmental conditions. Therefore, sediment exposures were also included to represent realistic environmental conditions where \textit{H. azteca} either burrow directly in the sediments (sediment only), or inhabit suspended vegetation (sediment with basket). Finally, a 3 ppt exposure was included because \textit{H. azteca} are able to inhabit slightly saline environments. Under these conditions, we expected the higher ion concentrations to impact the behavior of the NPs, thus potentially altering their toxicity.
Figure 1: Exposure conditions for the ZnO NP toxicity tests. Hyallela azteca were exposed to each treatment under four different exposure conditions. In all exposure conditions, the chemical treatments (ZnSO_4, ZnO NPs or transformed ZnO NPs) were added to the exposure media prior to adding the H. azteca. (A) Water-only conditions where H. azteca were exposed to each treatment using standard 96-h toxicity test conditions. H. azteca were provided with a small piece of nylon mesh as substrate. (B) 3 ppt salinity exposure, similar to A., but exposure media was augmented with Instant Ocean (Blacksburg, VA, USA) to obtain a salinity of 3 ppt. (C, E) Sediment conditions where H. azteca were provided with 22 g of clean sediment. (D, F) Sediment-basket conditions where H. azteca were placed within a nylon mesh “basket” suspended above 22 g of clean sediment.

Preliminary tests were conducted to determine the maximum salinity that did not impact long-term survival and growth of the H. azteca. Although US EPA and Environment Canada methods suggest that H. azteca can survive treatments up to 15 ppt salinity, we found that 6 ppt impacted H. azteca growth. Therefore, we chose to use 3 ppt salinity in our tests, which has a sufficient ionic strength to cause aggregation and rapid sedimentation of ZnO NPs. In addition, we conducted preliminary tests to determine the minimum amount of sediment that would not alter the toxicity of ZnSO_4 when compared with 50 ml of sediment. A reduced amount of sediment was desirable to (1) reduce the amount of burrowing of the H. azteca in the tests that may allow them to avoid the exposure, and (2) reduce the amount of sediment needed for analytical tests.

For all tests, test solutions were prepared in Duluth-100 media or Duluth-100 media supplemented with 3 ppt salinity. Stock solutions (1.0 g Zn L\(^{-1}\)) of each NP were prepared fresh by adding the appropriate mass of NP to 25 ml of milli-Q water. NPs were thoroughly suspended for 5 min using a Tissue Tearor (Biospec Products, Bartlesville, OK) rotor-type tissue homogenizer at maximum speed. Using this approach, the average hydrodynamic diameter of the ZnO NP suspensions was determined by dynamic light scattering (DLS) to be 650 ± 88 nm. The appropriate amount of stock solution was added to Duluth-100 media using a pipet immediately following homogenization and this was allowed to stir for 5 min. before preparing test solutions by serial dilution. All test suspensions were prepared by adding the appropriate amount of the more concentrated suspension by pipette and allowing the suspension to stir for 5 min before aliquoting to test beakers or preparing the next test suspension. ZnSO_4 stock solutions (Sigma-Aldrich, St. Louis, MO) were prepared by adding the appropriate amount of ZnSO_4 to milli-Q and stirring until fully dissolved. The ZnSO_4 stock solutions were placed in an amber bottle and stored at 4°C for up to 2 months. Test solutions of ZnSO_4 were prepared in a similar manner to the ZnO NP suspensions. ZnSO_4 was chosen
for the Zn control because sulfate was already present in the Duluth-100 exposure media and addition of ZnSO₄ did not substantially change the concentration of sulfate already present.

After exposure suspensions (200 ml for water only, 175 ml for sediment exposures) were aliquoted into 300 ml tall form beakers, 10 juvenile *H. azteca* were added to each beaker. Each exposure condition had four replicate beakers and a minimum of five concentrations and an unexposed control were tested for each chemical. Exposure beakers were placed in a 25° C environmental chamber for 96-h. After 48-h, beakers were removed and if possible, survivors were observed and counted without removing the animals from the beakers. After 96-h, surviving *H. azteca* were recorded and water, surviving animals, and sediments were collected. Temperature, pH, DO, conductivity, water hardness, and alkalinity were measured at the beginning and end of each exposure. Because ammonium levels may result from bacterial activity in the sediment exposures, ammonium was also measured at the end of the exposures to ensure that levels did not contribute to toxicity. In all exposures, temperature ranged between 22.0-24.5° C, DO ranged between 6.0-8.3 mg/L and pH ranged between 7.7-8.5. Conductivity ranged between 377-413 μS/cm, water hardness ranged between 100-120 mg/L, and alkalinity ranged between 85-95 mg/L in freshwater treatments. The following values were higher in the salinity treatments: conductivity: 5.67–5.78 mS/cm, water hardness: 650-810 mg/L, and alkalinity: 91-100 mg/L. Ammonium was 0 ppb in the treatments without sediment and ranged between 600-1000 ppb in treatments with sediment, well below levels that induce toxicity in *H. azteca*.48

Salinity treatments- Duluth-100 media was adjusted to 3 ppt with Instant Ocean Sea Salt (Spectrum Brands, Blacksburg, VA). Salinity was adjusted by adding approximately 2.9 g/L of Instant Ocean until the conductivity equaled 5.6 mS/cm².

Sediment treatments – Sediment was collected from Lake Massapoag in Sharon, MA, in December 2015, autoclaved to remove all microbial activity, and stored at 4° C. Sediment characterization was performed by the University of Kentucky Regulatory Services lab. The data are given in Table 1. Sediment pH was determined using a 1:1 slurry of sediment in DI water. Organic matter (based on carbon content) and total nitrogen content were measured using a Dumas combustion elemental analyzer. Cation exchange capacity (CEC) was determined using the NH₄Cl saturation-exchange method in neutral acetate buffer. Exchangeable element concentrations were determined by ICP-OES analysis of NH₄Cl-exchanged cations. Elemental concentrations were determined using a Mehlich III extraction
followed by analysis using ICP-optical emission spectroscopy (OES). Prior to the addition of exposure media, 22 g of sediment was added to each beaker. Baskets were constructed with 200 micron nylon mesh and designed to sit a few mm above the sediments. Baskets were clamped to the side of each beaker and provided adequate room for *H. azteca* to swim.

**Table 1.** Composition and Characteristics of Sediments.

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**Sample collection and Zn analysis**

*Water samples* – 2 ml samples of exposure water were collected at the initiation and conclusion of each test. Test initiation samples were collected while test suspensions were stirring prior to aliquoting into test beakers. Samples collected at the conclusion of the test represented a pooled sample from all replicates. Water was collected with a pipet after gentle stirring, pooled into a single beaker, and stirred for 5 min prior to collecting the 2 ml samples. To determine the amount of dissolution in the exposures, duplicate samples were collected. One sample “total Zn” was preserved upon collection with the addition of trace metal grade HNO₃, while the second sample, “dissolved Zn,” was centrifuged for 1.5-h at 21,330 x g. The top 1.8 ml was
removed from the centrifuged sample, placed in a metal-free cyrovial and preserved with nitric acid. Zinc concentrations in both “total Zn” and “dissolved Zn” water samples were determined by ICP-MS after open-vessel, microwave-assisted digestions using HNO$_3$ following US EPA methods 3005A$^{42}$ and 200.8.$^{43}$

Sediments – After *H. azteca* and baskets, if present, were removed from the beakers, sediment was collected using a 25 ml hand pipettor. Sediment was collected into a 15 ml metal-free centrifuge tube and stored at 4$^\circ$C until analysis. To determine total Zn concentrations in sediment, about 0.25 g of oven-dried sediment samples were weighed and digested using closed-vessel microwave with concentrated HNO$_3$ following US EPA method 3052.$^{49}$ The Zn concentrations in the digestates were determined by ICP-MS following EPA method 200.8.$^{43}$

**Bioaccumulation in *Hyalella azteca***

*H. azteca* were collected at the conclusion of each test for bioaccumulation measurements. All surviving individuals from each treatment were placed in a plastic weigh boat and washed three times with milli-Q water. Animals were then placed in a metal-free cyrovial and the tubes were dried at 60$^\circ$C overnight. Animals were not depurated in clean water prior to collection. Although this means that unabsorbed Zn in the gut of *H. azteca* was included in the total accumulated Zn, it provided a better prediction of the amount of Zn that could be transferred to higher trophic levels.

Only surviving *H. azteca* from the treatments were collected for total Zn analysis. For the majority of the samples, a total of 9-10 individuals were successfully collected from each replicate and were collectively weighed and analyzed for total Zn. At the highest concentrations tested in each treatment, mortality affected the number of individuals available for bioaccumulation analysis, and with some samples only containing 2-3 individuals. The *H. azteca* samples were oven-dried at 60$^\circ$C. The dried samples were weighed using an ultra-microbalance and transferred into a 1.5 ml fluoropolymer microcentrifuge tube. Ultra-pure HNO$_3$ (Aristar Ultra, VWR, Radnor, PA, USA) were added to each vial. The vial was loosely capped and heated on a hot-block at 100$^\circ$C for 2-h until a clear digest was obtained. The Zn concentration in the digestates was analyzed by ICP-MS as described above and normalized to the weight of the combined individuals in each replicate.

**Statistical analysis**
Toxicity data - Survival data were fit to non-linear regression models using the DRC package (3.0-1) in R (v. 3.4.0) to construct dose-response curves and calculate LC50 values. Log-logistic and Weibull models provided the best fit for the data based on comparison of the most commonly used models in DRC. Goodness of fit was confirmed for each model using "modelFit." LC50 values, confidence intervals, and standard error were calculated using the "ED" command.

LC50 values were compared across the different treatments using the LC50 ratio method described in Wheeler et al. The DRC command "comped" was used to determine if log transformed 95% confidence intervals for the LC50 ratio crossed 0. If the confidence intervals include 0, the null hypothesis is not rejected and the two LC50 values are equal. If the confidence intervals do not include 0, the LC50 are significantly different (p<0.05).

Bioaccumulation data were evaluated as means ± SD. Statistically significant differences among means were determined using one-way ANOVA followed by the Student-Newman-Keuls procedure being used for post hoc multiple comparisons.

Results:

Synthesis and Characterization of Transformed ZnO NPs

We obtained pure zincite-structured ZnO NP powder as confirmed by powder XRD (Fig. S1). The Zn content as measured by ICP-MS was 79.2% as compared to the theoretical percentage of 80.3%, indicating relatively high purity. The primary particle size of the as-synthesized ZnO NPs was 22.4 ± 2.8 nm (Fig. 2A). The p6-ZnO NPs were 44.5% Zn; the p8 ZnO NPs were 74.9% Zn; and the sZnO NPs were 59.7% Zn. The theoretical Zn percentages for Zn$_3$(PO$_4$)$_2$ and Zn$_3$(PO$_4$)$_2$·4H$_2$O and ZnS are 50.8, 48.5, and 67.1%, respectively. The Zn, O, P and S content of the particles was confirmed by EDS (Fig. S2). This demonstrates relatively complete conversion of Zn to a mixture of Zn$_3$(PO$_4$)$_2$ and Zn$_3$(PO$_4$)$_2$·4H$_2$O for the p6-ZnO NPs and a thin shell of amorphous Zn$_3$(PO$_4$)$_2$, for the p8 treatment, as we previously demonstrated using a combination of TEM, Zn K-edge X-ray absorption near edge structure spectroscopy (XANES) and $^{31}$P magic angle spinning nuclear magnetic resonance ($^{31}$P MAS-NMR). However, we found that in this case, there was less residual ZnO present in the p6-ZnO NPs product as compared to our previous work as demonstrated by the Zn content, XRD data, and TEM images (Fig. 2). The primary particle size of the ZnO starting material in our previous study was larger (34.2 ± 6.8 nm) than in this study (22.3 ± 2.8 nm). This is one possible explanation for the more complete conversion of Zn from the particles to Zn$_3$(PO$_4$)$_2$ in the p6 treatment. The solubility of particles generally increases with decreasing particle size, and this transformation involves
dissolution of ZnO and reprecipitation of $\text{Zn}_3(\text{PO}_4)_2$. The TEM data, Zn content, and XRD data demonstrate relatively complete conversion of ZnO to ZnS in the s-ZnO NP treatment (Fig. 2D). Their composition and morphology (clusters of ultra-fine ZnS NPs) are as described in Ma et al.¹²⁹

![Transmission electron micrographs of images of (a, ZnO NPs) and aged ZnO NPs (b, p6-ZnO NPs, c, p8-ZnO NPs, d, s-ZnO NPs). Scale bars represent 200 nm.](image)

**Figure 2.** Transmission electron micrographs of images of (a, ZnO NPs) and aged ZnO NPs (b, p6-ZnO NPs, c, p8-ZnO NPs, d, s-ZnO NPs). Scale bars represent 200 nm.

**Dissolution and sediment deposition**

Zn concentrations were determined in water and sediments following the 96-h exposures. The total and dissolved Zn measured in the water are reported in Table S1. Measured total Zn was close to nominal values in the freshwater and saltwater exposures (ranged between 75-112% in
FW; 72-104% in SW); however, in the presence of sediment, the total Zn in water ranged from 6-23% of nominal values. In the sediment exposures, almost 90% of the Zn was deposited on or sequestered to the sediments (Table S2). Zinc concentrations in exposures with or without baskets were similar in both the water and sediments, thus suggesting that the burrowing activity of the *H. azteca* did not impact Zn distribution. In addition, sediment absorption/deposition was similar across all ZnO NP exposures and ZnSO₄ exposures.

In the water only exposures, the as-synthesized ZnO NPs, p6-ZnO NPs, and p8-ZnO NPs underwent almost complete dissolution at concentrations below 1.0 mg/L over the 96-h exposure (Table S1, Fig. 3). In the sediment exposures, these NPs also showed a similar or even higher concentration of dissolved Zn compared to ZnSO₄; although, there is an overall decrease in the amount of dissolved Zn likely due to precipitation (which is expected at thermodynamic equilibrium at the pH values of the exposure media), or absorption onto suspended sediment particles. In contrast, the s-ZnO NPs had a much lower rate of dissolution (~20%) in all the exposures (Fig. 3).
Figure 3: Dissolution of ZnO NP expressed as the percent of dissolved Zn compared to total Zn measured at the conclusion of the 96-h exposures. (A) Freshwater, water-only treatment, (B) Salinity treatment with 3 ppt exposure media, (C) Sediment treatment containing clean sediment (D) Sediment-basket treatment where H. azteca were suspended in a nylon mesh basket above clean sediment. Concentrations are expressed as mg Zn L\(^{-1}\). Error bars represent standard deviation (n=4).

Toxicity test results
Non-linear regression analysis was used to construct concentration-response relationships for each treatment in the different exposure conditions. All exposure concentrations are expressed as mg Zn L\(^{-1}\). Overall, the shape of the concentration-response curves were similar for all treatments (Fig. 4); however, differences in the toxicity of ZnSO\(_4\) and the environmentally-transformed ZnO NPs were shown by the shift of the curves to the right or left in some exposure conditions. For example, in the freshwater exposures (Fig. 4A), the concentration-response curves for the ZnO NPs and both phosphate-transformed particles were shifted toward the left compared with ZnSO\(_4\), thus revealing that these NPs are more toxic than Zn\(^{2+}\) alone. The reduced toxicity of the s-ZnO NPs was also apparent by the right shift of the dose-response curves in the sediment exposures (Fig. 4C and D).
Figure 4: Concentration response curves illustrating the comparative toxicity of ZnSO₄ and each ZnO NP treatment under the four different exposure conditions. (A) Freshwater, water-only treatment, (B) Salinity treatment with 3 ppt exposure media, (C) Sediment treatment containing clean sediment, (D) Sediment-basket treatment where H. azteca were suspended in a nylon mesh basket above clean sediment. Dose response curves were fitted to survival data using non-linear regression models.

Concentration-response curves were also used to determine lethal median concentration (LC50) values for each treatment in all four exposure conditions. In the freshwater conditions, the phosphate particles exhibited the highest toxicity (Table 2) with LC50 values less than half the LC50 value for ZnSO₄. As seen previously,⁵ the as-synthesized ZnO NPs were also significantly more toxic than ZnSO₄ under these conditions. However, the differences between the treatments decreased in the other exposure conditions. In saltwater, only the ZnO NP and p6-ZnO NP were less toxic than ZnSO₄ and in both sediment exposures none of the particles
were more toxic than ZnSO$_4$. In contrast, s-ZnO NPs showed decreased toxicity in the presence of sediment with an LC50 two to four times higher than ZnSO$_4$ and the other particles.

The presence of sediment reduced the toxicity of all treatments including the ZnSO$_4$ and the NP exposures. Because the reduction of toxicity was seen in both sediment treatments, the sediments likely acted as a sink for the toxicants, possibly sequestering Zn$^{2+}$ from the exposure media or heteroaggregating with the particles. All of the treatments showed increased toxicity when *H. azteca* were placed in a basket above the sediment compared with the sediment only exposures. This implies that the *H. azteca* were not exposed to the NPs as they scavenge on the sediment surface. Instead, as the *H. azteca* burrowed in the sediments, the sediments acted as a refuge, limiting the exposure of the *H. azteca* to Zn$^{2+}$ or the NPs.

<table>
<thead>
<tr>
<th></th>
<th>Freshwater</th>
<th>Saltwater (3 ppt)</th>
<th>Sediment</th>
<th>Sediment- basket</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnSO$_4$</td>
<td>0.26 (0.22-0.30)$^a$</td>
<td>0.66 (0.45-0.86)$^a$</td>
<td>2.35 (1.56-3.14)$^a$</td>
<td>0.94 (0.80-1.01)$^a$</td>
</tr>
<tr>
<td>ZnO NP</td>
<td>0.18 (0.14-0.22)$^b$</td>
<td>0.23 (0.16-0.30)$^b$</td>
<td>1.55 (1.26-1.84)$^a$</td>
<td>0.96 (0.74-1.19)$^a$</td>
</tr>
<tr>
<td>p6-ZnO NP</td>
<td>0.13 (0.10-0.15)$^c$</td>
<td>0.30 (0.24-0.35)$^b$</td>
<td>2.58 (0.60-4.57)$^a$</td>
<td>0.75 (0.53-0.96)$^a$</td>
</tr>
<tr>
<td>p8-ZnO NP</td>
<td>0.11 (0.10-0.13)$^c$</td>
<td>0.36 (0.00-1.15)$^{a,b}$</td>
<td>1.57 (1.23-1.90)$^a$</td>
<td>0.96 (0.50-1.41)$^a$</td>
</tr>
<tr>
<td>s-ZnO NP</td>
<td>0.29 (0.26-0.32)$^a$</td>
<td>0.48 (0.43-0.52)$^a$</td>
<td>7.12 (5.48-8.76)$^b$</td>
<td>2.27 (1.98-2.57)$^b$</td>
</tr>
</tbody>
</table>

Table 2: Comparative toxicity of ZnSO$_4$ and ZnO NPs under different exposure conditions. The lethal median concentration (LC50) values in mg Zn/L are shown with 95% confidence intervals for ZnSO$_4$ and each nanoparticle under four different exposure conditions. Sediment exposures were conducted in freshwater allowing *H. azteca* to freely burrow in sediment (sediment) or placed in a mesh basket above the sediment preventing them from burrowing (sediment-basket). For each condition, different superscript letters represent statistically different LC$_{50}$ values based on the LC$_{50}$ ratio method described by Wheeler et al. (2006). Numbers in bold are used to highlight differences from the ZnSO$_4$ treatment.

Bioaccumulation

Following the 96-h exposures, total Zn was measured in the surviving *H. azteca* to compare the amount of bioaccumulation across ZnO NP exposures and among the different treatments. Zn concentrations were also measured in *H. azteca* prior to test initiation to determine background levels of this essential metal. Average Zn concentrations in control *H. azteca* following the 96-h exposures ranged from 60-62 mg Zn kg$^{-1}$ dry mass, which were not different from initial values (59 + 10 mg Zn kg$^{-1}$ dry mass). This indicates that *H. azteca* did not accumulate additional Zn from the untreated media or sediments during the exposure in the control treatment. However, in all the ZnSO$_4$ and ZnO NP exposures, Zn accumulated in *H. azteca* to concentrations that were significantly higher than initial values. These concentrations also increased relative to the
increasing concentrations of Zn in the exposures (Fig. 5). In general, there was little difference in the amount of Zn accumulated in *H. azteca* between the different ZnSO₄ and ZnO NP exposures at each concentration. The one exception was the much higher bioaccumulation seen for the s-ZnO NPs in the saltwater treatment at all tested concentrations (Fig. 5B).

Bioaccumulation occurred to a greater extent in the water only exposures compared with the sediment exposures (Fig. 5). Interestingly, in the sediment exposures, *H. azteca* which were held in a basket and unable to borrow in the sediment accumulated more Zn than those able to scavenge and burrow in the sediment despite the higher Zn concentration in the sediment compared to the water.

![](image)

**Figure 5: Bioaccumulation of Zn measured in *H. azteca* following 96-h exposures to ZnSO₄ or environmental transformed ZnO NPs.** (A) Freshwater, water-only treatment, (B) Salinity treatment with 3 ppt exposure media, (C) Sediment treatment containing clean sediment (D) Sediment-basket treatment where *H. azteca* were suspended in a nylon mesh basket above clean sediment. Error bars represent standard deviation (n=4). Missing bars in some treatments (i.e. saltwater 1.0 mg L⁻¹, sediment-basket, 3.0 mg L⁻¹) are because there was no survival in the exposure at that concentration.

**Comparison of bioaccumulation and toxicity**
Whole-body Zn concentrations partially explained the differences in toxicity seen in each of the treatments. Across all the treatments, we generally observed high mortality (> 40%) when whole-body Zn concentrations reached 200-500 mg Zn kg\(^{-1}\) dry mass. Bioaccumulation occurred to a greater extent in the freshwater treatment where toxicity was greatest. In addition, the sediment-basket treatment caused a higher level of Zn bioaccumulation than in the sediment treatment, which also matched the trend in toxicity. Although the bioaccumulation data support the differences in toxicity across the freshwater, saltwater, and sediment treatments, it is not able to explain particle specific differences in toxicity. In the freshwater exposure where we see the greatest difference in toxicity to the different particles (Table 2), bioaccumulation does not show a similar trend (Fig. 6). For example, the phosphate-aged particles (p6-ZnO NPs and p8-ZnO NPs) are more toxic than ZnSO\(_4\) but these particles are not accumulated to a greater extent. In all the treatments, the toxicity of the s-ZnO NPs was 2-5 times lower than the other NPs (Table 2), but in many of the treatments, the Zn bioaccumulation is similar to the other particles and in the saltwater treatment, the s-ZnO NP exposure results in the highest bioaccumulation (Fig. 6).

**Figure 6: Relative toxicity and bioaccumulation of Zn ions and ZnO NPs in different exposure scenarios.** (A) Freshwater, water-only treatment, (B) Salinity treatment with 3 ppt exposure media, (C) Sediment treatment containing clean sediment (D) Sediment-basket treatment where *H. azteca* were
suspended in a nylon mesh basket above clean sediment. In each figure, bioaccumulation increases toward the top of the figure and toxicity increases toward the right. Toxicity and bioaccumulation were summarized from Table 2 and Figure 5 and are shown relative to the different treatments (Zn ions or the different ZnO NPs). Treatments that group together had similar bioaccumulation and toxicity in the given exposure, while treatments that are separated had a distinct bioaccumulation and/or toxicity pattern. Direction of arrows illustrates the difference in toxicity and bioaccumulation of the majority of the particles in comparison with the Zn ion. Circles represent similarities in toxicity and bioaccumulation between NPs and the Zn ions. In each figure, s-ZnO NPs have a distinct pattern in comparison with the other particles.

Discussion:
Recent studies have shown that ENMs undergo complex environmental transformations after entering aquatic systems,\textsuperscript{8,18} emphasizing the importance of studying environmentally-realistic exposures to transformed NPs. In the present study, we reconfirmed that \textit{H. azteca} is more sensitive to ZnO NPs compared to Zn\textsuperscript{2+} in water only exposures, and that under some conditions, environmentally-transformed particles are more toxic. However, in the presence of sediment, there are no differences and only one type of transformed ZnO NP, the s-ZnO NP, is less toxic than Zn\textsuperscript{2+}. ZnO NPs were less toxic in saltwater and sediment exposures, suggesting that water-only exposures overestimate the risk to \textit{H. azteca}. The differences across the exposure conditions were well explained by the decrease in Zn bioaccumulation; however, bioaccumulation did not explain the differences in the toxicity across the ZnO NPs.

\textit{Environmental transformations of ZnO NPs altered toxicity}

Despite large differences in solubility constants for the phosphate-transformed ZnO NPs, we observed similar levels of dissolution compared with pristine particles. In addition, particle-specific differences in toxicity (e.g., LC\textsubscript{50} values) could not be attributed to the dissolution or bioaccumulation data, which was measured at the end of the 96-h exposures. Transformation of ZnO NPs in the presence of phosphate reduces their solubility;\textsuperscript{52} however, the structure and composition of the transformation products is strongly dependent upon the pH at which the reaction takes place.\textsuperscript{23} We used phosphate-transformed ZnO NPs following the methods of Ranthnayake et al. at pH 6 and pH 8.\textsuperscript{23} At pH 6, the ZnO NPs were transformed into larger sheets consisting primarily of hopeite-like Zn\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} and other Zn\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} phases. In the pH 8 treatment, particles had a ZnO-Zn\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} core-shell structure, where the shell consists of amorphous Zn\textsubscript{3}(PO\textsubscript{4})\textsubscript{2}.\textsuperscript{23} Because Zn\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} has a very low solubility, we predicted low dissolution of p6-ZnO NPs and p8-ZnO NPs. However, we observed similar dissolution in the exposures to the as-synthesized ZnO NPs particles (Fig. 3), and toxicity of the p6-ZnO NPs and p8-ZnO NPs were similar or greater than the toxicity of the pristine ZnO NPs (Table 2, Fig. 6). The similarity in dissolution may be reflective of the low exposure concentrations in the study.
Specifically, given a Ksp value of $9 \times 10^{-33}$ for $\text{Zn}_3(\text{PO}_4)_2$ in DI water, the dissolved Zn concentration at saturation would be 0.030 mg L$^{-1}$. This suggests that differences in dissolution kinetics, which were not reflected in the present study, may be important. For example, if dissolution is more rapid, the area under the concentration versus time curve is greater than if it is slower, resulting in a greater time-integrated exposure to dissolved ions.

In contrast, sulfidized particles (s-ZnO NPs) had lower dissolution and lower toxicity compared with the ZnO NPs and pZnO NPs. Lower dissolution is expected. Given a Ksp value of $3 \times 10^{-23}$ for ZnS in DI water, the dissolved Zn concentration at saturation would be $1 \times 10^{-9}$ mg L$^{-1}$. The s-ZnO NPs used in this study were produced following the methods of Ma et al. by aging ZnO NPs in the presence of sulfide.$^{29}$ These particles had a structure consisting of clusters of ultra-fine ZnS. They were smaller than ZnO NPs (2.5-5 nm), had reduced dissolution, and increased aggregation compared to pristine particles.$^{29}$ These expectations were met in our s-ZnO NPs exposures. Zn dissolution was < 20% in almost all the s-ZnO NPs exposures and they exhibited reduced toxicity compared with the other NPs.

While transformation processes are important for environmental ENM exposures, there are still relatively few studies that have investigated the toxicity of environmentally-transformed NMs. One area of focus has been on how wastewater treatment plants (WWTPs) influence chemical and physical transformations of NPs,$^{53}$ and the resulting toxicity of effluents and biosolids containing transformed NPs. Chronic toxicity and metal uptake was investigated in the legume *Medicago truncatula* when grown on biosolids amended with ENMs. They found that biosolids amended with ZnO NPs resulted in higher Zn accumulation in plants and increased chronic toxicity, although the speciation of Zn in the biosolids was similar for NP-amended and bulk/dissolved metal-amended biosolids.$^{24}$ Several additional studies have investigated the toxicity of sulfitidized Ag NPs, which are the major Ag end product from chemical transformations in WWTPs.$^{53}$ Sulfitidation of AgNPs has been shown to reduce its toxicity to a range of aquatic and terrestial species including fish,$^{25, 54}$ aquatic plants,$^{25}$ and the nematode *Caenorhabditis elegans*.$^{25, 55}$ Reductions in toxicity are correlated with reduced dissolution of Ag$^{+}$ and lower bioaccumulation.$^{25, 55}$ However, other studies in terrestrial plants and arthropods have emphasized that sulfitidized Ag NPs are still bioavailable and have been shown to accumulate in these species.$^{56, 57}$ These results are similar to our findings for s-ZnO NPs. Sulfitidation of the ZnO NPs decreased dissolution and toxicity, but still resulted in a similar level of bioaccumulation to the other ZnO NPs in most treatments. Therefore, there a potential for s-ZnO NPs to be transferred to higher trophic levels, and it is not clear what form of Zn the predators that consume *H. azteca* would be exposed to.
Given the relatively low solubility of ZnS particles, it is likely that most effects were caused by particle-specific mechanisms. Similarly, the greater toxicity of the particles compared with equivalent concentrations of dissolved Zn, also suggest particle-specific mechanisms. There are possible effects of particles caused by damage to external body surfaces that are not related to dissolution and uptake of ions. For example, toxicity to the gut epithelium as seen with C. elegans exposed CeO NPs may alter digestive processes. Similarly, Starnes et al. demonstrated that Ag₂S nanoparticles damage the C. elegans cuticle and cause toxicity in the absence of uptake of Ag into the internal tissues. Particles which are internalized and remain intact can also cause effects such as unfolded protein responses. Finally, phototoxicity is a possibility for ZnO NPs.

**Salinity and sediment reduces the toxicity of ZnO NPs**

The ionic strength of natural waters is known to impact the stability of NPs causing greater aggregation and sedimentation of particles. ZnO NPs were shown to undergo aggregation and rapid sedimentation at NaCl concentrations above 15 meq L⁻¹ (or 0.9 ppt). In the present study, particle concentrations were far too low to measure aggregation by dynamic light scattering. Although it is predicted that increased particle size and sedimentation will decrease ZnO NP bioavailability, because H. azteca are epibenthic species, we hypothesized that sedimentation would increase their level of exposure and possibly the toxicity of the particles. However, the toxicity of Zn²⁺ decreases in seawater or brackish water given greater competition of cations such as Ca²⁺ for sites of uptake as well as greater concentrations of ligands which bind Zn²⁺. As expected, the toxicity of ZnSO₄ was reduced 2-fold in the saltwater exposures. Most of the ZnO NPs particles also exhibited reduced toxicity. Because salinity did not increase the toxicity of the ZnO NPs, it appears that increased sedimentation does not pose a risk for H. azteca and that the larger aggregate particle size may reduce absorption and bioavailability. This was reflected in the reduced bioaccumulation in the saltwater treatment compared to the freshwater. However, dissolution of the ZnO NPs was similar in the saltwater exposures and the freshwater exposures, suggesting that the main impact in the salinity treatment was the presence of additional cations that compete for uptake.

The presence of sediment reduced concentrations of Zn in the water column across all the exposures, which resulted in decreased toxicity in both the sediment-basket and sediment only exposures. Experiments by Li et al. demonstrated that ZnO NPs bind to sediment particles and show low mobility through sand packed columns. Phosphate increased the mobility of Zn suggesting that phosphate transformed ZnO NPs may have a lower affinity for
sediment. Despite this prediction, the total Zn measured in the water column was similar across the exposures and represented approximately 10% of the nominal Zn added. The remaining 90% of Zn was accounted for in the sediment measurements. The Zn partitioning was similar, but slightly lower, to a previous study that found 2% Zn in the water column and 97% in the sediments. The increased partitioning to the sediments was likely due to the high salinity of their exposure media.\textsuperscript{31} In the sediment-basket treatment, the toxicity across all treatments was reduced about 10-fold in comparison to the freshwater only treatment, which correlates with the 10-fold reduction in total Zn found in the water column. Toxicity was further reduced in the sediment only treatment where \textit{H. azteca} were able to scavenge on the sediment surface and burrow. Instead of increasing their exposure and toxicity, the presence of sediment appeared to act as refuge enabling the amphipods to escape from the exposure as shown in the reduced bioaccumulation in this treatment (\textbf{Fig. 5}). Overall, these results strongly suggest that \textit{H. azteca} are exposed to ZnO NPs through the water column and not by scavenging on or within the sediment.

Previous studies investigating the toxicity of ZnO NPs to sediment-dwelling organisms focused on bioaccumulation in clams, polychaetes, and crustaceans. Two studies in marine systems used isotopically-labeled Zn to investigate partitioning and bioaccumulation in marine organisms. Both studies revealed rapid sedimentation of ZnO NPs with increased concentrations found in the top layers of the sediment.\textsuperscript{30, 31} Uptake of isotopically labeled Zn was seen in the clam \textit{Scrobicularia plana} and ragworm \textit{Nereis diversicolor} exposed to ZnO NPs and was correlated with chronic toxicity (increased GST activity, reduced feeding); however, this level of Zn was only about 10% of the Zn that accumulated in these species from their natural environment.\textsuperscript{30} Considering the low concentration used in their study (similar to the 0.3 mg L\textsuperscript{-1} exposure in our study), the levels of bioaccumulation were similar. Larner et al. used isotopically labeled \textsuperscript{68}ZnO NPs to trace their distribution in a saltwater-sediment exposure. Amphipods accumulated more \textsuperscript{68}Zn in the ZnO NP exposures than in bulk ZnO exposures, but bioaccumulation was similar to the ZnCl\textsubscript{2} exposure.\textsuperscript{31} Two additional studies investigated the toxicity of sediment exposures to crustaceans. Hanna et al. showed that dissolved Zn\textsuperscript{2+} in pore water released from ZnO NP-amended sediment was sufficient to cause the observed toxicity to the marine amphipod \textit{Leptocheirus plumulosus},\textsuperscript{32} suggesting that similar to our study, Zn uptake is through the water column or pore water. Josko et al. exposed \textit{Heterocypris incongruens} to ZnO NPs in two sediments with different amounts of organic carbon content. The sediment with lower organic content (i.e., sandier) had higher bioavailable Zn and caused increased mortality.
The authors suggested that organic matter causes immobilization of the metals in the sediments and makes them less bioavailable.62

Toxicity data suggest complex route of uptake

For metals, *H. azteca* primarily respond to the concentrations of metals found in the overlaying waters, and not metals bound to sediments.63 Therefore, for Zn$^{2+}$, we expected that bioaccumulation and toxicity would be strongly correlated with water column concentrations of Zn. However, for metal oxide NPs, the route of exposure is less clear. *H. azteca* are found primarily on the sediment surface preferring epiphytic algae as a primary food source.63 If sedimentation of NPs occurs, we would expect *H. azteca* to ingest these particles as they scavenge on the sediment surface. Cross et al.18 also explains that in ponds and slow moving rivers, NP deposition will lead to high concentrations in the “benthic boundary layer,” or the top layer of sediment that is resuspended and mixed through bioturbation and may result in a very high level of exposure for epibenthic organisms like *H. azteca*. Dietary exposure has also been shown to be a significant source of NP exposure for sediment dwelling organisms. For example, the freshwater snail *L. stagnalis* was able to assimilate over 85% of ZnO delivered through a diatom food source.64 Many benthic and epibenthic organisms feed on the biofilm that develops on surficial sediment,65 which retains ZnO NPs and aids in the accumulation and transfer of ZnO NPs to predators.66 When taken together, these studies suggest that the higher toxicity of the ZnO NPs compared to Zn$^{2+}$ could be due to sedimentation and higher exposure at the sediment surface. However, results from our present study contradict this prediction.

Several lines of evidence strongly suggest that *H. azteca* are accumulating Zn through the water column. First, in the saltwater treatment, we expected an increase in the sedimentation of ZnO NPs due to the higher ionic strength. In this treatment, we observed a decrease in toxicity, suggesting that the sedimentation and removal of NPs from the water column was related to decreased toxicity. Secondly, the 5-10-fold reduction in toxicity in the sediment with basket treatment compared to freshwater treatment corresponds well to the reduction of Zn in the water column to about 10% nominal in the sediment treatments. Finally, the sediment with basket treatments resulted in higher toxicity to ZnSO$_4$ and all ZnO NP exposures when compared to the sediment only treatment. These organisms were not exposed to the settling NPs, but only to those suspended or dissolved in the water column.

Many studies have suggested that ZnO NP toxicity is governed primarily through dissolution and subsequent exposure to Zn$^{2+}$.39 For example, ZnO NP toxicity was primarily related to Zn$^{2+}$ dissolution in and early study with the green algae *Pseudokirchneriella*
Several studies in aquatic crustaceans supported this mechanism of toxicity, but it was not found to be universal and often depended on the specific particles investigated and the environmental parameters of the exposure media.\textsuperscript{3}

Since dissolution was high in all the ZnO NP exposures except for the s-ZnO NPs which exhibited reduced toxicity, our results suggested that dissolution was playing a large role in NP toxicity. However, this could not explain the greater toxicity seen in the ZnO NP exposures compared to ZnSO$_4$ in the water only exposures. In addition, dissolution was a poor predictor of bioaccumulation, which is most apparent when comparing the ZnSO$_4$ and s-ZnO NPs. In most treatments, the bioaccumulation of the s-ZnO NPs was equivalent or greater than in the ZnSO$_4$ exposures despite the low level of s-ZnO NP dissolution. In summary, although the water column is the primary route of exposure for the ZnO NPs, dissolution does not account for the differences in toxicity. Other factors must be involved that govern the differential toxicity. Because all the measurements in the present study were taken after 96 hours, the differences in dissolution and bioaccumulation may occur at early time points, and these differences could drive the differential toxicity of the ZnO NPs. It is also possible that this is coupled with particle-specific modes of toxicity. Other studies have shown particle-specific effects, even when high levels of dissolution have been observed. For example, Lopes et al. found chronic effects to feeding and growth in NP exposed \textit{Daphnia magna} not observed in the Zn$^{2+}$ treatments despite high levels of dissolution.\textsuperscript{69} Given the high sensitivity of \textit{H. azteca}, understanding the factors that contribute to ZnO NP toxicity in this species will enable us to predict which aquatic organisms are most susceptible to ZnO NPs and likely other ENMs as well.

Conclusions:

Once NPs enter the aquatic environment, they undergo physical and chemical transformations that alter their toxicity. ZnO NPs can react with phosphate and sulfide within wastewater treatment facilities or in aquatic environments that are enriched for phosphate. This study investigated whether transformation processes alter the toxicity of ZnO NPs to one of the most sensitive aquatic species, the epibenthic crustacean \textit{H. azteca}, under different environmental exposure scenarios. Phosphate-transformed particles were at least as toxic as pristine particles and Zn ions, and under some conditions they caused enhanced toxicity, while sulfide-transformed particles were generally less toxic. All ZnO NPs were taken up by \textit{H. azteca} and bioaccumulated in this important prey species, suggesting that \textit{H. azteca} may act as vector for trophic transfer to fish.
Toxicity results from the different exposure scenarios provided new insight into the route of exposure and potential uptake mechanisms for the transformed ZnO NPs. Sediment exposures reduced the toxicity and bioaccumulation of all ZnO NPs and Zn\textsuperscript{2+}, consistent with the overlying water being the primary route of exposure for NPs. However, different levels of dissolution and bioaccumulation could not fully explain the differences in toxicity across the particles. For example, the sulfidized ZnO NPs showed the least toxicity across all exposure scenarios, but had similar or higher levels of bioaccumulation. Phosphate-transformed particles showed high levels of dissolution, but were twice as toxic as Zn ions in the water-only exposures. These results suggest that other mechanisms are responsible for governing toxicity, possibility related to differences in uptake kinetics and internal absorption. For example, we could propose that ZnO NPs provide a mechanism of enhanced uptake and accumulation within the gut of \textit{H. azteca}, similar to a "Trojan-horse"-like mechanism.\textsuperscript{70} Differences in the internal solubility of the particles and absorption into tissues may then govern particle specific toxicity.

Uptake kinetics studies may shed light on these proposed mechanisms explaining the differential toxicity of the NPs. Recent studies have utilized stable isotope methods to track the bioaccumulation and elimination of ZnO NPs over time allowing for calculations of uptake and elimination rate constants.\textsuperscript{15, 31, 64} Similar studies in \textit{H. azteca} would allow us to understand differences in the rate of uptake of the different particles as well as how much of the particles are actually absorbed by the organisms. The sediment community is expected to experience some of the highest levels of NP exposure; therefore, understanding the relative toxicity of environmentally realistic NP exposures to this community is critical for NP risk assessment. Our study suggests that the processes governing NP uptake and absorption maybe key for predicting risks to the sediment community, and studies focusing on the relevant risk of environmentally-transformed particles will provide increased realism to our risk assessments.

\textbf{Conflict of Interest Statement:}
The author declare no conflicts of interest.

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TOC entry:

Transformations of ZnO NPs under different environmental conditions alters their toxicity to sediment-dwelling crustaceans