Impact of Engineered Zinc Oxide Nanoparticles on the Individual Performance of *Mytilus galloprovincialis*

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**Abstract**

The increased use of engineered nanoparticles (ENPs) in consumer products raises the concern of environmental release and subsequent impacts in natural communities. We tested for physiological and demographic impacts of ZnO, a prevalent metal oxide ENP, on the mussel *Mytilus galloprovincialis*. We exposed mussels of two size classes, 0.1–2 mg l⁻¹ ZnO ENPs in seawater for 12 wk, and measured the effect on mussel respiration, accumulation of Zn, growth, and survival. After 12 wk of exposure to ZnO ENPs, respiration rates of mussels increased with ZnO concentration. Mussels had up to three fold more Zn in tissues than control groups after 12 wk of exposure, but patterns of Zn accumulation varied with mussel size and Zn concentrations. Small mussels accumulated Zn 10 times faster than large mussels at 0.5 mg l⁻¹, while large mussels accumulated Zn four times faster than small mussels at 2 mg l⁻¹. Mussels exposed to 2 mg l⁻¹ ZnO grew 40% less than mussels in our control group for both size classes. Survival significantly decreased only in groups exposed to the highest ZnO concentration (2 mg l⁻¹) and was lower for small mussels than large. Our results indicate that ZnO ENPs are toxic to mussels but at levels unlikely to be reached in natural marine waters.


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**Introduction**

Increasing production of engineered nanoparticles (ENPs) for use in a variety of applications has raised concern about their potential ecological impacts [1,2]. The increased reactivity and other special properties associated with the small size of ENPs make them ideal for application in a variety of consumer products, such as cosmetics, electronics, clothing, and tires, and as manufacturing aids in the form of catalysts, conductors, and semi-conductors [1,3]. As a result, the rate at which ENPs are being released into the environment is increasing [4]. Yet there is a growing body of evidence demonstrating that ENPs are toxic and may pose ecological risks. For example, metal and metal oxide ENPs reduce the growth rates of bacteria [5], freshwater algae [6], and marine phytoplankton [7,8], and survival of fish [9,10] and crustaceans [11]. While these studies show that ENPs are potentially hazardous, many of them used short-term laboratory exposure tests with relatively short-lived species, which hinders the use of the results in making ecologically relevant predictions. A compounding problem is our inability to measure accurately the typically low ENP concentrations in the environment. Despite those uncertainties, environmental loading models [12] suggest that most ENPs are not substantial environmental risk factors at present.

An important role of ecotoxicology is to assess the hazard potential of anthropogenic contaminants before they become real environmental problems. One way forward is to test the influence of ENPs on ecologically-important organisms, such as keystone or ecological engineer species [13] in life-cycle experiments. However, many such species are long-lived, so it is only feasible to determine the impact of ENPs on the performance of individuals during relatively small portions of their life histories, and then use the information obtained with those studies to model the potential impact over entire life spans of the animals, with the caveat that different life stages may have different susceptibility and therefore responses to the same type and level of contaminant [14]. To better understand the potential impacts of ENPs on populations of long-lived organisms, we can couple laboratory-based studies with modeling, thereby allowing us to simulate the effects of ENPs on populations based on the results of individuals.

Here we test whether ZnO ENPs impact the physiological performance and survival of the marine mussel, *Mytilus galloprovincialis*, a coastal marine ecosystem engineer that helps maintain water quality through filter-feeding, builds biogenic reefs that support biodiversity, and is an important prey species to many coastal marine predators [15,16]. ZnO ENPs are present in a number of cosmetics, sunscreens, and coatings [1,17] and therefore, may potentially be released into marine environments...
in biologically meaningful quantities. While the effects of ZnO ENPs on mussels are unknown, Zn has been shown to decrease enzyme activity in mussel tissue [18,19] and increase metabolism during recovery in clean water [20]. Mussels accumulate Zn when exposed to particulate or dissolved forms [21], and concentrate Zn especially in cells along the gut and in the mantle and gills [22], with smaller animals having higher body burdens of Zn compared to larger ones [23]. Zn tissue concentration is negatively correlated with growth rate in mussels [24] and survival decreases with increasing environmental Zn concentrations [25]. Based on what is known about Zn toxicity to mussels, we test the hypotheses that mussels exposed to ZnO ENPs will 1) increase respiration rate after exposure, 2) accumulate Zn throughout the experiment, 3) decrease growth and survival, and 4) show size dependence during the experiment, with smaller mussels having more pronounced effects than larger mussels. We fit descriptive regression models that describe the size and dose dependence of the various physiological and demographic rates.

**Methods**

**Mussels**

*Motilus galloprovincialis* with shell lengths of approximately 2–6 cm were obtained from Taylor Shellfish Farms (Shelton, WA, USA). Mussels were housed in flowing, sand-filtered seawater for 1 wk prior to the start of experiments. A total of 4,400 mussels were used in ZnO ENP exposures. We separated mussels into small (<4.5 cm total shell length) and large (≥4.5 cm total shell length) size classes and divided them into 40 aerated polystyrene tanks, each containing 5 l of sand-filtered seawater. Tanks of small mussels contained 120 individuals while tanks of large mussels contained 100 individuals. Mussels were allowed to acclimate to their tanks for 3 days prior to experiments. We exposed small and large mussels to ZnO ENPs at 0.1, 0.5, 1, and 2 mg l⁻¹ for 12 wk by adding ZnO ENPs along with feed to tanks 5 d each wk during recovery in clean water [20]. Mussels accumulate Zn when exposed to particulate or dissolved forms [21], and concentrate Zn especially in cells along the gut and in the mantle and gills [22], with smaller animals having higher body burdens of Zn compared to larger ones [23]. Zn tissue concentration is negatively correlated with growth rate in mussels [24] and survival decreases with increasing environmental Zn concentrations [25]. Based on what is known about Zn toxicity to mussels, we test the hypotheses that mussels exposed to ZnO ENPs will 1) increase respiration rate after exposure, 2) accumulate Zn throughout the experiment, 3) decrease growth and survival, and 4) show size dependence during the experiment, with smaller mussels having more pronounced effects than larger mussels. We fit descriptive regression models that describe the size and dose dependence of the various physiological and demographic rates.

**ENPs and Suspension Preparation**

ZnO ENPs were obtained from Meliorum Technologies (Rochester, NY, USA) and characterized by the University of California Center for the Environmental Implications of Nanotechnology (UC CEIN) as spheroid, 100% zincite and 20–30 nm in diameter [26,27]. We prepared stock suspensions for experiments by adding ZnO ENPs to purified water (Barnstead Nanopure, Thermo Fisher Scientific, Waltham, MA, USA, resistivity >18 MΩcm) to make a 1 g l⁻¹ suspension and sonicating in a bath sonicator for 10 min. We diluted this suspension in filtered seawater (0.45 μm) containing 10 mg l⁻¹ algae to prepare a 100 mg l⁻¹ suspension of ZnO ENPs. We then sonicated this suspension for 10 min, added commercially prepared phytoplankton (Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA), which includes *Isochrysis* sp., *Pavlova* sp., *Thalassiosira weissflogii*, and *Tetraselmis* sp., and used this mixture in exposure treatments. Enough phytoplankton was added to attain a final cell count of 2×10⁶ cells ml⁻¹ in each tank.

**Respiration measurements**

We determined respiration rates of mussels after 12 wk of exposure (i.e., at the end of the experiment) for 12 mussels from each concentration and size class. Individual mussels were placed into a respirometer containing clean, sand-filtered seawater, a water pump to ensure mixing, and an optical oxygen probe with built-in datalogger (D-Opto, Envco, NZ). The respirometer was submerged in flowing seawater to maintain temperature, sealed, and the oxygen concentration in the chamber was measured once per minute for 1 h. We then removed mussels from the chamber and measured oxygen concentration for 1 h more to account for any other biological activity in the chamber. We measured mussel length and dry weight of tissue after dissection from the shell. These mussels were then analyzed for Zn content as described below.

**Bioaccumulation of zinc**

We removed five mussels from each tank every 2 wk throughout the study and froze them for Zn analysis. Frozen mussel samples were thawed, dissected and separated into somatic tissue and gonad. Samples were dried at 60°C for 72 h. Samples were weighed to the nearest 0.1 mg, digested in concentrated trace-metal-free HNO₃ (Sigma-Aldrich Co., St. Louis, MO, USA) for 2 h at 90°C in capped glass vials and diluted to 10% acid using purified water. We then determined Zn content of tissue and gonad samples using inductively coupled plasma atomic emission spectroscopy (ICP-ES, Thermo ICAP 6300, Thermo Fisher Scientific). Blank and standard solutions were run every 10 samples.

**Growth and survival**

We determined individual mussel growth by marking 20 individuals in each tank and measuring the total length (TL) of the mussels’ shell over the course of the study. The outer shell of each mussel was roughened with sandpaper and numbered with white paint. We measured TL of labeled mussels using digital calipers (±0.01 mm), along the longest axis of the mussel. TL was measured prior to exposure, after 6 wk, and after 12 wk of exposure. Growth rates were calculated for individual mussels and averaged among replicates. We counted mussels in each tank after 6 and 12 wk of exposure and corrected for sampling to determine survival.

**Statistical Analysis**

We tested whether mussel respiration rate, Zn somatic tissue concentration, Zn gonad tissue concentration, rate of Zn accumulation in somatic tissue, and growth rate varied as a function of ZnO ENP concentration using multiple ordinary least squares (OLS) regression models. Mussel size (small or large), ZnO ENP concentration, and the interaction of these terms were factors in the analysis for all models except Zn concentration in gonad, as gonads were only analyzed from large mussels. We predicted that increased exposure to ZnO ENPs would increase respiration rate post exposure, increase Zn loading in tissues as well as the rate at which Zn was accumulated, and decrease growth of mussels throughout the experiment. We also predicted that larger mussels would be able to tolerate higher concentrations of ZnO ENPs than smaller mussels, thus reducing the impacts of these ENPs on physiological rates. To test these predictions we constructed multiple regression models for each physiological parameter as follows:

\[ Y = \beta_0 + \beta_1 \text{Conc} + \beta_2 \text{Size} + \beta_3 \text{Conc} \times \text{Size} + \epsilon \]

where \( Y \) is the physiological parameter of interest (respiration rate, Zn tissue concentration, rate of Zn accumulation, or growth), \( \beta \) is the physiological parameter value of the control group of large
mussels, *Conch* is the ZnO ENP concentration, *Size* indicates whether the mussel was small or large, and *ε* is the error not explained by the model. The coefficients, *β*, are estimates of the impact of the independent variable on the dependent *Y*. We removed the interaction term if it was not statistically significant and ran the model. When the interaction term was statistically significant, we ran separate linear regression models for small and large mussels to determine the relationship between the physiological parameter and nominal ZnO ENP concentrations. We square-root transformed ZnO ENP concentration for the respiration rate model because the response variable, respiration rate, was a non-linear function of ZnO ENP concentration. We also compared the tissue dry weight to shell TL ratio from mussels at the beginning of the experiment with that of mussels at the end of the experiment to determine if mussels gained or lost weight during the study. This analysis was conducted using OLS regression similar to the above, with the tissue dry weight to shell TL ratio as the dependent variable and shell TL and time (beginning or end of the experiment) as independent variables. For all regression models reported herein, residual and quantile-quantile plots were examined to ensure homogeneity of variance and normal distribution of data.

We tested whether survival varied as a function of ZnO ENP concentration, mussel size, or their interaction using a two-way ANOVA with mussel size and ZnO ENP concentration as fixed factors. We predicted that survival would decrease with increasing ZnO ENP concentration but that this relationship would be different for small and large mussels causing a significant size x ZnO ENP interaction. We used Tukey’s HSD test post-hoc to determine which groups significantly differed and Levene’s test to ensure homogeneity of variance between groups. R statistical software (The R Foundation for Statistical Computing, version 2.10.1) was used for all analyses.

**Results**

**Respiration**

Volume specific respiration rate of mussels was consistently higher for small mussels than large mussels (Figure 1). Respiration rate of mussels significantly varied as a function of ZnO ENP concentration and mussel size (Table 1, OLS: *r*² = 0.24, *p* < 0.0001). Respiration rate of mussels generally increased with increasing ZnO ENP concentration (OLS: *t*₂, 113 = 2.27; *p* < 0.05) and was significantly higher for small mussels than large (OLS: *t*₂, 115 = 5.67, *p* < 0.0001).

**Bioaccumulation**

Mussels exposed to ZnO ENPs accumulated Zn in both somatic and reproductive tissues (Figure 2). Zn concentration in somatic tissue increased as a function of ZnO ENP concentration but not mussel size (Table 1, OLS: *r*² = 0.58; *p* < 0.0001). Mussels exposed to 2 mg l⁻¹ ZnO ENPs for 12 wk had approximately three fold more Zn in somatic tissue than the control group (Figure 2A).

Similarly, Zn concentrations in gonad increased with increasing concentrations of ZnO ENPs (OLS: *r*² = 0.51; *p* < 0.001). Large mussels exposed to 2 mg l⁻¹ ZnO ENPs for 12 wk had approximately 3.8 times more Zn in gonad than control mussels (Figure 2B).

Rate of Zn accumulation throughout the experiment showed different trends for small mussels than for large mussels (Figure 3). Small mussels accumulated Zn 10 times faster than large mussels at 0.5 mg l⁻¹ ZnO ENPs but large mussels accumulated Zn four times faster than small mussels at 2 mg l⁻¹. The effect of ZnO ENP concentration on the rate of Zn accumulation significantly varied as a function of mussel size (OLS: *t*₂, 36 = 3.42; *p* < 0.01), therefore, separate models were run for small and large mussels. For small mussels, the rate of Zn accumulation did not depend on ZnO ENP concentration (Table 1, OLS: *r*² < 0.0001, *p* > 0.9), but was highly variable for mussels exposed to >0.1 mg l⁻¹ ZnO ENPs. For large mussels, Zn accumulation rate increased with increasing concentration of ZnO ENPs (OLS: *r*² = 0.58, *p* < 0.001).

**Growth and survival**

The increase in growth, as measured by shell total length (TL), for mussels exposed to ZnO ENPs for 12 wk was less than that of mussels in control groups, although growth was relatively low for all groups (Figure 4). Mussel shell growth significantly varied as a function of ZnO ENP concentration and mussel size (Table 1, OLS: *r*² = 0.12; *p* < 0.0001). Small mussels in the 2 mg l⁻¹ exposure group grew 41% less, and large mussels grew 47% less, than the corresponding control group. After 12 wk of exposure to ZnO ENPs, mussel shell growth decreased 0.19 mm for every 1 mg l⁻¹ of ZnO ENPs (OLS: *t*₂, 326 = −3.87, *p* < 0.001). Mean shell growth for small mussels was 20–107% greater than large mussel growth during the experiment. Mussels had lower tissue dry weight to shell TL ratios with increasing shell TL (OLS: *t*₂, 43 = −7.43, *p* < 0.001). Additionally, at the beginning of the experiment mussels had a lower tissue dry weight to shell TL ratio than mussels at the end of the experiment (OLS: *t*₂, 43 = 2.88, *p* < 0.01).

Mean survival of mussels in control groups corrected for sampling was 94% after 6 wk and 91% after 12 wk, and was similar for small and large mussels in all groups except for the highest exposure group (Figure 5). After 6 wk of exposure to 2 mg l⁻¹ ZnO ENPs, survival was 91% for large mussels but only 59% for small mussels. After 12 wk of exposure to 2 mg l⁻¹ ZnO ENPs, 62% of large mussels survived while only 23% of small mussels survived. The effect of ZnO ENP concentration on survival after 6 wk significantly varied with mussel size (two-way ANOVA: *F*₁, 30 = 16.45; *p* < 0.0001) and a similar relationship was seen after 12 wk of exposure (two-way ANOVA: *F*₁, 30 = 13.84; *p* < 0.0001). Small mussels exposed to 2 mg l⁻¹ ZnO ENPs had
significantly lower survival compared to all other groups after 6 wk of exposure (Tukey HSD: \( p < 0.0001 \)). After 12 wk of exposure, small and large mussels exposed to 2 mg l\(^{-1} \) ZnO ENPs had significantly lower survival compared to all lower concentrations, and small mussels in this highest exposure group had significantly lower survival compared to the large group at the same exposure concentration (Tukey HSD: \( p = 0.001 \)).

**Discussion**

We predicted that mussels exposed to ZnO ENPs would increase respiration rates post exposure, increase total tissue Zn, and decrease growth and survival compared with control organisms, and that these impacts would be more pronounced in small mussels. After exposing *M. galloprovincialis* to ZnO ENPs for 12 wk, we observed increases in respiration rates and Zn concentration in tissues. These impacts were related to decreases in growth and survival, and suggest that mussels were expending energy to combat the effects of excess environmental Zn but were

**Table 1. Multiple OLS regressions examining effects of ZnO ENP concentration and mussel size on physiological parameters.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Intercept</th>
<th>Tissue [Zn]</th>
<th>Zn accumulation rate (small)</th>
<th>Zn accumulation rate (large)</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration rate</td>
<td>5.02 (1.29)**</td>
<td>138.39 (23.61)**</td>
<td>7.37 (2.90)*</td>
<td>1.00 (1.96)</td>
<td>0.63 (0.06)**</td>
</tr>
<tr>
<td>Conc</td>
<td>2.64 (1.17)*</td>
<td>125.58 (18.35)**</td>
<td>0.00 (2.83)</td>
<td>9.46 (1.92)**</td>
<td>-0.19 (0.05)**</td>
</tr>
<tr>
<td>Size</td>
<td>6.95 (1.23)**</td>
<td>40.48 (27.12)</td>
<td>0.19 (0.05)**</td>
<td>0.32 (0.06)**</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>17.77</td>
<td>24.46</td>
<td>&lt;0.01</td>
<td>24.38</td>
<td>21.81</td>
</tr>
<tr>
<td>DF</td>
<td>113</td>
<td>36</td>
<td>18</td>
<td>18</td>
<td>326</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.24</td>
<td>0.58</td>
<td>&lt;0.01</td>
<td>0.58</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Parameter values reported with standard error in parentheses. For the Zn accumulation rate model, the Concentration x Size interaction term was significant so large and small mussels were run in separate models. ZnO ENP concentration was square root transformed for the respiration rate model.

**Figure 2. Bioaccumulation of Zn in mussels exposed to ZnO ENPs.** Mean Zn concentration per gram dry weight ±1 SE in somatic tissue (A) and gonad (B) of mussels after 12 wk of exposure to ZnO ENPs. Zn concentrations are shown for tissue in small (black) and large (gray) mussels. Zn concentration in somatic tissue of both small and large mussels increased as a function of ZnO ENP concentration (OLS: Somatic Zn = 138.39+125.58(Concentration)+40.48(Size), \( r^2 = 0.58 \)). Zn concentration in gonad of large mussels increased as a function of ZnO ENP concentration (OLS: Gonad Zn = 81.18+66.88(Concentration), \( r^2 = 0.51 \)). Zn concentration in gonad from small mussels is not shown due to minimal gonad biomass in this group.

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**Figure 3. Zn uptake by mussels exposed to ZnO ENPs.** Mean rate of Zn uptake by dry weight into somatic tissue ±1 SE for small (black) and large (gray) mussels exposed to ZnO ENPs for 12 wk. Zn accumulation rate did not vary as a function of ZnO ENP concentration in small mussels (OLS: Zn rate = 7.37+2.98×10\(^{-3}\)(Concentration), \( r^2 < 0.0001 \)) but increased as a function of ZnO ENP concentration in large mussels (OLS: Zn rate = 1.00+9.46(Concentration), \( r^2 = 0.58 \)).

doi:10.1371/journal.pone.0061800.g003
phytoplankton, each mussel in our experiment received between 2 \times 10^5 and 1 \times 10^6 cells d^{-1} at the beginning of the experiment and more as the experiment progressed due to mussel mortality and sampling. Previous work suggests that mussels of the size class used in our experiment can ingest as much as 5 \times 10^5 to 3 \times 10^5 cells h^{-1} depending on flow rate and phytoplankton concentration in the water [31]. However, food limitation and slow growth is common in natural populations of mussels [32–34], unlike the typical laboratory condition of ad libitum feeding. Furthermore, ZnO ENPs decrease population growth of marine phytoplankton [8], making it likely that phytoplankton productivity and mussel food supplies will be reduced in zones of ZnO ENP contamination. Taken together, our results suggest that increased ZnO ENP concentrations will increase stress in marine mussels, and that this stress may be further exacerbated under food-limited conditions.

Respiration rates of mussels increased as a function of ZnO ENP concentration after 12 wk of exposure. However, this relationship was more pronounced for small mussels than for large mussels. This difference agreed with our finding of lower survival in the small mussel group exposed to 2 mg l^{-1} ZnO ENPs compared with the large group. We found no statistically significant relationship between respiration rate of mussels as a function of Zn tissue concentrations (results not shown), which is probably the result of high variability of Zn tissue concentrations in mussels in our study, similar to that reported by many others [26–30]. Decreased growth rates in the presence of ZnO ENPs combined with increased respiration after exposure suggest that mussels were using energy to detoxify Zn, remove Zn from tissues, or repair damage that resulted from high concentrations of Zn instead of using this energy to produce new tissue or shell.

Shell growth was very low for all mussels in our study (<1 mm in 12 wk), and somatic tissue in relation to shell length decreased during the study, suggesting that mussels were food limited. Assuming each mussel in each tank consumed the same amount of phytoplankton, each mussel in our experiment received between 2 \times 10^5 and 1 \times 10^6 cells d^{-1} at the beginning of the experiment and more as the experiment progressed due to mussel mortality and sampling. Previous work suggests that mussels of the size class used in our experiment can ingest as much as 5 \times 10^5 to 3 \times 10^5 cells h^{-1} depending on flow rate and phytoplankton concentration in the water [31]. However, food limitation and slow growth is common in natural populations of mussels [32–34], unlike the typical laboratory condition of ad libitum feeding. Furthermore, ZnO ENPs decrease population growth of marine phytoplankton [8], making it likely that phytoplankton productivity and mussel food supplies will be reduced in zones of ZnO ENP contamination. Taken together, our results suggest that increased ZnO ENP concentrations will increase stress in marine mussels, and that this stress may be further exacerbated under food-limited conditions.
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