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Accumulation of Nanoplastics in *Biomphalaria glabrata* Embryos and Transgenerational Developmental Effects

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Abstract: (1) Background: Nanoplastics are emerging environmental pollutants with potential toxic effects on aquatic organisms. This study investigates the toxicity of NPs in *Biomphalaria glabrata*, a freshwater snail species widely used as a bioindicator species in ecotoxicology studies.; (2) Methods: We exposed three generations (F0–F2) of *B. glabrata* snail embryos to different sizes of polystyrene nanoparticles and assessed responses.; (3) Results: We observed severe effects on F0 to F2 *B. glabrata* embryos, including size-dependent (30 to 500 nm) increases in mortality rates, size and dosage-dependent (1 to 100 ppm) effects on hatching rates with concentration-dependent toxicity in the 30 nm exposure group. The F2 generation embryos appear to be most responsive to detoxification (CYP450) and pollutant metabolism (HSP70) at 48-h-post-treatment (HPT), while our developmental marker (MATN1) was highly upregulated at 96-HPT. We also report a particle-size-dependent correlation in HSP70 and CYP450 mRNA expression, as well as enhanced upregulation in the offspring of exposed snails. We also observed significant reductions in hatching rates for F2.; (4) Conclusions: These findings indicate that F2 generation embryos appear to exhibit increased stress from toxic substances inherited from their parents and grandparents (F1 and F0). This study provides valuable insights into the impact of plastic particulate pollution on multiple generations and highlights the importance of monitoring and mitigating plastic waste.

Keywords: *Biomphalaria glabrata*; nanoplastics; transgenerational effect; embryos

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1. Introduction

Nanoplastics (NPs) have become a global environmental concern, with an ever-increasing body of evidence attesting to their negative impacts on organisms, including humans [1]. The freshwater snail *Biomphalaria glabrata* is a benthic macroinvertebrate and an important indicator species. Recent studies have underscored the concerning possibility of nanoplastic accumulation in the eggs of *B. glabrata*, and the potential for transgenerational developmental effects [1–3]. NPs are emerging environmental pollutants that can have detrimental effects on various species [1,3–5]. These particles have been found to accumulate in the tissues and organs of aquatic species, including freshwater snails like *B. glabrata* [1,3]. Concerning findings suggest that exposure to nanoplastics can lead to bioaccumulation and trophic transfer, potentially impacting the health and development of both individual organisms and entire ecosystems [3,5].

Studies have shown that microplastics and nanoplastics can induce a range of adverse effects, such as altered feeding behavior, reduced growth and reproduction, and even transgenerational impacts [3]. For example, Jeyataj noted in *Environmental Pollution*, that freshwater snails are negatively impacted by polypropylene which has shown to affect antioxidant parameters and oxidative stress in the proteins and lipids due to the increase in free radical production [6]. Further, Silva et al. demonstrated that exposure to small-sized nanoplastics can cause significant toxicity and developmental disruption in the chironomid, *Chironomus riparius* [4]. Previous study reports that NPs accumulate in the gastropod *Lymnean stagnalis* during embryo development. Although there is a maintained metabolism under NPs exposure, the NPs treatment decreased the egg clutch laying number [7]. The bioaccumulation of Mps or NPs in more than 15 gastropod species has been detected in different tissues, including the respiratory system [8], foot, visceral sack [9], and intestine [10]. There is evidence showing that bioaccumulation of MPs can lead to negative effects on *Crepidula onyx* snail larvae, such as the impact on growing sizes and juveniles' development [11]. Besides, abnormal shell formation of *Potamopyrgus antipodarum* snail embryos is observed under MPs exposure [12]. Oxidative stress, tissue damage in the stomach, and even the harmful impact on gastropods' gut microbiota could also be induced by NPs exposure in the gastropod species [13,14]. However, there are a lack of studies to understand the NPs embryotoxicity in gastropods, in which smaller plastics could penetrate and accumulate more effectively.

The toxic effects of plastic particulates on a range of snail species, specifically, have been investigated with various toxic effects being observed [15]. Some common findings include bioaccumulation in the gut, gills, head, liver [16], intestines, upregulation of apoptosis marker CASP-3, total inhibition of shell formation [12], effects on feeding, foraging and the gut microbiome [13]. Overall, the toxic effects on NPs including oxidative stress, inflammation, immune dysfunction, dysbiosis, and general systemic toxicity are well characterized in many species [17]. However, transgenerational toxicity is an important emerging field. The goal of this study was to assess the ecotoxicity, end fates, and transgenerational effects of NPs in freshwater environments using *B. glabrata*.

Although the density of polystyrene (PS) approaches that of water at 25 °C, other factors, such as the salinity of the water, NP sizes, biofouling, particle size, surface charges, and surface energies can influence position of PS-NPs in the water column or sediment [18,19]. It has also been reported that $CaCl_2$ can promote sedimentation of negatively charged PS-NPs, increasing their sedimentation dramatically [19]. Environmental factors are known to alter the density of microplastics [20], which has devastating implications for benthic habitats. Our laboratory has conducted studies for other projects in which we have incubated PS-NPs in samples of natural water and found them to agglomerate and settle to the bottom of the vials over time. Therefore, although the mechanisms responsible for settling and end-fates are highly complex, and remain to be fully elucidated, it is likely that PS-NPs are a threat to benthic invertebrate species.

The transgenerational effects of nanoplastics and microplastics are a particular area of concern, as these particles may have the ability to disrupt the normal development and functioning of organisms, with consequences that may be passed down through multiple generations. It is already known that NPs bioaccumulate within organisms and parental exposure can facilitate exposure to unexposed subsequent filial generations [21]. NPs with environmental contaminants adsorbed onto their surfaces can be transported in gametes and may build up in the lipids of the embryos [22,23]. Anomalous developmental effects can manifest in indirectly exposed subsequent generations [24,25].

Previous transgenerational studies have revealed effects in other freshwater species, affecting the hatching and mortality rates in *Danio rerio* [26] as well as localization within

the yolk sac [22]. Water fleas and copepods in general appear notably sensitive to the transgenerational effects of plastic fragments. *Daphinia magna*, exhibited reductions in reproduction and growth over the F1–F3 generations [27], increased mortality rates in F1 and F3 [28], and transfer of NPs from F0 to F1 and F2 [29]. In *Daphinia pulex*, inhibitory effects on antioxidant responses and altered growth rate and reproduction in F2 were observed [30]. Increased oxidative stress and altered antioxidant responses in F1 and F2 generations of *Moina macrocopa* have also been reported [31]. A transgenerational study in a snail species is lacking in the scientific literature, at present. This study sought to determine whether transgenerational exposure to nanoplastics affects subsequent generations in mortality, hatching rate and gene expression.

2. Materials and Methods

2.1. Snail Husbandry

B. glabrata snails (BB02 strain) were received as a gift from Dr. Daniel Wagner at Rice University in Houston, TX, USA. Snails were reared at the laboratory in two-quart, 7 in. × 11 in. Pyrex® pans filled with 1.0 L of filtered snail aquarium water (n = 15 per pan). The tanks were stored in an incubator set to 25 °C with alternating 12-h light and dark cycles. Snail aquarium water was prepared by mixing 0.5 g of Instant Ocean® Reef Crystals® reef salt 1.0 g sodium bicarbonate, and 500 mg amoxicillin in 5.0 L of reverse osmosis (RO) water stirred for a minimum of three hours to overnight to allow for complete dissolution. Before use, the snail water was filtered using a 1 L, 0.2 µm polyether sulfone (PES) membrane attached to vacuum filter. Snails were fed a diet of spinach and green leaf lettuce which was changed every other day. Fecal matter was removed every two days using an electric pipettor and fresh snail water was added to maintain water levels at 1.0 L per tank. Four-month-old, pigmented individuals between 8.0 and 12.0 mm in shell diameter, weighing between 0.24 g and 0.32 g were used for egg production. Snails laid egg masses on Styrofoam floats. Studies show that Styrofoam does not leach chemicals from ambient temperatures up to 50 °C [32]. With the difficulty in egg clutch collection, Styrofoam was deemed to be the safest alternative that provided a texture that was closest to what can be found in their natural environment. Snail egg masses were collected and randomized into groups with approximately 150 (±12) individual snails per treatment group Three generations of snails were studied in these experiments, with full gene expression, mortality and hatching experimentation performed on generations F0 and F2.

2.2. Latex Bead Preparation

Polystyrene nanoplastics (PS-NPs) were selected as the contaminant due to the ease of absorbance of PS-NPs in lettuce and other possible natural food sources for *B. glabrata.* [33]. Three sizes of PS-NPs used were 30 nm, 500 nm, and 1.0 µm. All testing was done with PS-NPs at concentrations of 1.0 ppm, however, some additional hatching and mortality testing in the F2 generation were done with increased concentrations (10 ppm and 100 ppm) to look for dose-dependent effects. The PS-NPs were carboxylate-modified, fluorescently-tagged, spherical latex beads composed of polystyrene in aqueous suspension (purchased from Sigma Aldrich, St. Louis, MO, USA; Product Number: 30 nm–L5155, 500 nm–L2380, 1.0 µm–L4655). These beads have zeta potential values ranging from −44.5 to −58 in the literature, which suggests good colloidal stability and monodispersity in water [17,34]. The stock of each size was diluted in approximately 50 mL of filtered snail aquarium water for treatments and vortexed prior to administration The control group was treated with filtered snail water only. All treatments began at ~15 h post fertilization (HPF) and lasted six days, or 144 h post treatment (HPT). The egg masses were observed for a total of 15 days when

the last embryo either hatched or was determined deceased via lack of movement and a lack of heartbeat.

2.3. Transgenerational Preparations

In the wild, egg masses are laid on a hard substrate or submerged vegetation, while in the laboratory, they may lay eggs on Styrofoam, glass walls, lettuce, or the shells of others. Styrofoam floats were placed in the control tank after 7:00 pm for snails to lay their eggs on and were collected approximately 15 h later, while still in the blastula stage of development. Each embryo was randomly assigned to either a treatment group or control and placed in a petri dish, as before, with approximately 150 individual embryos per group, which left a total of 640 animals to serve as controls. Egg masses were rinsed with fresh, filtered, snail water three times before the excess water was removed. Next, for the treatments, the petri dish was filled to 20 mL with aqueous PS-NP maximizing the air interface to allow for gas exchange. Treatment groups were exposed to 1 ppm concentrations for the main study. We also tested groups with exposure to 10 and 100 ppm to check for dose-dependent effects on mortality and hatching. Controls were treated similarly; however, they were filled with snail aquarium water only. Eggs were then incubated at 25 °C with 12-h light and dark cycles. The egg masses were monitored and underwent daily observations. At the six-day mark, the aqueous PS-NP solution was removed, and the egg masses were rinsed with clean, filtered snail water three times before filling approximately halfway with clean snail water. The juveniles were then monitored and kept in the petri dishes until their first egg mass was laid. At this point, the juveniles were placed in clean two-quart Pyrex® pans filled with 1.0 L of filtered snail water. These tanks are then labeled with their hatching date, date of their first egg clutch lay, fluorescent color tag and the size of polystyrene contaminant. For subsequent generations, egg masses were taken from their respective groups. For example, the second generation previously treated with 30 nm particles at 1 ppm was taken and treated with 30 nm PS-NPs at 1 ppm, as before. All three test groups of each generation were treated in the same manner with no deviations. All exposure experiments lasted six days, with observations spanning 15 days.

2.4. Quantification via Fluorescence Assay

To estimate the total amount of PS-NP accumulation in the egg masses, PS-NPs remaining in water samples after incubation with snail embryos were quantified using a Cytation5 spectrophotometry plate reader (BioTek Instruments, Winooski, VT, USA). A standard curve was created with three known NP concentrations (0.1, 1, and 10 ppm). The concentrations of PS-NPs in each collected water sample were calculated using the equation generated from the standard curve. It follows that the PS-NPs no longer remaining in the water have adhered to or entered the snail eggs. The fluorescently labeled PS-NPs were added to *B. glabrata* embryos culturing water at a concentration of 1 ppm. Water samples were collected every 24 h for six days, encompassing the major embryonic development period. Water samples were moved to 96-well plates and the intensity of the fluorescent signal was quantified using the plate reader.

2.5. Visualization of Fluorescent NPs

To visualize the accumulation and uptake of PS-NPs over the treatment period, an egg mass from each treatment group and control group was collected every 24 h. A standard, inverted microscope (Olympus Co., Tokyo, Japan) equipped with a mercury lamp and a CCD camera was used to monitor the accumulation of the fluorescent PS-NPs on an/or in the egg mass. We did not observe fluorescent signals suggesting adhesion to the well plates, if it did occur, it was not the predominant target. Commercial PS-NPs are coated with a surfactant to make them water soluble and highly colloidally stable. An AX/AX R

with NSPARC confocal fluorescent microscopy system (Nikon, Japan) was to obtain more detailed images of surface accumulation and z-scans were performed to monitor the vertical migration of PS-NPs into the snail egg chorion. 1 ppm fluorescent yellow-green latex beads of carboxylate-modified polystyrene with an excitation wavelength of λ_{ex} = 470 nm and an emission wavelength of λ_{em} = 505 nm was used to monitor the penetration of NPs under confocal microscope.

2.6. Gene Expression

Prior to treatment, each sample had the number of embryos recorded per clutch and photographed with the microinjection microscope. The egg clutches were suspended in aqueous PS-NPs and allowed to settle to the bottom of the 24-well plate. At the 48-HPT mark, six egg masses were collected per test group; control, 30 nm, 500 nm, and 1.0 µm; in 500 µL of TRIzol® (acid-guanidium-phenol) reagent with 1.0 mm glass beads, wrapped in parafilm, and stored at −80 °C for use in gene expression analysis. Each 24 h after 48-HPT, six egg masses per test group were collected until 144-HPT. The 24 HPT mark was proven to show no relevant increase in mortality or gene expression, and was thus discarded in all subsequent experiment runs. They were not used for subsequent comparisons due to 48 other samples being used during the experiment as well as the controls for each day, concentration and size for the primary experiments. RNA extractions were performed following standard extraction and purification procedures. After RNA extraction, the purity and quality ($\lambda_{260/280}$ values) of each sample were evaluated, and RNA was quantified in ng/µL using an Eppendorf BioSpectrometer (Eppendorf Biotech, Hamburg, Germany). RNA samples of over 500 ng/µL were diluted in molecular water for cDNA synthesis, which was performed using the Invitrogen SuperScript III reverse transcription system with a random hexamer primer (Invitrogen Co., Carlsbad, CA, USA). Samples were processed in the Thermocycler (Thermo Fisher Scientific, Waltham, MA, USA) according to the predetermined timeline for the SuperScript III transcription kit, and product cDNA was stored at −20 °C for downstream processing.

The relative gene expression of two known xenobiotic stress responses and developmental genes were quantified using the Thermo Fisher QuantStudio 3 (Thermo Fisher Scientific, Waltham, MA, USA) qPCR instrument. To test the relative gene expression changes between NP treatment groups and control groups, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH was used as a reference gene to test the relative gene expression changes of the following genes: cytochrome P450 (CYP32A1), heat shock protein 70 B2-like (HSP70), and matrillin-1 (MATN1). The primers for this study were designed through Integrated DNA Technologies' custom primers website (IDT, USA), using the available sequences from the NCI gene bank.

2.7. Toxicity Bioassay

Every 24 h post-treatment, egg masses from each treatment were monitored under a stereomicroscope to determine development stage, embryo mortality rate (EM) and hatching rate (HR) [35]. This was continued until the final egg either hatched or died. The EM (%) was calculated by dividing the number of deceased embryos by the total number of embryos per clutch and then multiplying by 100 [35]. Deceased embryos were identified by negative movement and heartbeat, or disintegration of the body [36]. HR (%) was calculated by dividing the number of hatched snails by the total number of embryos per clutch and then multiplying by 100 [35]. The hatched snail must have completely exited the egg mass and not just the egg capsule to be counted as hatched.

2.8. Graphical and Statistical Analyses

Statistical analyses of gene expression were performed using RStudio, Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA, USA. Fluorescent quantification, hatching rate, mortality rate, and qPCR data were nonparametric thus a one-way ANOVA was performed for significance followed by Shapiro–Wilk test to test for normality. The difference in treatment groups over each time point were evaluated using pairwise one-way ANOVAs. Mortality and hatching rates were analyzed using log-rank (Mantel-Cox) tests, logrank tests for trend, and Gehan-Breslow_Wilcoxon tests and graphed using Prism GraphPad version 4.2.3 for Windows, Boston, MA, USA.

3. Results

3.1. Quantification via Fluorescence Assay

We sought to quantify the particles that adhered to or entered the snail embryos by subtracting the amount remaining in the water by their fluorescence. This method worked well and revealed a clear downward slope in concentration for the larger particles (500 nm and 1 μm), but only a 21% of 1 ppm reduction in the 30 nm particle group over time, see Supplementary Information Figure S1. This is likely due to the inherent limitations of the equipment since particles were clearly observed in and on the embryos.

3.2. Nanoplastic Bioaccumulation and Migration

Fluorescent imaging of egg masses exposed to fluorescently tagged PS-NPs showed bioaccumulation of PS-NPs on the surface of the egg masses across all three treatments (Figure 1). Though these images could not serve as a clear indicator for NP migration through the outer membrane and uptake by the embryo, however, increased PS-NP density on the surface could be verified by observing the increased diameter of fluorescent signals. Figure 1 shows a visual comparison of the treatments over each 24-h time point and how bioaccumulation of NP on the outer surface of the egg mass increases with exposure time. The 30 nm treatment had a single florescent signal at 24 HP, but by 96 HPT many more PS-NPs can be visualized as well as clusters of NPs which can be observed as an increased luminescent diameter of the fluorescent signals. The 30 nm NPs appeared to agglomerate in the water, with fewer signals observed between the larger agglomerates. Fluorescent images of different particle size treatments (at 1 ppm) vs time are summarized in Figure 1. The 500 nm treatment at 24-HPT was sparsely covered in NPs with a few clusters. By 96-HPT there were many more clusters with many small signals in-between. At 24-HPT treatment of 1.0 μm, the egg mass had only an area with clusters of NPs. At 96-HPT the surface is widely covered in NPs and has larger clusters than seen in the previous treatments. During a preliminary experiment, we did observe a juvenile treated with 10 ppm of 30 nm NPs emerge with fluorescent NP signals (Figure 2) which suggests that NPs can be taken up by the embryos during development. We did not observe any brightly fluorescent embryos in the 1 ppm treatment groups.

Although we were able to determine that the particles were adhering to the snail eggs, we were unable to verify whether they were penetrating the eggs using fluorescence microscopy alone. Confocal images revealed accumulation of the NPs within the snail embryos throughout, including the center and bottom of the snail embryo (Figure 3). Figure background was adjusted slightly to correct for artifacts from snail movement (snail was not anesthetized). From the confocal images, we could conclusively verify that the NPs are able to penetrate the snail eggs and exert effects within the developing snail.

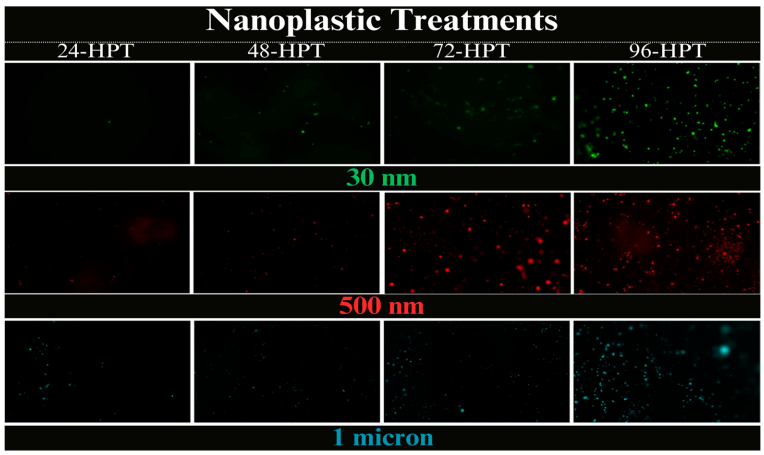

Figure 1. Fluorescent images comparing treatments: particle size increases with row vs. exposure time increasing with columns from right to left. All treatment concentrations are 1 ppm. Fluorescent signals appear beginning at 24 HPT, and bioaccumulation continues through 96 HPT.

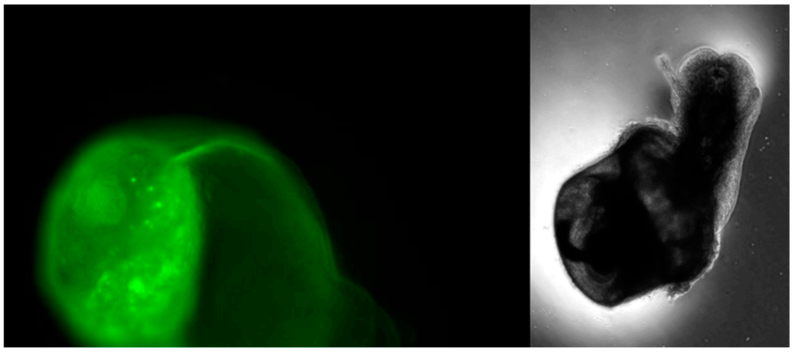

Figure 2. Fluorescence microscopy image of juvenile snail treated with 10 ppm of 30 nm NPs during first 6-days of embryonic development, emerged from egg with fluorescent emission (**left image**) and brightfield image of the same snail (**right image**).

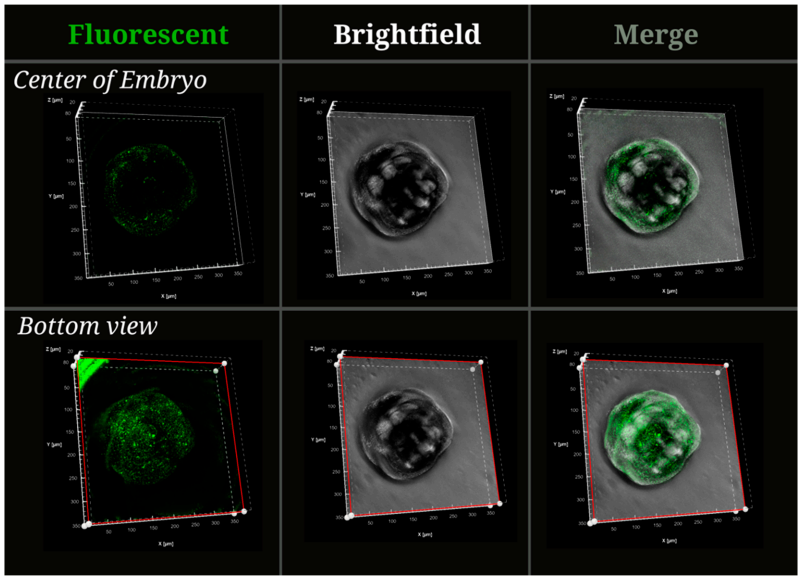

Figure 3. Confocal microscopy images of snail embryos showing full-scan still images of the center of the embryo (**top row**) and bottom-view scans of the embryo (**lower row**); both locations show still images of the fluorescence (**left column**), a brightfield image of the embryo (**center column**), and merged images (**right column**). Fluorescent particles clearly accumulated within the embryo and not just on the outside of the egg.

3.3. Hatching Rates

Although we observed a clear, concentration dependent reduction in the overall hatch totals for the F2 snails treated with 30 nm PS (Figure 4), we did not observe this trend for any of the other particle sizes. Rather, we observed a non-linear effect. When comparing the hatching rates for the F0 and F2, it is immediately apparent that the reductions in hatching rates, evident in the 1 µm group are not as drastic in the F2 generation. It is possible that we may have selected out the most susceptible individuals from our population by F2. However, we observed a consistent reduction in hatching rate when comparing the F0 and F2 generation groups, with significantly reduced overall hatching rates in the treated groups of F2 (Figure 5A,B). However, we do observe a clear reduction in the hatching rates for all groups, in fact, hatching appears to plateau after day 7, with the 500 nm group in the F2 generation exhibiting the most drastic reduction in hatching rates. These effects were not apparent in F0. With for the 500 nm PS, we found that both the 1 ppm and 10 ppm concentrations resulted in a hatch rate of ~72%, and although the 100-ppm concentration resulted in a 96% hatch rate, we observed a spike in the hatching on day 7 that was not observed in the other treatment groups or the control (Supplementary Information Figures S2–S4). This apparent manifestation of toxicity must cause some alterations in the developmental timeline initially, but F0 animals appear to recover and catch-up with controls by study end. We do observe a higher hatching rate on the first day of hatching for the 1 ppm and 10 ppm groups, 28% and 23%, respectively, and a lower hatching rate of 3% for the 100 ppm groups compared to 12% for controls (Supplementary Information Figures S2–S4). The hatching rate remains depressed in the 1 ppm and 100 ppm groups through day 14, however, in the 10-ppm group the hatch rates are on par with controls throughout the experiment. It might be possible that agglomeration is favored at higher concentrations, but more work is needed to understand this observation. The hatching rate curves were highly statistically significant when compared using a log-rank (Mantel-Cox), logrank test for trend, or Grehan-Breslow-Wilcoxon test which returned p-values < 0.0001 for all tests for both generations compared to controls (F0: 1 µm PS-NP and F2 all treatments).

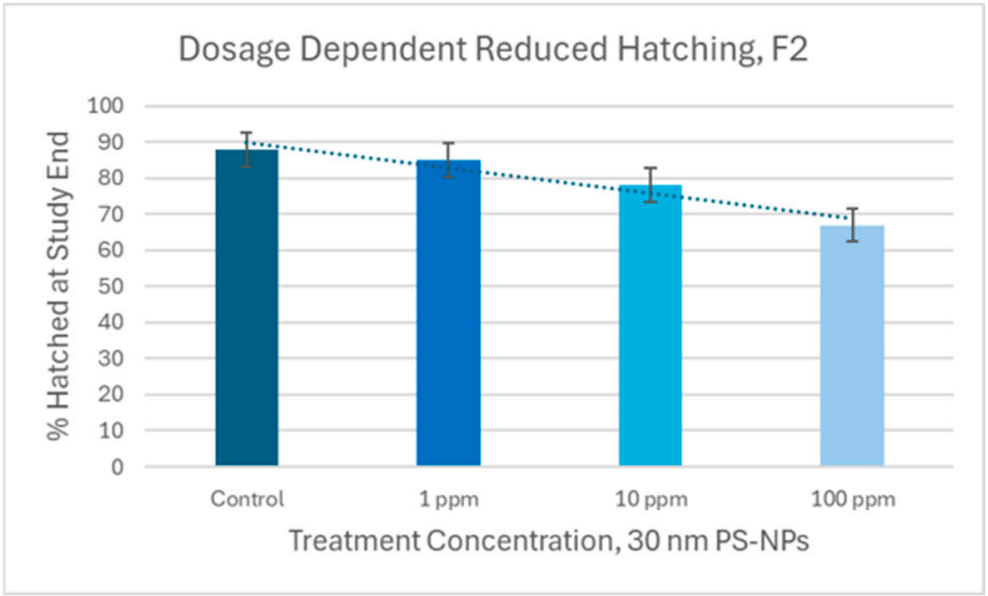

Figure 4. Dose-dependent reduction in hatching rates for 30 nm NP exposures.

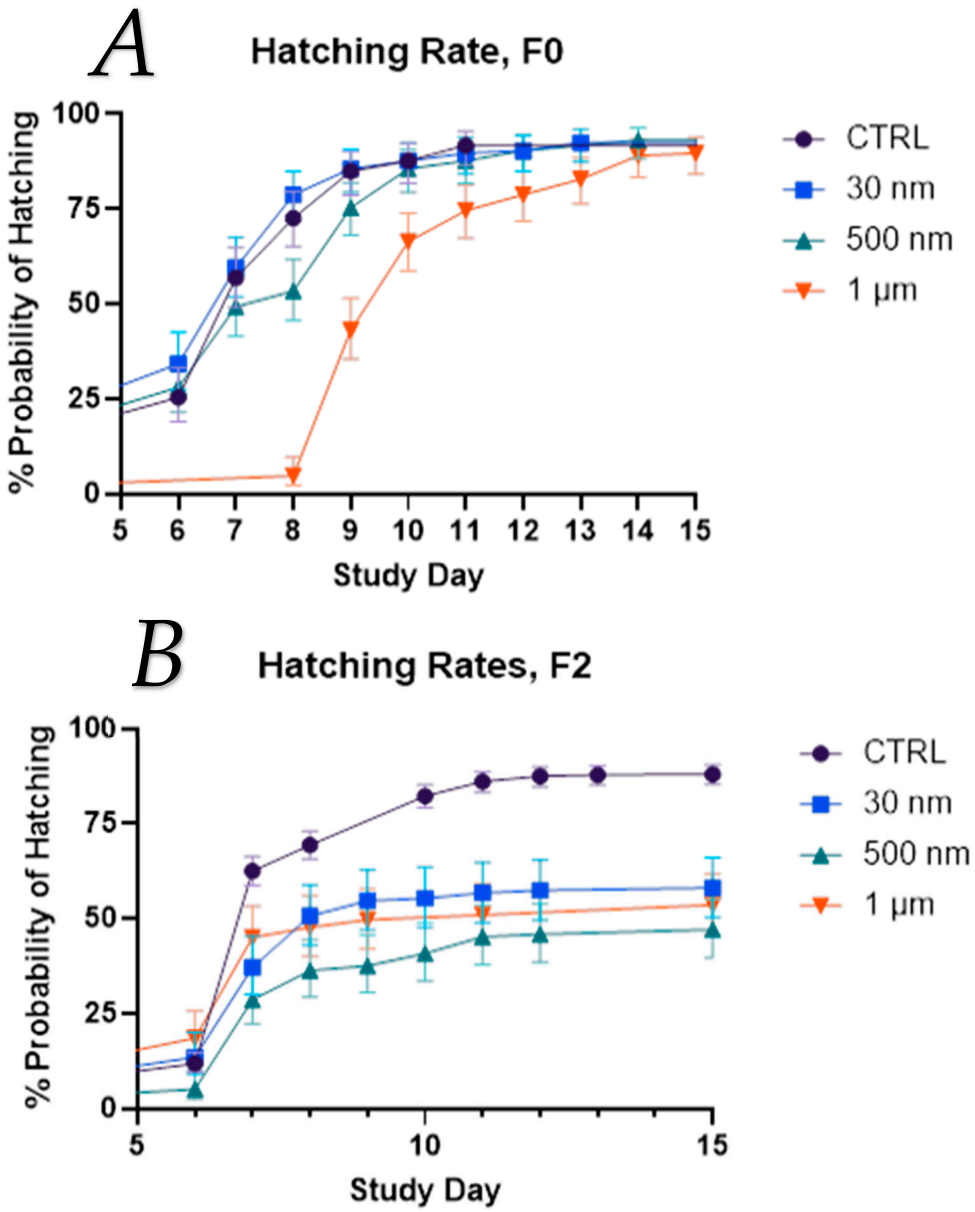

Figure 5. (**A**) The hatching rate of F0 generation snails showed significantly reduced hatching in the 1 µm group on days 6–10 and continued with slight reductions through day 15 ($p < 0.001$). We also saw slight reductions in the hatching rates for the 500 nm treatment group on day 8 only. (**B**) The hatching rate of F2 generation snails showed statistically significant reduced hatching rates ($p < 0.001$) at all timepoints for snails treated with particles. All treatment concentrations were 1 ppm. Error bars are 95% CI.

3.4. Mortality Rates

As with the hatching rates, the only dosage dependence we observed for total mortalities was with the 30 nm particles (Figure 6). The 500 nm particles demonstrated maximum mortality at 10 ppm peaking on days 6–7 and plateauing after that while the mortality rates started higher than average in the 10-ppm treatment group but did not increase with increased dose (Supplementary Information Figure S6).

We also noticed a similar mortality rate for 1 µm treated snails at concentrations of 1 ppm and 100 ppm (Supplementary Information Figure S7). At the investigational dose of 1 ppm, we observed sharp increases in mortalities in all the treated snail embryos on day seven, that was suspiciously absent from the control groups (Figure 7). We have noticed in other studies that as the embryos get closer to their hatching time, the seem to become

more sensitive to environmental toxicants (*unpublished data*). However, the F2 generation does not express this increased slope between days 7–8, likely because we have selected out the most susceptible individuals from our population. Historical averages for mortalities during this time tends to be 5–10% (*unpublished data*). Therefore, only 500 nm particles in the F2 generation exceeded the historical average mortality rates for the laboratory. Neither of the survival curves reached statistical significance when compared using a log-rank (Mantel-Cox), logrank test for trend, or Grehan-Breslow-Wilcoxon test which returned a F0 *p*-values of 0.4866, 0.2399, and 0.4923, respectively and F2 *p*-values of 0.4366, 0.5574, and 0.4219, respectively.

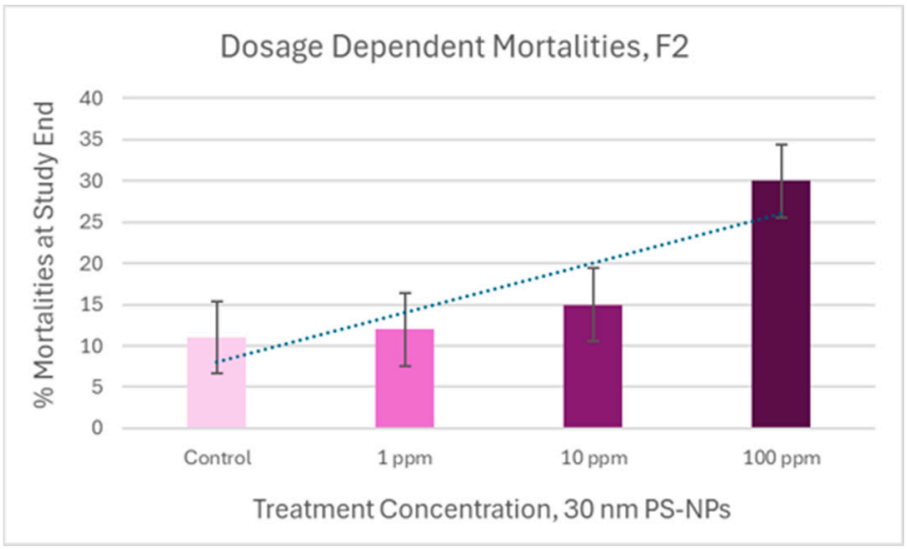

Figure 6. Mortality rates show dose-dependence for the 30 nm treatments groups in the F2 generation.

3.5. Relative Gene Expression

Any alterations in gene expression were most likely to be apparent at 48-h post-treatment, with some visible changes at 96-h. We observed upregulation in *HSP70* and *CYP450* at 48-h post-treatment in both F0 and F2 generations of snails. Additionally, we observed upregulation of *CYP450* in the F0 generation at 96-hpt, but upregulation of CYP450 (Figure 8) at 48-hpt only for snail embryos treated with the 30 nm particles. The expression levels of genes related to pollutant metabolism (*HSP70*) and detoxification (*CYP450*) were detected separately. In the F0 generation, the 48-h old embryos only exhibited significant upregulation pattern of HSP70 in the 500 nm treatment group compared to the control group (1.66 ± 0.23-fold increase), and there was no significant change in HSP70 expression level after 24 h development among different size of nano plastics treatment. The expression pattern of *CYP450* was significantly downregulated in the 1 µm nanoplastics treatment group (0.541 ± 0.373-fold of control group) during 48-h development stage, but its expression recovered after 24 h development. On the contrary, the smaller size nano plastic treatment group (30 nm) resulted in significant upregulation of *CYP450* at the 96-h developmental stage (1.3 ± 0.2-fold of control group). As the major protein resisting parasite infections during snail embryonic development, the encoded gene *MATN* only showed significant upregulation in the 30, and 500 nm-NPs treated 96 h-old snail embryos (1.25 ± 0.25, 1.35 ± 0.09-fold of control with 30 and 500 nm NPs treatment, respectively), but there was no significant changes in the *MATN* expression level in any other NP treatment group during 48 h embryonic development stage in F0.

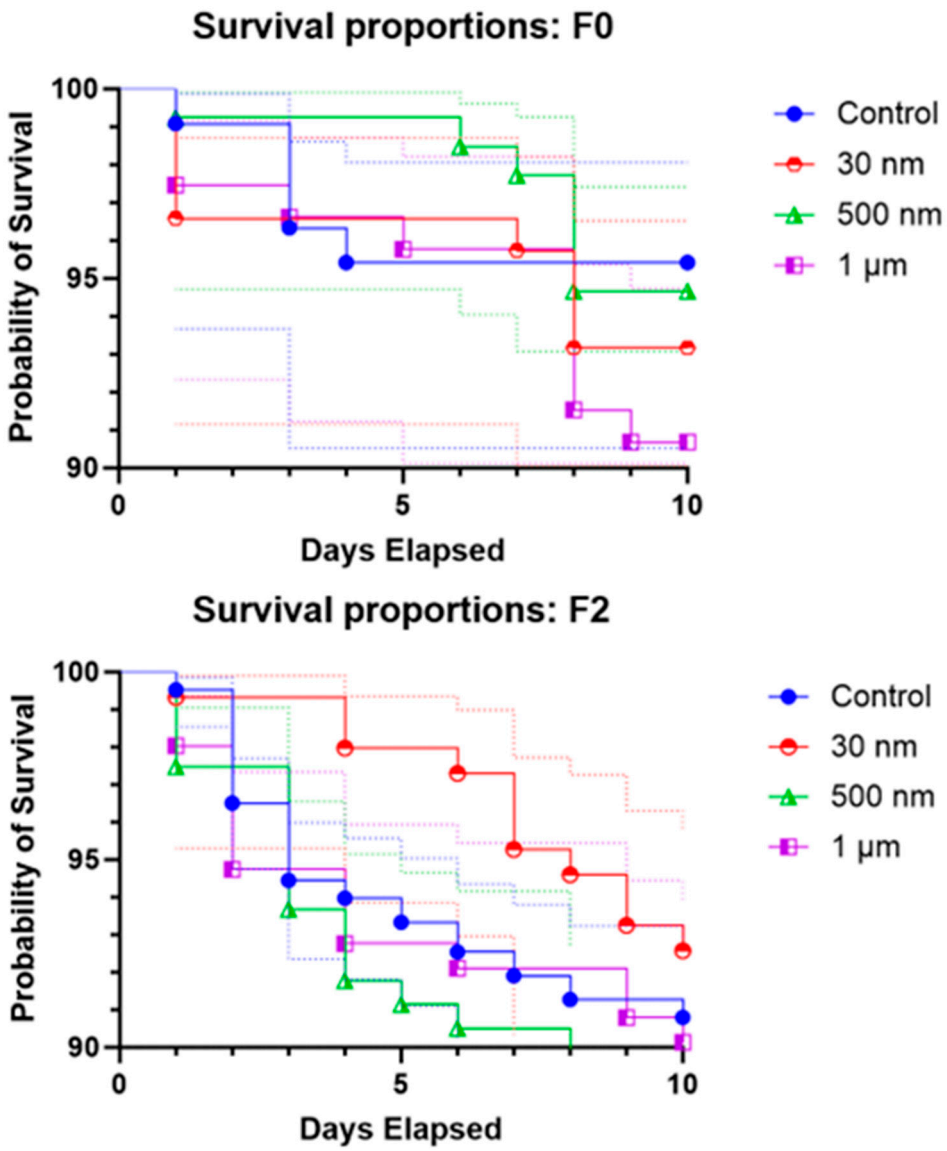

Figure 7. Survival curves for F0 (**upper graph**) and F2 (**lower graph**), shows a spike in mortalities in the F0 generation on day 8 that is absent from the F2 curves. We also see that most mortalities are in the 1.0 µm group in the F0, and the 500 nm group in F2. Dotted line error bars are 95% CI. Neither survival curve reached statistical significance, as evidenced by overlap of the error bars. Historical averages are ~5–10% mortalities for controls.

Among the genes evaluated, the overall upregulation of CYP450 and HSP70 in the F2 generation were clearly observed on the transcript analyses (Figure 8). The transaction analysis result revealed a size-dependent increase in HSP70 mRNA expression in 48-h-old embryos (Figure 8). Upregulation patterns of HSP70 were only detected in the offspring of 30 and 500 nm-NPs treated snails (5.25 ± 2.31, 5.91 ± 4.35-fold of control with 30 nm and 500 nm NPs treatment, respectively). HSP70 is a multifunctional protein that protects cells from stress by preventing protein misfolding and repairing or degrading damaged proteins. Therefore, the upregulation of HSP70 in the F2 generation embryos indicated that the early embryo development of F2 generation might still experience stress from toxic substances inherited from their parents through maternal nutrient supply in the eggs. In a previous study, the NPs with diameter around 30 nm were detected in maternally exposed F1 embryos of zebrafish and affected their antioxidant system [12]. Similarly, the expression levels of CYP450 were significantly upregulated in all treatment groups during 48-h embryonic developmental stage (3.73 ± 1.07, 5.65 ± 2.11, and 3.60 ± 1.49-fold

of control with 30 nm, 500 nm, and 1 um NPs treatment, respectively). It is suggested that the F2 embryos might still detoxify the foreign NPs from their parents to adapt to a NP-rich chorion environment compared with the control group. After the adaptation or further bioactivating with the NPs, the expression level of CYP450 recovered in 96-HPT embryos. The expression pattern of MATN1 during 48-h embryonic developmental stage only upregulated in the 30 nm group. However, significant upregulations of MATN1 were detected in 96-h old embryos among all three treatment groups (17.7 ± 10.21, 15.8 ± 12.68, 19.9 ± 17.11-fold of control) with 30 nm, 500 nm, and 1 µm NPs treatment, respectively. Therefore, the snails appear most susceptible to alterations in this specific marker of embryonic development by this toxicant at 96-HPT.

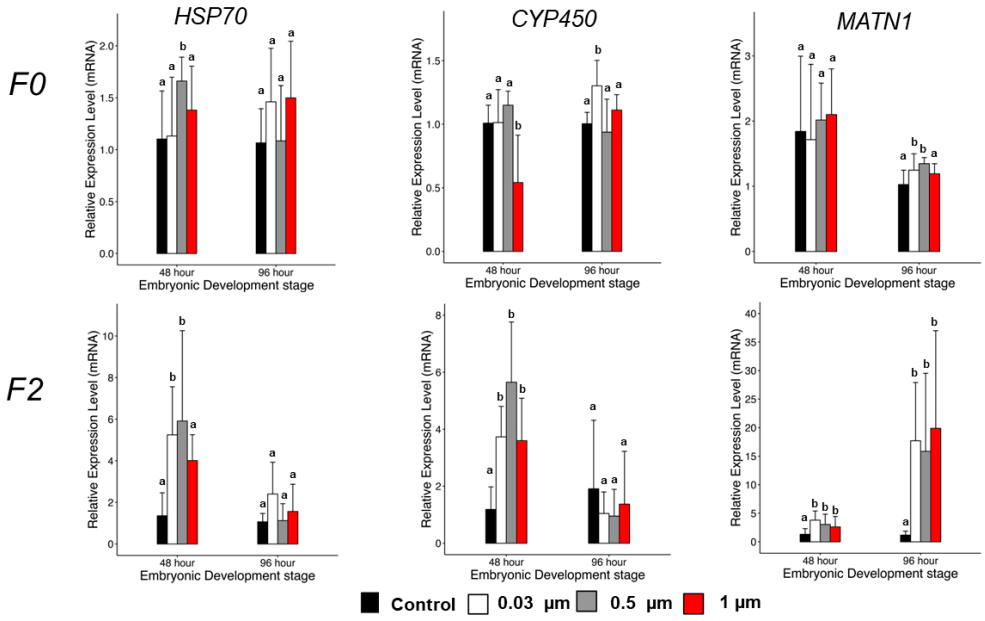

Figure 8. Relative Gene Expression $2^{\Delta\Delta Ct}$ for HSP70 (heat shock protein) leftmost column, CYP450 (cytochrome oxidase enzyme) center column, and MATN1 matrilin protein, rightmost column for F0 generation (**top row**) and F2 generation (**bottom row**). We see upregulation of HSP70 in both F0 and F2 and 48-h. This is more pronounced in the F2 generation. We also see significant upregulation of CYP450 in F0 at both 48 and 96-h, and in F2 at 96 h, once again this upregulation is higher in the F2 generation. We also observe significant upregulation of MATN1 in F0 at 96-h and in F2 both at 48-h as well as 96-h (n = 6). Error bars represent the standard deviation (SD).

4. Discussion

The accumulation of microplastics in *Biomphalaria glabrata* eggs and their potential transgenerational effects is a concerning environmental issue that warrants further investigation. Our results demonstrate the ability of nanoplastics to accumulate in *B. glabrata* eggs, with potential impacts on development and hatching success. These findings are consistent with previous studies that have reported adverse effects of nanoplastics and microplastics on various aquatic organisms, including reduced growth, altered behavior, and reproductive impairment [2–5].

The observed transgenerational effects of nanoplastics on *B. glabrata* development are particularly alarming, as they suggest the potential for these pollutants to have long-lasting impacts on the health and fitness of freshwater ecosystems. Additionally, we also noted reduced adverse effect levels in later generations suggesting selective breeding of the more resistant animals. Interestingly, we appear to have selected for those with a higher fold-change in gene modification of HSP70 and CYP450 in F2, with a six-fold increase in gene expression for F2 vs. a less than 0.2–2-fold change for F0. The mechanism behind

these transgenerational effects remains to be fully elucidated but may involve epigenetic modifications and alterations in key developmental pathways.

Matrilin genes, or cartilage matrix proteins (CMP), are a protein family associated with the formation of extracellular matrices of different tissues. Specifically, the MATN-1 gene used in this study is mainly expressed in cartilage [37]. A matrilin-like protein sequence was identified from *B. glabrata* and is like MANT from *Mus musculus* [38], therefore, it is likely relatively conserved. Furthermore, other researchers observed this gene expression during the early development stages of *B. glabrata* [39]. We selected this gene coding for MANT1 as a means of determining whether PS-NPs affect embryonic development. qPCR results yielded slight upregulation for the treatments in the F0 generation, however, we observed statistically significant upregulation (fold change ~20) of MANT1 in the F2 generation when treated at 1 ppm. Again, these findings suggest generational toxicant transfer leading to clear, enhanced effects on subsequent generations of snails in NPs of all sizes tested.

Heat shock proteins (HSPs), or stress response proteins, are a family of proteins that are over-expressed in response to both abiotic and biotic stimuli. HSP70 is an important protein within the HSP family that aid in an organism's ability to mitigate stress responses and cellular damage from the environment [40]. This gene was chosen to see how NPs in an aquatic environment might affect an organism's stress and cellular damage due to stressful environments. The only significant upregulation of gene HSP was at 48 HPT in both F0 and F2, while at 120 HPT the treatment groups were downregulated compared to the controls (*unpublished data*). HSP70 results in other species showed up-regulation of the stress response when exposed to NP at 1.0 ppm concentrations [41,42].

CYP450 is an ancient superfamily of enzymes that plays a significant role in metabolizing endogenous substances including vitamins, steroids, fatty acids, as well as xenobiotics/toxicants. Because the CYP450s can carry out a diverse range of monooxygenase activities, they allow for comprehensive protection from a large array of xenobiotics that organisms interact with. In fact, it can be transcriptionally activated by a range of different xenobiotics [43]. In research, the gene expression is used as a bioindicator for PAH contamination in various species including fish and aquatic invertebrates [44–46]. This gene was used to determine whether PS-NPs would induce a xenobiotic response. In the F0 generation, 48-hpt only the 500 nm group was statistically significantly elevated at 48-hpt, and the 1.0 μm and exhibited a trend of upregulation compared to the control, but not again during the treatment period. At 96-HPT, the 30 nm treatment group was upregulated vs control in the F0 generation. We also observed increased CYP expression in the F2 generation closer to 6-fold higher than control, vs. ~1.5-fold higher in F0, with no upregulation observed at 96-h. CYP expression in other studies showed upregulation of this gene at doses below 1.0 ppm [39]. This could be due to agglomeration of the particles at higher concentrations, leading to increased hydrodynamic size and subsequent reduced penetration.

5. Conclusions

Our study provides evidence of the accumulation of NPs in *Biomphalaria glabrata* embryos and the potential for these particles to have transgenerational effects on the development and fitness of this important freshwater snail. Although the NPs do not seem to have a concentration, or size dependent effect on mortality at sizes > 30 nm, they do alter gene expression with regards to detoxification pathways, stress, and embryonic development. Although we did not observe significant toxicity in survival assays, we found that 500 nm particles exerted the most significant effects on gene expression overall. We also observed severe delays in hatching for snails exposed to 1 μm NPs in the F0 generation. These

findings underscore the need for continued research and the implementation of effective strategies to mitigate the growing issue of nanoplastic pollution in aquatic environments.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/environments12010028/s1, Figure S1: Concentration of NPs remaining in water with snail egg masses; Figures S2–S4: Hatching rates for snail embryos exposed to increasing doses of 30 nm, 500 nm and 1 μm particles, respectively; Figures S5–S7: Mortality rates of F2 generation snails treated with increasing doses of 30 nm, 500 nm, and 1 μm particles, respectively.

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Conflicts of Interest: Leisha Martin declares corporate affiliation with MNT SmartSolutions, Inc. and LEI NanoTech.

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