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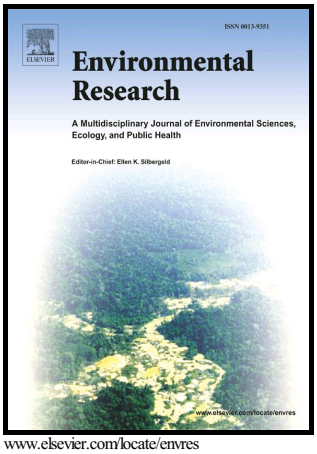

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Size dependent oxidative stress response of the gut of *Daphnia magna* to functionalized nanodiamond particles

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Abstract:

Nanodiamonds are a type of engineered nanomaterial with high surface area that is highly tunable and are being proposed for use as a material for medical imaging or drug delivery to composites. With their potential for widespread use they may potentiall be released into the aquatic environment as are many chemicals used for these purposes. In is generally thought that nanodiamonds are innocuous, but toxicity may occur due to surface functionalization. This study investigated the potential oxidative stress and antioxidant response of enterocytes in a freshwater invertebrate, *Daphnia magna*, a common aquatic invertebrate for ecotoxicological studies, in response to two types of functionalized nanodiamonds (polyallylamine and oxidized). We also examined how the size of the nanomaterial may influence

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toxicity by testing two different sizes (5 nm and 15 nm) of nanodiamonds with the same functionization. Adults of *Daphnia magna*, were exposed to three concentrations of each of the nanodiamond for 24 hours. We found that both 5 and 15 nm polyallylamine nanodiamond and oxidized nanodiamond induced the production of reactive oxygen species in tissues. The smaller 5 nm nanodiamond induced a significant change in the expression of *heat shock protein 70* and *glutathione-S-transferase*. This may suggest that daphnids mounted an antioxidant response to the oxidative effects of 5 nm nanodiamonds but not the comparative 15 nm nanodiamonds with either surface chemistry. Outcomes of this study reveal that functionalized nanodiamond may cause oxidative stress and may potentially initiate lipid peroxidation of enterocytes cell membranes in freshwater organisms, but the impact of the exposure depends on the particle size.

Keywords:

reactive oxygen species, nanodiamonds, gene expression, nanotoxicity, oxidative stress

1. Introduction

Diamond nanoparticles (DNPs) are a type of carbon nanomaterial that have lately received great attention due to their exceptional mechanical, optical, and tunable surface properties that makes them ideal for several applications such as lubricant additives, biomedical imaging, and drug delivery (Ho et al., 2015; Ivanov et al., 2012; Kaur and Badea, 2013; Mochalin et al., 2012; Pecoraro et al., 2018; Pecoraro et al., 2017; Vaijayanthimala et al., 2015; Zhu et al., 2012). Studies have found that these DNPs exhibit low toxicity or no toxicity when used in lab experiments employing cultured cells or model organisms (Zhu et al., 2012). However, given the tunability of

these NPs using there is a question as to if DNPs toxicity will change due to with changes to the initial particle size and surface functionalization.

Small DNPs (<10 nm) exhibit some level of toxicity when exposed to different living systems although studies have shown variable results. Toxicity endpoints such as cell proliferation, genotoxicity, apoptosis, and mortality were affected for several *in vitro* studies (Mytych et al., 2016; Schrand et al., 2007b). For example, genotoxic and mutagenic activity of DNPs was described on human peripheral cells (Dworak et al., 2014). Additionally, there is evidence that small DNPs have shown to present toxicity *in vivo* studies. For example, abnormalities and high mortality were present in the gastrulation and neurulation stages in African clawed frog embryos after exposure to 4 nm DNPs (Marcon et al., 2010). Moreover, freshwater Asian clams exposed to DNPs less than 6 nm produced an oxidative stress response followed by an increased lipid peroxidation (Cid et al., 2015). In addition, a significant increase in activity of oxidative stress enzymes was observed in house cricket exposed to 3.5 – 5.2 nm DNPs (Karpeta-Kaczmarek et al., 2016).

In contrast to the effects of small DNPs, it seems that larger DNPs (>50 nm) are innocuous to living cells or organisms. In this case *in vitro* studies have shown that large DNPs cause no toxicity to low toxicity in cultured cells (Keremidarska et al., 2014; Paget et al., 2014; Yu et al., 2005). For example, minor toxicity was apparent when fluorescent nanodiamonds (114.7 nm) caused low neuronal toxicity but interfere with neuronal morphogenesis (Huang et al., 2014). Similarly, *in vivo* studies have demonstrated that large DNPs produced no deleterious effects. Fluorescent DNPs (120 nm) did not alter longevity, reproduction, and stress responses of nematodes (Mohan et al., 2010).

Surface functionalization is known to influence the impact of a variety of nanoparticles however this effect can depend on the core of the material (Arndt et al. 2013). There have been limited studies as to the potential impacts of surface chemistry on DNPs (Burleson et al., 2009; Marcon et al., 2010; Moore et al., 2014; Schrand et al., 2007a). Outcomes of exposing functionalized DNPs to cultured cells or organisms have resulted in no toxicity to low toxicity (Burleson et al., 2009; Schrand et al., 2007b). For example, exposure of several human cell lines to carboxylated DNPs did not result in cytotoxicity or genotoxicity (Paget et al., 2014). Similarly, it was found that three different functionalized DNPs tested in Hela and HepG2 cells as drug carriers for daunorubicin showed less toxicity to both cell types than unfunctionalized counterparts (Moore et al., 2014). On the other hand, *in vivo* studies found that carboxylated DNPs have a potential embryotoxicity and teratogenicity to *Xenopus laevis* (Marcon et al., 2010). Determining the overall risk from these toxicity studies are still challenging because there is not enough information to understand the toxicity of functionalized DNPs or the mechanism by which they may interact with cells. Since there is some evidence of the potential for organism toxicity in limited studies it is necessary to find out how the interaction of size and surface chemistries may drive the toxicity response of DNPs.

Reactive oxygen species (ROS) and oxidative stress are natural cellular responses to a foreign object and are considered a potential indicator of toxicity in NPs studies (Kim et al., 2010; Meng et al., 2009; Nel et al., 2006; Pecoraro et al., 2018; Pecoraro et al., 2017). ROS production can indicate damage or the process of attempted repair inside a cell upon insult (D'Errico et al., 2018; Fu et al., 2014; Guerriero et al., 2003; Maurer-Jones et al., 2013). The continuous presence of a foreign particle like a NP interacting with cells may lead to a disturbance of redox

status resulting in oxidative stress (Lushchak, 2011). This perturbation may trigger other responses that can be protective for the organism and can be easily measurable (Rana and Kalaichelvan, 2013). To determine such protective responses, molecular biomarkers such as gene expression can quantify the level of oxidative stress in organisms previously exposed to NPs and provide a clear understanding of the possible mechanisms that are altered upon exposure of organisms to NPs (Chen et al., 1999; Eads et al., 2007; Klaper et al., 2014). For example, different gene expression profiles in *D. magna* exposed to silver nanoparticles and silver nitrate demonstrate the potential for different modes of toxicity (Poynton et al., 2012).

A vast concern is that the increasing use of DNPs in research and consumer products also be reflected in the amount of these NPs entering the different environmental matrices. Aquatic ecosystems are of special interest because studies have predicted that NPs will accumulate in surface waters as do many other emerging contaminants that began their life cycle in similar products (Benn and Westerhoff, 2008; Kaegi et al., 2008) yet there are unknown consequences for human and environmental health. In this work, we used *Daphnia magna,* a common model for ecotoxicological testing of nanomaterials, to determine levels of ROS production in the gut tissue in response to DNPs with two different functionalizations and two different sizes. The degree of antioxidant response in the form of *hsp70*, *cat* , and *gst* gene expression was measured along with measures of ROS production to examine the mechanisms that may be perturbed in relation to exposure. Efforts were focused on measuring the impact of DNPs on oxidative and cellular stress in daphnid guts using similar concentrations as recently demonstrated in Dominguez et al. (2015) where similar types of surface functionalization of nanogold particles

(AuNPs) caused a difference in the oxidative stress seen in the guts of daphnids. In the current study, we expand this research to determine how well the results with AuNPs predicts how a given surface functionalization will affect toxicity for another particle and examine the role of size in determining the impact of functionalization which we could not do with the AuNPs.

2. Materials and Methods

2.1. Preparation of nanodiamond particles and characterization

Monocrystalline synthetic nanodiamonds (15 nm) were obtained from Microdiamant USA Inc.; the carboxylated nanodiamond (5 nm) aqueous slurry from Adamas Nanotechnologies Inc., the poly(allylamine) hydrochloride (PAH, 14kDa) from Sigma, and a dialysis membrane from Spectrum Labs (MWCO 50 kDa, # 131384).

To obtain 15 nm oxidized diamond nanoparticles (OXI-DNP), diamond nanoparticles (DNP) were prepared by refluxing nanodiamond powder in a 3:1 mixture of H_2SO_4:HNO_3 for three days. (Caution: mixture is extremely caustic). After, acids were carefully diluted in Milli-Q water (18.2 MΩ-cm resistivity), and particles were isolated by centrifugation (5 min, 4,696 rcf). Particles were iteratively centrifuged and resuspended until pH was neutral, and particles no longer pelleted. From this solution, 15 nm particles were isolated by centrifugation (5 min, 14k rcf). The concentration of the oxidized stock was determined by gravimetric analysis. The small, 5 nm OXI-DNP were used as provided. DNP (15 nm and 5 nm) were functionalized with PAH by mixing a 1 mg/mL PAH in 1 mM NaCl stock solution with a 1 mg/mL solution in an equal volume ratio. This solution was sonicated and incubated overnight. DNP were dialyzed through at least 12 L MilliQ water and

characterized by dynamic light scattering (DLS) and Laser Doppler microelectrophoresis (Malvern Nano ZS).

Transmission electron microscopy was used to characterize the size and morphology of the diamond nanoparticles. To prepare for TEM, DNP stocks were sonicated briefly, diluted in methanol, and drop cast onto 200 mesh copper grids with Formvar and carbon supports (Ted Pella, Inc., Redding CA). The grids were quickly dried near an open 65 °C oven and images were collected on a Tecnai T12 transmission electron microscope at an operating voltage of 120 kV. Nanoparticles were manually sized using ImageJ as follows. For the 5 nm DNP, which exhibited a spherical shape, the diameter was measured by drawing a line across the width of the nanoparticles. For the 15 nm DNP, which had an irregular shape, the area of the nanoparticle was first determined and related to its diameter by assuming a circular shape and using the equation for surface area of a circle.

2.2. Toxicity assays

Daphnids used for this study were obtained from Aquatic BioSystems, Inc. Test animals were acclimated for 1 day, and kept at 20 ºC with a 16:8 light/dark cycle in 500 mL of moderately hard reconstituted water (MHRW) (USEPA, 2003). Daphnids fed on the green microalgae, *Pseudokirchneriella subcapitata*, three times per week. All test subjects were fasted for a minimum 12 hours before treatment. Adult female daphnids (3 to 4 weeks old) were exposed to one of three different concentrations (1, 10, and 50 µg/L) of DNP suspensions for 24 h. Control daphnids were exposed to MHRW with no DNP. Three independent experiments were conducted per each nanodiamond suspension. For each experiment one set of exposures was used to measure the production of reactive oxygen species (ROS) within the gut tissue in

response to DNPs toxicity, and the second one to determine gene expression patterns of key genes related to cellular stress and oxidative response.

2.3. ROS Measurement

We conducted four independent experiments with three replicates per concentration (1, 10, and 50 µg/L) for 5nm PAH-DNP, 15nm PAH-DNP, 5nm OXI-DNP, and 15nm OXI-DNP. Adult daphnids were individually exposed in small beakers with 20mL of DNP suspensions for 24 hours. A total of 48 daphnids were tested per each type of these DNPs. Negative controls were maintained in MHRW on each exposure date. Gut dissection, staining tissue and imaging were followed as previously described in Dominguez et al. (2015) to detect ROS production in gut tissues in response to DNPs toxicity. In brief, guts were dissected after exposing them to DNPs for 24 h and individually stained in 0.25 mL of 10 µM CMH2DCFDA dissolved in PBS 1X for 20 min in the dark. Guts were washed off by momentarily submerging the tissue in PBS 1X to remove dye excess, then tissues were individually mounted on a glass slide plus 20 µL Vectashield (Vector Laboratories) to prevent photobleaching, finally a cover slip was carefully placed. Stained guts images were acquired using laser scanning confocal mode with an Inverted Research Microscope Eclipse Ti (Nikon, Japan) with an excitation/emission 493-556 nm for fluorescein dye at 20X magnification. Fluorescent enterocytes were quantified using the automatic nuclei counter plug-in available on ImageJ software (Schneider et al., 2012). For statistical comparisons, we considered the average number of fluorescent cells per gut for each concentration.

2.4. Quantification of stress genes by real-time PCR

We set up this experiment by exposing three adult daphnids were in small beakers with 60 mL of DNPs suspension for 24 hours. Two independent experiments were conducted with four replicatesper concentration (1, 10 and 50 µg/L) per each DNP type. Negative controls in MHRW were kept in MHRW without DNPs suspensions. After 24 h, gut tissues were removed, pooled (n=3), flash frozen in liquid nitrogen, and stored at -80 ºC until RNA extraction. RNA was isolated from these samples using RNAeasy Micro Kit (Qiagen) and treated with DNase I before cDNA synthesis. Reverse transcription was performed using 0.25 µg RNA, 10 µl 2X RT reaction mix, 2 µl RT enzyme mix, followed by addition of RNase free water in a total volume of 20 µl using SuperScript™ III First-Strand Synthesis Supermix (Life Technologies).

Quantitative real-time PCR (qRT-PCR) was performed using a StepOnePlus™ Real Time PCR System (Applied Biosystems) with 1 µl of cDNA for target gene in a volume of 20 µl containing 10 µl iTaq Universal SYBR® Green supermix 2X (Bio-Rad), 7 µl nuclease free water, 1 µl of forward primer (10 µM), and 1 µl reverse primer (10 µM) as described in Dominguez et al. (2015). Real-time PCR conditions were 95 °C for 10 min, 95 °C for 15 sec and 62 °C for 30 secs for 40 cycles. The gene expression levels of target genes: *glutathione S-transferase* (*gst*), *catalase* (*cat*), and *heat shock protein 70* (*hsp70*) were normalized to a corresponding endogenous control: *actin* (*act*) values and presented as fold expression change from control using the $\Delta\Delta C_t$ method. All primers used in the qPCR study are presented in Table 1. There are multiple genes or metabolites that can be measured for oxidative stress and ROS and we chose these three genes to investigate the potential impact of DNP at different points in the cellular and oxidative stress pathway to better determine where the nanomaterials may be acting. The gene

expression of *gst* in particular was chosen as it is a metabolite to protect cells against peroxides (Veal et al., 2002). It is known that *gst* genes have a broad substrate specificity which helps to protect cells against a range of toxic chemicals (Salinas and Wong, 1999) including reactive oxygen species (Kiruthiga et al., 2007; Letelier et al., 2010). For environmental studies, *gst* is considered as a good biomarker for oxidative stress for aquatic organisms such as fish (Karadag et al., 2014; Nabi et al., 2017; Yadav et al., 2015), daphnids (Borgeraas and Hessen, 2002; Kim et al., 2009; Wu et al., 2011) and has been shown to have a concentration-dependent increase in relationship to stress and damage upon chemical exposure (e.g. Kim et al., 2009).

2.5. Statistical analyses

SigmaPlot version 13.0 (Systat Software Inc., San Jose, CA) software for Windows was used for statistical analysis. ROS and gene expression data were tested for normality and variance homogeneity. Based on this statistical analysis, ROS data was evaluated by Kruskal-Wallis One-way analysis of variance on ranks with Dunn's multiple comparison to test for differences among treatments ($p < 0.05$) and gene expression (data as log2 transformed) by One Way ANOVA with Tukey's test for multiple comparison.

3. Results

3.1. Characterization of DNPs

Dynamic light scattering was used to determine the size and number mean of the diamond nanoparticles. The Z-average of nanodiamonds were 29.9 ± 0.4 nm for 5 nm OXI-DNP, 72 ± 3.1 nm for 5 nm PAH-DNP, 26.4 ± 0.2 nm for 15 nm OXI-DNP,

and 53.4 ± 0.3 nm for 15 nm PAH-DNP. The number mean of the diamond nanoparticles were as follows 9.3 ± 1.2 nm for 5 nm OXI-DNP, 32.5 ± 14.8 nm for 5 nm PAH-DNP, 16.1 ± 1.4 nm for 15 nm OXI-DNP, and 34.3 ± 1.5 for 15 nm PAH-DNP. To measure the charge of the functionalized nanodiamonds we employed Laser Doppler Microelectrophoresis. As expected, functionalization of the diamond particles changed the electrical charge from negative to positive. The zeta potential values of the nanodiamonds were -44.2 ± 1.8 mV for 5 nm OXI-DNP, 54.2 ± 1.5 mV for 5 nm PAH-DNP, -35.6 ± 1.1 mV for 15 nm OXI-DNP, and 65.3 ± 7.5 mV for 15 nm PAH-DNP (**Figure 1 a and b**). Values above +30 mV or below -30 mV are considered stable colloids due to electrostatic repulsion (Riddick, 1968). All the results from the nanodiamond characterization are summarized in Table 2. From TEM images, the 5 nm DNP exhibit a spherical morphology whereas 15 nm DNP exhibit a non-spherical morphology. Size analysis shows the diameter of the 5 nm DNP to be 5.1 ± 1.7 nm (n=507) and the diameter of 15 nm DNP to be 14.9 ± 6.4 nm (n=269) (**Figure 1 c and d**).

3.2. Effect of DNPs on ROS production and gene expression

Large functionalized DNPs caused the highest production of ROS. The 15 nm PAH-DNP and 15 nm OXI-DNPs caused gut cells to generate ROS in a dose-response manner (**Figure 3 c and d**). However, ROS generation was only significant at the highest concentration (50 µg/L) (15 nm PAH-DNP; P=0.002, H=15.3, 15 nm OXI-DNP; P<0.001, H=23). Surface functionalization also influenced the degree of ROS generated. When comparing ROS responses of large DNPs by concentration, it is noted that 15 nm OXI-DNP produces more ROS than the positively charged 15 nm PAH-DNP.

Smaller particles did generate some ROS, 5 nm OXI-DNP in particular caused enterocytes to generate ROS, but this effect is markedly lower than ROS levels produced by large DNPs. However, ROS response was significantly different at 10 and 50 µg/L (P=0.004, H=13.2). There was not a significant difference in ROS production in response to 5 nm PAH-DNP at any concentration with respect to control (P=0.061, H=7.3).

Despite the fact that larger particles caused greater ROS generation, smaller DNPs caused greater alterations in gene expression response related to oxidative stress than larger particles. The levels of *hsp70* transcripts were significantly upregulated at 50 µg/L for 5 nm PAH-DNP (P=0.004, H=13) and 5 nm OXI-DNP (P=0.008, H=11.7) with 2.0 and 1.9-fold increase respectively from control after 24 h of exposure. Treatment of 15 nm PAH-DNP did not cause a significant regulation of *hsp70* at any concentration **(Figure 4)**. In general, the expression levels of *gst* differed among all treatments. The *gst* transcripts were significantly expressed after exposure to 50 µg/L 5 nm OXI-DNP (P=0.045, H=8.042) treatment, resulting in a 1.2-fold increase. Neither of the DNPs wrapped with PAH caused significant regulation of *gst* transcripts **(Figure 5)**. Finally, none of the functionalized DNPs triggered significant changes in *cat* transcripts.

Surface functionalization made a difference in the ROS responses of daphnid guts. Both small and large OXI-DNPs induced enterocytes to produce more ROS than PAH-DNPs by ~30% and ~20%, respectively (**Figure 3**). For the gene expression study, small 5 nm OXI-DNPs significantly induced *gst* expression at high dose (50µg/L). Differential responses were observed for *hsp70* expression in response to surface functionalization of DNPs. Three out of four functionalized DNPs significantly upregulated this gene (5 nm and 15 nm OXI-DNPs and 5 nm PAH-

DNP). As indicated before, surface functionalization did not impact the expression of *cat* gene.

4. Discussion

The outcomes of this study suggest that the levels of ROS production and its antioxidant response in the gut tissue of *D. magna*, are affected by the DNP exposure. Both size and type of functionalization of DNP impact its effects on gut cells.

Our results indicate that the size of the nanomaterial has an impact on the production of ROS in the daphnid gut cells. In addition, this size dependent impact is related to gene expression related to oxidative stress, cellular protection against toxic chemicals, and metabolic function. In our study, large DNPs (15 nm) caused gut cells to produce more ROS that increased with increasing doses of exposure where 5 nm particles did not (**Figure 3 c and d**). However, this response was only significant for high 15 nm DNP concentration after 24-hour exposure. Previous *in vitro* and *in vivo* studies have reported minimal impact to low impact in terms of toxicity of DNPs but these were particles greater than 50 nm. In addition most of the endpoints in these previous studies involved cell proliferation assays, phagocytes activity, and survival (Burleson et al., 2009; Huang et al., 2014; Karpukhin et al., 2011; Yu et al., 2005). Likewise, longevity, reproductive potential, and stress response were not affected by DNPs exposures in nematodes (Mohan et al., 2010).

However, *in vitro* studies using small DNP (< 50 nm) reported some mild effects on several endpoints including oxidative stress (Solarska et al., 2010), genotoxic and mutagenic activity (Dworak et al., 2014), cell survival (Adach et al., 2016), and impairment of nucleolar activity (Mytych et al., 2014), but most authors suggest

DNPs are mostly biocompatible with cell lines. Our previous research as well as others suggests that small DNPs may increase oxidative stress *in vivo* studies (Cid et al., 2015; Dominguez et al., 2015; Karpeta-Kaczmarek et al., 2016); however, the mechanism for this toxic endpoint needs to be elucidated.

Interestingly, antioxidant response, measured as mRNA expression of *hsp70*, *gst*, and *cat* genes, was more impacted by small DNPs and reveal a different pattern when compared to the oxidative stress response for this time period of exposure. Although there was a noticeable dose-response trend for *hsp70* expression particularly triggered by small DNPs, it was not significant for all concentrations. Transcripts of *hsp70* were substantially upregulated in the 50 µg/L DNP exposures, but only in 5 nm DNP where we did not observe significant ROS production. Previous research demonstrated that gold NPs functionalized with PAH modulate the expression levels of *gst* in *D. magna* gut cells (Dominguez et al., 2015) which may suggest that an electrostatic effect occurs when positively charged NPs interact with negatively charged cellular membranes (Cho et al., 2009). Somewhat unexpectedly, only 5 nm OXI-DNP exposure at high dose caused a significant regulation of *gst* in this study. The expression of *cat* was also not significant despite oxidative stress. The differences observed between the cellular ROS levels and the gene expression responses could indicate a sensitivity of these measures to timing of the measurement and length of exposure. There is some possibility that changes in the expression of these genes for the 15 nm particles happened during an earlier timepoint and that the ROS measurements indicate that these cells have been overcome and are now showing ROS generation as stress. It is also possible that there are different cellular mechanisms at work when exposed to smaller particles versus larger particles and that ROS production in cells is not correlated with the

gene expression measures. However in our previous study these measures directly correlated with each other which suggests that in this case an alternate mechanism rather than alternate timepoint may be responsible for the impacts we see.

Our results also point that surface functionalization of DNPs also plays an important role in the ROS levels and gene expression seen in this study, although its effect appears to have a minor impact when compare to size effect. Oxidized DNPs appear to cause more damage than positively charged polymer wrapped particles. Both OXI-DNPs appear to upregulate expression of *hsp70* and *gst*, and this modulation was significant for 5 nm OXI-DNP in both cases.

It was unexpected that the 5 nm PAH-DNP treatment did not cause gut tissues to produce any significant ROS levels at any concentration. The 5 nm PAH-DNP treatment generated less than 50% of the ROS levels at the higher concentration when compared to 15 nm PAH-DNP. In our previous work we found that functionalized PAH-Au NPs (4 nm) produced significant ROS levels at all tested concentrations (Dominguez et al., 2015), we expected to see a similar effect in this study. We hypothesize that aggregation state may impact our results. Although the ζ-potential value for 5 nm PAH-DNP was +30 mV in pure media, which is considered a value that demonstrates colloidal suspension stability (Riddick, 1968), it is possible that DNP began to aggregate in the presence of the daphnids in the media, which emit various organic materials as pheromones and waste. Uptake of DNPs by *D. magna* may be a product of good DNP stability that results in a greater availability of NPs in suspension (Petersen et al., 2010). It is possible that PAH-DNPs sustained a significant aggregation/agglomeration state, and it should be carefully considered and evaluated in future *in vivo* DNP studies. Another possibility may be related to

the actual core material itself. Although we attempted to replicate the synthesis protocols to create analogous particles to our previous study it is possible that the interaction of the ligands with the core material created inherently different particle types that interacted differently with the organism and its tissues. This demonstrates the importance of not only the surface chemistry of the particle but the core as well as has been shown in our previous research (Arndt et al. 2013).

5. Conclusions

We conducted a comparison study to determine the effects of two types of functionalized DNPs on oxidative stress and antioxidant response. Our findings offer evidence that functionalized DNPs may impact small invertebrates such as *D. magna*, once these NPs are taken up from the aquatic environment. In addition, this study shows an apparent effect of size on the generation of ROS in daphnid gut cells. The results of the gene expression analysis of indicate that *hsp70* and *gst* may be affected by DNP exposures. More work is needed to assess the possible interaction of functional groups attached to DNPs with biomolecules present inside the daphnid gut environment.

Disclosure summary:
Authors have nothing to declare

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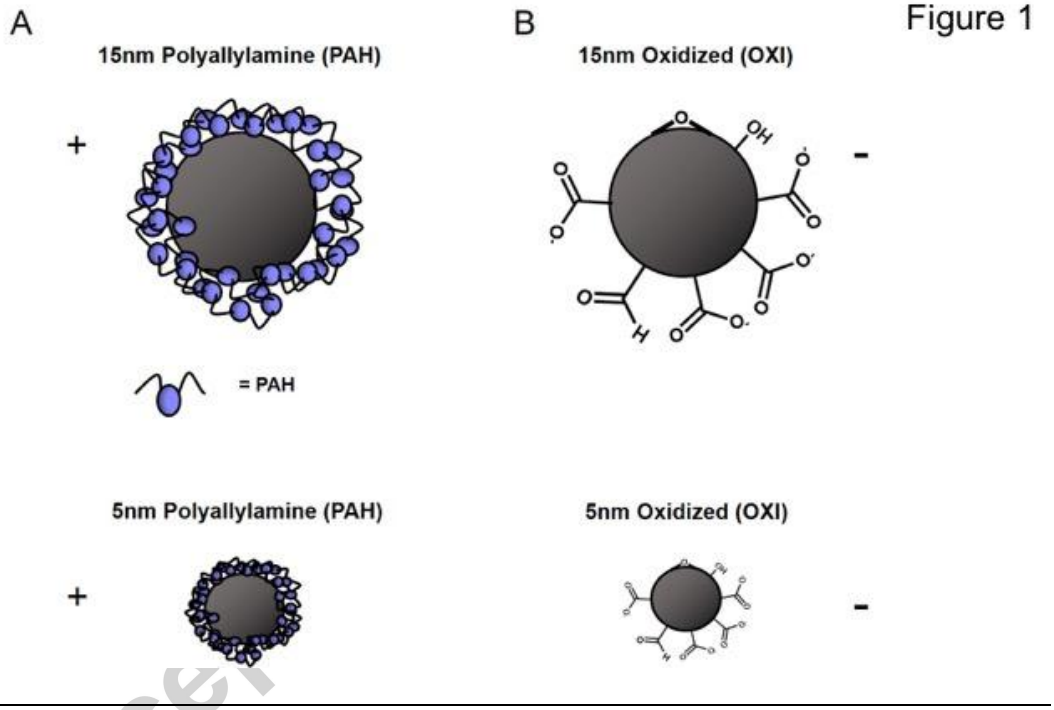

Figure 1

Figure 1

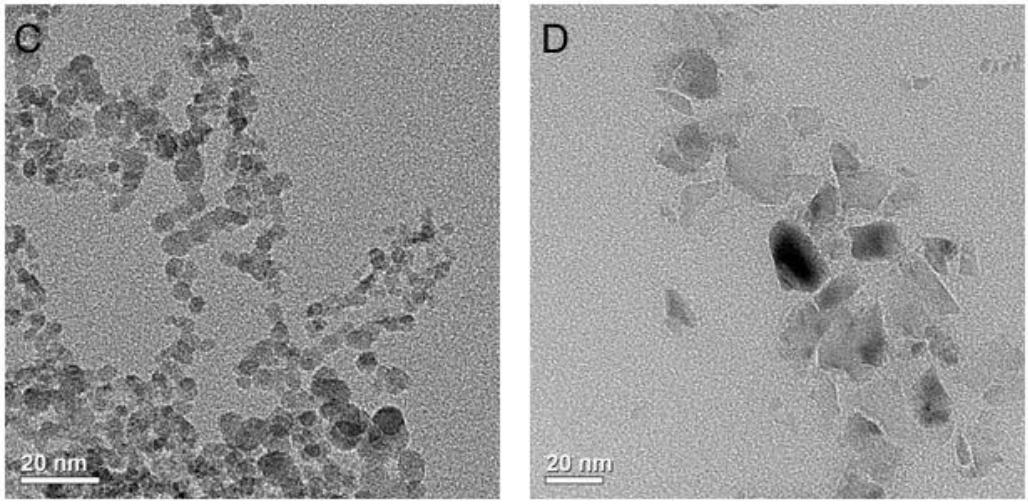

Figure 1. Types of functionalized diamond nanoparticles used in this study. A) 15 nm and 5 nm polyallylamine diamond nanoparticles (PAH-DNP), B) 15 nm and 5 nm oxidized diamond nanoparticles (OXI-DNP), and a representative TEM image of C) 5 nm DNP and D) 15 nm DNP.

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Figure 2

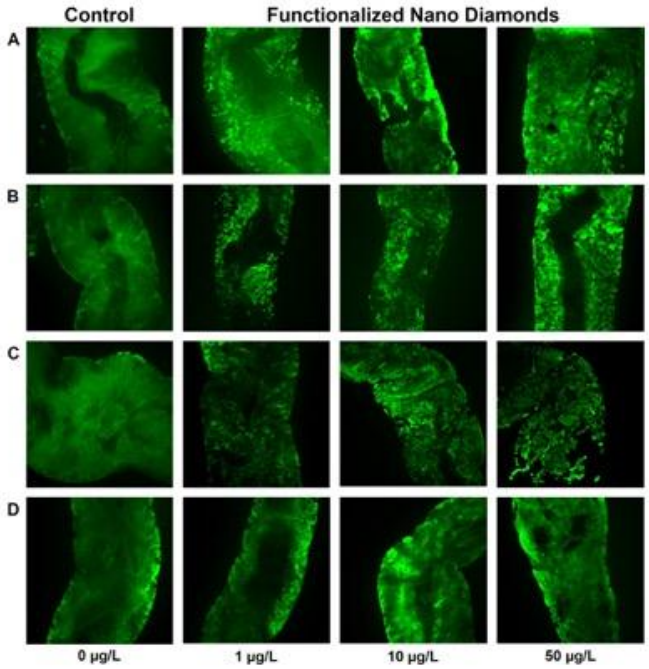

Figure 2. Diamond nanoparticles ingested by *D. magna* induced ROS activity after 24 h exposure. A) 5 nm PAH-DNP. B) 5 nm OXI-DNP. C) 15 nm PAH-DNP. D) 15 nm OXI-DNP. ROS production is indicated by a bright green fluorescent enterocyte. Confocal microscopy was used to acquire images at 20X magnification.

Figure 3

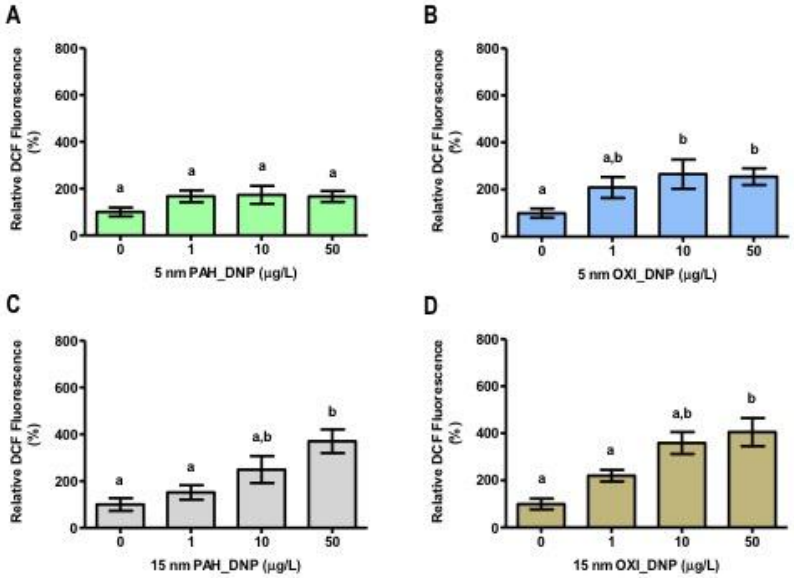

Figure 3. ROS levels in *D. magna* gut tissue exposed to functionalized DNPs. Data were graphed as mean±SE of labeled enterocytes by DCF after 24 h exposure. A) 5 nm PAH-DNP. B) 5 nm OXI-DNP. C) 15 nm PAH-DNP. D) 15 nm OXI-DNP. Letters represent significant differences for each treatment group after Kruskal-Wallace One-way analysis of variance on ranks with Dunn's multiple comparison test statistical analysis ($p < 0.05$).

Heat shock protein 70 (hsp70) expression

Figure 4

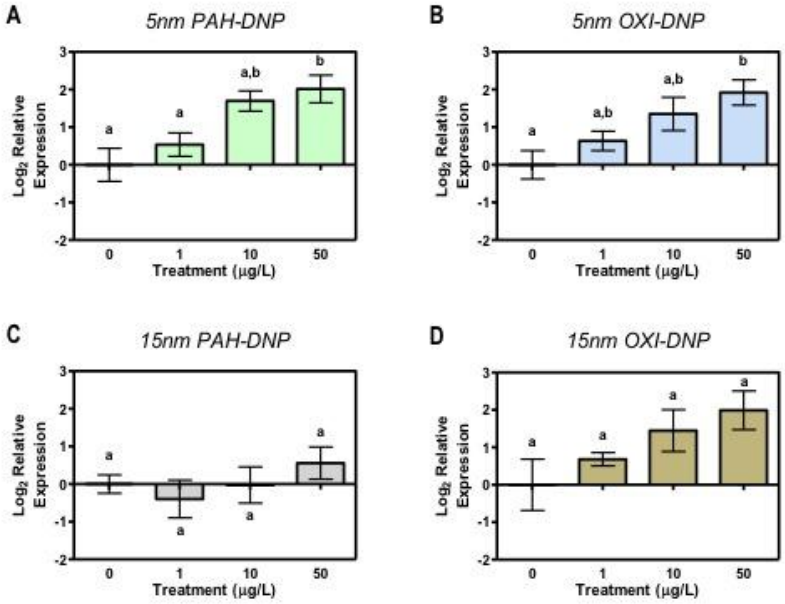

Figure 4. Expression levels of heat shock protein 70 *(hsp70)*. Data were graphed as mean± SE of fold expression change from control. A) 5 nm PAH-DNP. B) 5 nm OXI-DNP. C) 15 nm

PAH-DNP. D) 15 nm OXI-DNP. Letters represent significant differences for each treatment group after one-way ANOVA with Tukey's multiple comparison test statistical analysis ($p < 0.05$).

Glutathione-S-transferase (gst) expression

Figure 5

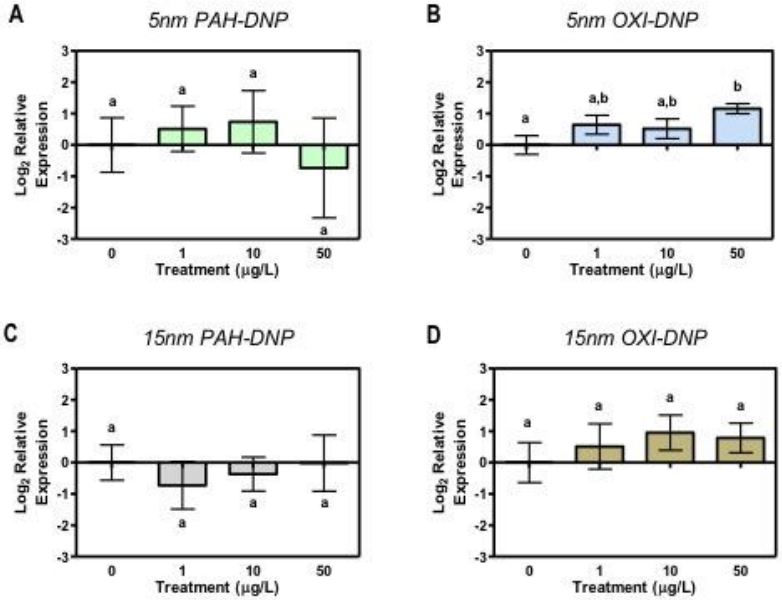

Figure 5. Expression levels of glutathione S-transferase *(gst)*. Data were graphed as mean± SE of fold expression change from control A) 5 nm PAH-DNP. B) 5 nm OXI-DNP. C) 15 nm PAH-DNP. D) 15 nm OXI-DNP. Letters represent significant differences for each treatment group after one-way ANOVA with Tukey's multiple comparison test statistical analysis ($p < 0.05$).

Catalase (cat) expression

Figure 6

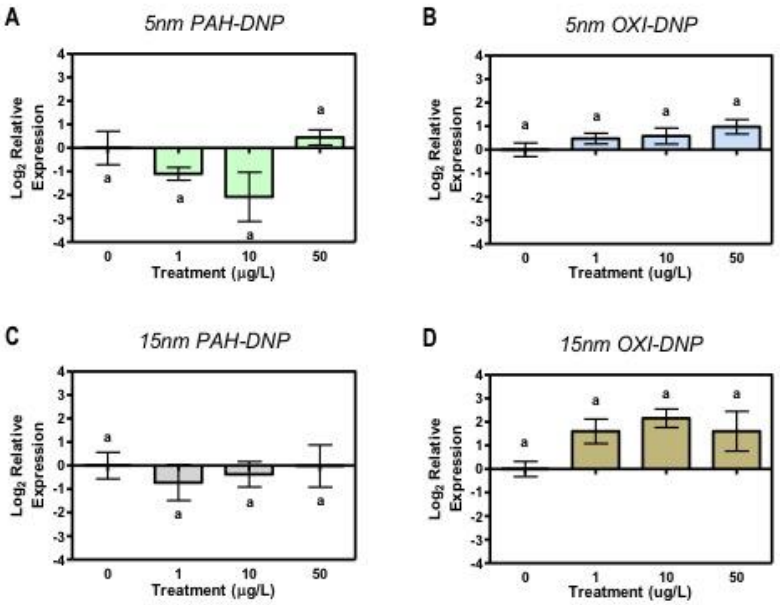

Figure 6. Expression levels of catalase *(cat)*. Data were graphed as mean± SE of fold expression change from control. A) 5 nm PAH-DNP. B) 5 nm OXI-DNP. C) 15 nm PAH-DNP. D) 15 nm OXI-DNP. Letters represent significant differences for each treatment group after one-way ANOVA with Tukey's multiple comparison test statistical analysis ($p < 0.05$).

Table 1. Primer sequences used for the qRT-PCR study

Primer name	Primer sense	Size bp	Sequences from 5' to 3'	Accession No.
gst	Forward	20	CAACGCGTATGGCAAAGATG	ES408246.1
	Reverse	21	CTAGACCGAAACGGTGGTAAA	
cat	Forward	21	CAGCATCATCGGCAGTTAGTT	GQ389639.1
	Reverse	21	CTGAAGGCAAACCTGTCTACT	
hsp70	Forward	21	CCTTAGTCATGGCTCGTTCTC	EU514494.1
	Reverse	20	TCAAGCGGAACACCACTATC	
act	Forward	21	CCTCCACCTCTTTGGAGAAAT	AJ292554.1
	Reverse	21	CAAGAATGAGGGCTGGAAGAG	

Table 2. Nanodiamond characterization.

Particle Name		Z-Average (nm)	Number Mean	Zeta Potential (mV)
5 nm	OXI-DNP	29.9 ± 0.4	9.3 ± 1.2	-44.2 ± 1.8
5 nm	PAH-DNP	72.0 ± 3.1	32.5 ± 14.8	54.2 ± 1.5
15 nm	OXI-DNP	26.4 ± 0.2	16.1 ± 1.4	-35.6 ±1.1
15 nm	PAH-DNP	53.4 ± 0.3	34.3 ± 1.5	65.3 ± 7.5

Research Highlights

1. We examined the effects of 2 sizes and 2 types of functionalized diamond nanoparticles.
2. Our results suggest a correlation between size and ROS production.
3. 5 nm DNPs instigate expression of *hsp70* and oxidized DNPs cause greater stress than PAH-ND particles.