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Investigation of the Toxicity of Amine-coated, Carboxyl-coated and Polyaniline-coated FeO Magnetic Nanoparticles in *Caenorhabditis elegans*

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Abstract

Since the introduction of nanotechnology there has been an increase in the use of nanoparticles (NPs) in the development of biosensors for the detection of bacterial pathogens. Consequently, research exploring their potential toxicity has also increased as some have been shown to be harmful depending on their size, shape, and chemical composition making it imperative that we understand their possible harm to humans and the environment. In this study we investigated the potential toxicity of three differently coated FeO Magnetic NPs (MNPs), amine, carboxyl, and polyaniline, on the *Caenorhabditis elegans* (*C. elegans*) nematode. Briefly, *C. elegans* were exposed to the singular coated-MNPs types at a concentration of 100 µg/mL and assessed for physiological effects on their metabolism, reproduction, longevity, and oxidative stress resistance. Exposure to singular coated-MNPs corresponded with a statistical decrease in their metabolic and acute oxidative stress resistance abilities, and revealed a trend towards lower reproduction and longevity. Taken together, these results add to the growing evidence that FeO coated-MNPs have an *in vivo* toxic effect on *C. elegans*. These findings advocate for a need to take safety precautions when discarding FeO coated-MNPs as they may pose a toxic health hazard to our environment and health.

Introduction

The introduction of nanotechnology has lead to an increase in the research and industrial application of NPs in fields related to energy, medicine, safety, and defense [1]. In particular, since the 2001 distribution of *Bacillus anthracis* through the United States postal system, much attention has been directed towards the use of DNA or antibody based biosensors for the biological detection of pathogens that incorporate the use of NPs [2-5]. Consequently, the more these biosensors are synthesized and used, the larger impact they may have on our environment as they start to accumulate as waste and decompose. Hence, there is a growing need to investigate and monitor the potential nanotoxicity of NPs to both our health and environment. Towards that end, some studies have shown that NPs, such as platinum NPs, are beneficial in their ability to reduce oxidative stress [6], however many others have shown NPs to reduce this ability [7-9]. Conversely, some studies have shown that the NPs themselves can impart toxicity, increase cellular oxidative stress, and damage [10]. For instance, many toxicological studies have been conducted using the *C. elegans* soil nematode as they would most likely be in first contact with decomposing NPs at waste sites. For example, using the *C. elegans* animal model it has been revealed that NPs synthesized from various materials, such as zinc, aluminum, titanium, silver, gold, and silica to name a few, can cause harmful effects on living organisms such as reduced egg production, number of off spring, and growth [11-14]. In particular, a study comparing the toxicity of silver-NPs to silver ions, found that while silver ion exposure did not impact growth, it resulted in reduced reproduction potential as seen with silver-NP exposure, although the silver-NP exposure had bigger reduction in reproduction [11].

Another important NP that is increasingly used in the development of biosensors is the Magnetic Nanoparticle (MNP) [15]. Many of these MNPs are synthesized from FeO (i.e., iron III oxide or ferric oxide) and contain a shell coating that serves as a binding surface. Not only have the MNPs been shown to be toxic by both *in vivo* and *in vitro* studies [16], but also a recent study has demonstrated that the Dimercaptosuccinic Acid (DMSA) shell coating of a FeO MNP additionally had toxic properties [17]. Therefore, this study aims to investigate the toxic potential of three differently coated FeO -MNPs (amine-MNPs, carboxyl-MNPs, and polyaniline-MNPs) that have been used in the development of DNA and antibody based biosensors for the detection of bacterial pathogens [2-5,18,19]. Using the *C. elegans* model, that has been extensively studied in toxicological investigations from the molecular to organismal level [20,21], we will evaluate the potential toxicity of these three differently coated-MNPs by assessing their impact on the worm's metabolism, reproduction ability, longevity, and resistance to oxidative stress. We hypothesize that similar to DMSA-coated FeO MNPs, *C. elegans* exposure to amine-MNPs, carboxyl-MNPs, and polyaniline-MNPs will result in overall lowered physiological health outcomes.

Materials and Methods

Nanoparticles

For these studies we used three different types of coated FeO -MNPs: amine-coated MNPs, carboxyl-coated MNPs, and polyaniline-coated MNPs. The MNPs were synthesized and provided by Dr. Evangelyn C. Alocilja (Michigan State University, East Lansing, MI, USA), which have been characterized previously [18,19]. Briefly, coated iron-core MNPs were determined to be approximately between 50 to 100nm in

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size by transmission electron microscopy. The electrical properties of the MNPs were investigated by four-point probe measurements and I-V measurements and had a resistivity of 0.385 Ωcm and showed ohmic behavior. MNPs were prepared for exposure to *C. elegans* in the following manner: the coated-MNPs were reconstituted in sterile water, and added to the *C. elegans* food source *Escherichia coli* OP50 (OP50) at a concentration of 100 μg/mL (100 mg/L), as a previous study investigating the toxicity of coated FeO -MNPs demonstrated harmful effects using this concentration [17]. The prepared MNP-OP50 mixture was added to experimental 35mm plates containing solid Nematode Growth Media (NGM) seeded with a spot (20 μL) of MNP-OP50 food mixture (experimental plates).Control 35 mm plates consisted of NGM seeded with a spot of OP50 only. *C. elegans* were then placed onto these plates for all studies conducted. Each plate contained 25 worms, and worms were transferred to fresh plates every 3 days.

Maintenance of *C. elegans*

Wild type Bristol N2 *C. elegans* were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA) and maintained as frozen stock until needed. All synchronized cultures were grown on 60 mm solid NGM plates seeded with a spot (100 μl) of OP50 for food [22]. Reproductive adults were placed onto fresh 60 mm NGM plates and allowed to lay eggs for 2-4 hrs, producing age-synchronized groups. Working cultures were maintained at 22 ± 1°C.

Fertility Assessment

Egg Production Assessment: Individual 1-day old adults, grown starting from the egg stage on experimental or control plates, were transferred into individual wells on a 96 well microtiter plate containing 20% bleach (sodium hypochlorite, NaOCl) and incubated for 8 min, to dissolve the cuticle. Remaining eggs were counted at 50X magnification using a K400 Motic dissection microscope (Martin Microscope Co., Easley, SC, USA). Each group (experimental and control) contained a total of 24 worms, per trial. A total of 4 trials were conducted. All groups were maintained at 22 ± 1°C.

Egg Laying Rate Assessment: Individual 1-day adults, grown starting from the egg stage on experimental or control plates were transferred to fresh 35 mm NGM plates seeded with 20 μL of OP50 only, and allowed to lay eggs for 4 hrs. At the end of the 4 hr period, eggs released were counted at 50X magnification using a K400 Motic dissection microscope (Martin Microscope Co., Easley, SC, USA). Egg rate was calculated as eggs laid/hr. Each group (experimental and control) contained a total of 4 worms, per trial, and 4 trials were conducted. All groups were maintained at 22 ± 1°C.

Defecation Assessment

Individual 2-day old adults, grown starting from egg stage on experimental or control plates, were transferred to fresh 35 mm NGM plates seeded with 20 μL of OP50 only, and allowed to acclimate for 5 min. Observation of pBoc movements, the interval between the obvious posterior contractions that load the rectum for defecation [23], were recorded over a period of 10 minutes (600s) using a K400 Motic dissection microscope (Martin Microscope Co., Easley, SC, USA). Defecation rate was calculated as the average pBoc movements/600s. Each group contained a total of 4 worms, per trial, and a total of 4 trials were conducted.

Longevity Assessment

Synchronized worms at 3-days of age (L4 stage) were transferred to 35mm experimental or control plates and then transferred daily to fresh

plates until the cessation of egg laying to avoid confounding generations. All groups were maintained at 22 ± 1°C. Worms were monitored over a 20-day period and scored as dead if they did not respond to a touch stimulus. Animals that showed bagging, exploded or crawled off the plates were censored. Each group contained a total of 100 worms over 4 plates (25 worms per plate), per trial. A total of 4 trials were conducted.

Oxidative Stress Resistance Assessment

Temporary Oxidative Stress Resistance: Synchronized worms at 3-days of age (L4 stage) were transferred to 35 mm experimental or control plates for 48 hrs, after which they were exposed to a temporary oxidative stress environment by being transferred to fresh 35 mm NGM plates containing a final concentration of 40 μM juglone (5-hydroxyl-1, 4-naphthoquinone, Sigma-Adrich) within the 20 μL of OP50 and incubated for 24 hrs. Juglone is a quinone that generates superoxide anion (O_2-) from molecular oxygen during metabolism [24]. The juglone was added to OP50, mixed, applied directly to the NGM plates which were then allowed to dry for 20mins, after which worms were transferred onto these plates for immediate exposure. Following the exposure, groups were transferred back to fresh corresponding experimental or control plates. Groups were transferred daily until the cessation of egg-laying to avoid confounding generations. Worms were monitored for the next 16 days and scored as dead if they did not respond to a touch stimulus. Animals that showed bagging, exploded or crawled off the plates were censored. All groups were maintained at 22 ± 1°C. Each group contained a total of 100 worms over 4 plates (25 worms/plate), per trial. A total of 3 trials were conducted.

Acute Oxidative Stress Resistance: Synchronized worms at 3-days of age (L4 stage) were transferred to 35 mm experimental or control plates for 48 hrs after which they were exposed to an acute oxidative stress environment by transferring worms to 35 mm plates containing 472 μM juglone within the NGM. The plates were prepared by dissolving juglone in 100% ethanol and immediately mixing it into liquefied NGM at 54°C. The mixture was poured into plates and after solidification they were seeded with 20 μL of OP50 and allowed to dry in a fume hood. Plates were used within 3 hrs of preparation. Survival was monitored every 30 min for the next 4 hrs after the initial transfer and scored as dead if they did not respond to a touch stimulus. Animals that showed bagging, exploded or crawled off the plates were censored. Each group contained at total of 100 worms over 4 plates (25 worms/plate), per trial. A total of 3 trials were conducted.
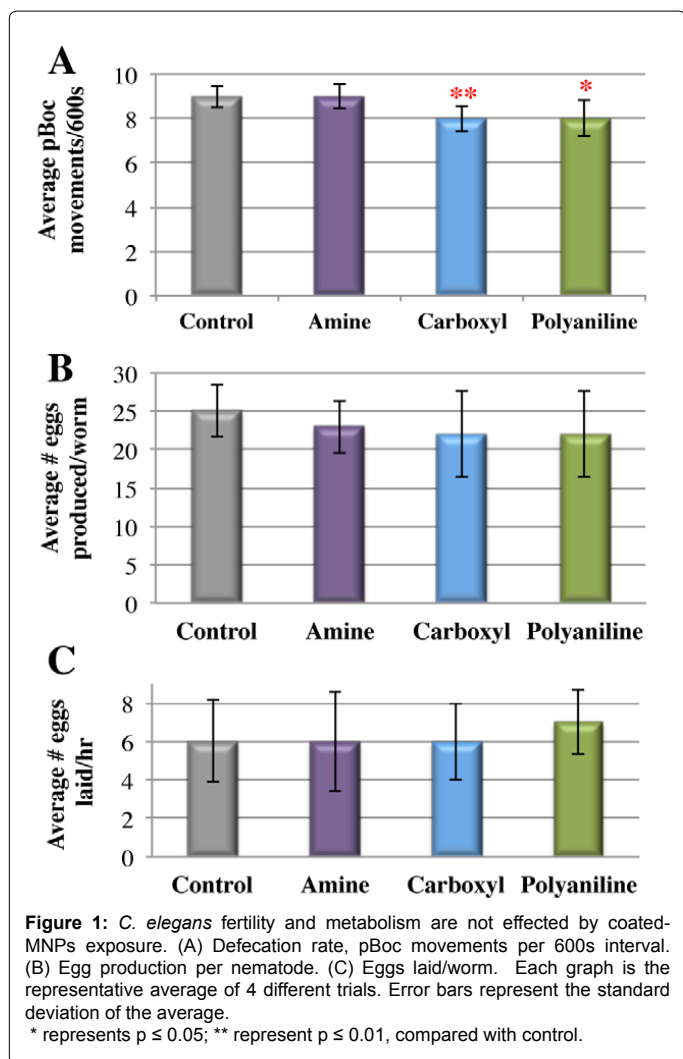
Statistical Analysis

Two-tailed Student's *t*-Test of equal variance was used for all experiments. A p-value ≤ 0.05 was considered to be statistically significant.

Results

Impact on Metabolic Health

As a first step towards determining the potential physiological impact of differently shell coated-MNP exposure on *C. elegans*, we assessed their ability to defecate normally as previous studies have shown that the defecation rate is a good outcome measure for metabolic health [25-27]. The defecation rate was measured by counting the number of pBoc movements, which represent the second stage of the defecation motor program, over 10 minutes (600s). Our results revealed that while there was no difference in the defecation rate between the control and amine-MNPs groups, there was a statistical decrease in defecation within the carboxyl-MNPs and polyaniline-MNPs groups (Figure 1A)

Figure 1: *C. elegans* fertility and metabolism are not effected by coated-MNPs exposure. (A) Defecation rate, pBoc movements per 600s interval. (B) Egg production per nematode. (C) Eggs laid/worm. Each graph is the representative average of 4 different trials. Error bars represent the standard deviation of the average.
* represents $p \leq 0.05$; ** represent $p \leq 0.01$, compared with control.

Both the carboxyl-MNPs (p=0.003) and polyaniline-MNPs (p=0.04) exposed groups had a defecation rate of 8pBoc movements/600s, which was 11% below that of the control and amine-MNPs groups, 9 pBoc movements/600s.

Impact on Reproductive Health

As previous mentioned, MNPs on their own have been shown to have toxic effects [16], and recent studies have also demonstrated their nanoparticles can negatively affect the reproductive abilities of various animal models [14,28,29]. Therefore, in order to determine if these differently coated-MNPs had any impact on reproductive health, we conducted two fertility assays on 1-day old adults: eggs produced within a single worms (Figure 1B) and the egg-laying rate (Figure 1C). Exposure of coated-MNPs at 100 µg/mL to *C. elegans* was not associated with any impact on their ability to produce or lay eggs as compared to controls. MNPs-exposed worms on average contained 22 eggs compared to 25 eggs in controls. Similarly, the average egg-laying rate was 6 eggs/hr for the control, amine-MNPs, and carboxyl-MPNs groups, while the polyaniline-MNPs had a rate slightly higher, but not significant, of 7 eggs/hr.
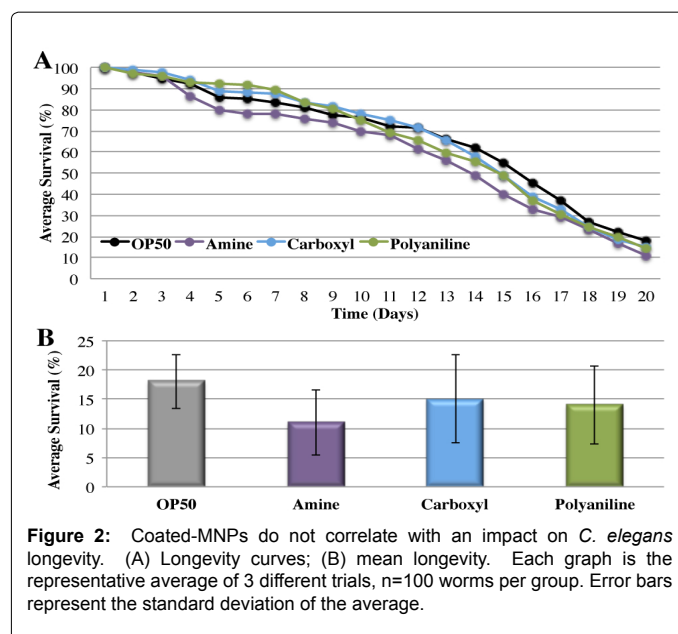
Impact on Longevity

As the exposure to carboxyl-MNPs and polyaniline-MNPs

demonstrated an effect on the defecation rate, our next step was to investigate if the coated-MNPs would have any impact on the longevity of the *C. elegans*, which normally live to approximately 20 days [30]. Beginning at the L4-stage and continuing all they way through the 20-day assay, our findings indicate that a continual exposure to coated-MNPs at 100 µg/mL did not correlate with any significant change on longevity (Figure 2A). On average the survival rate at the end of the assay was 18% for control, 11% for amine-MNPs, 15% for carboxyl-MNPs, and 14 % for polyaniline-MNPs (Figure 2B).
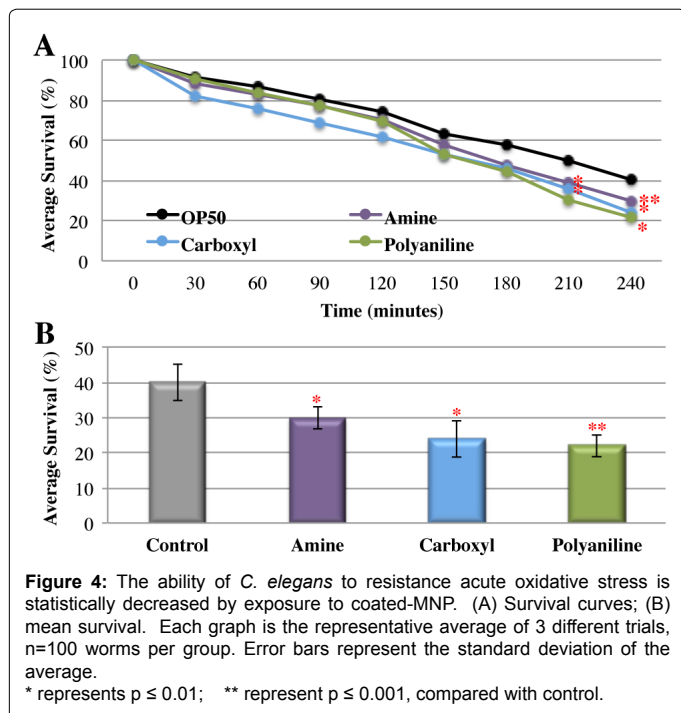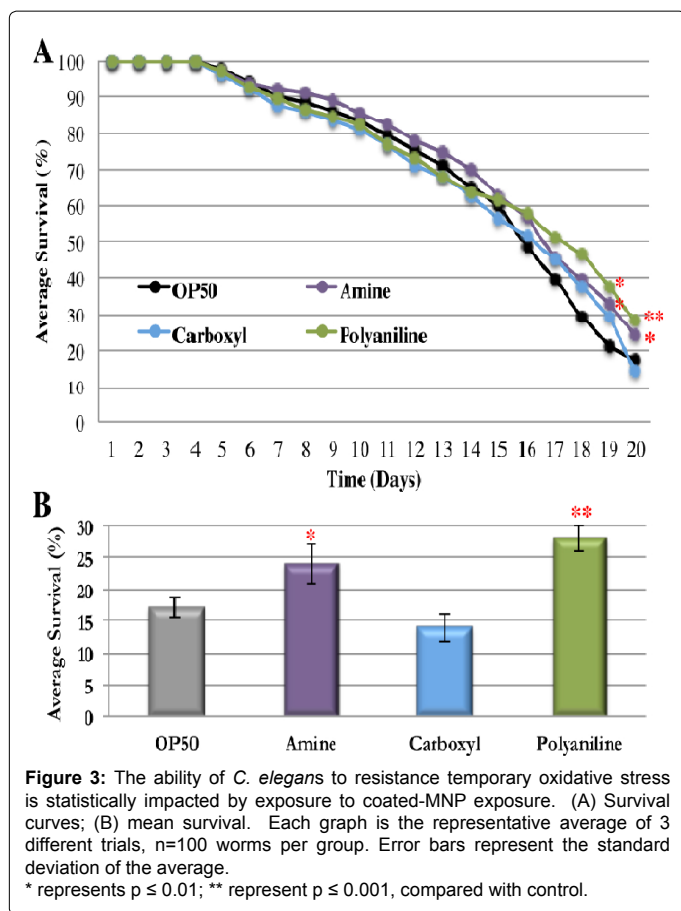
Impact on Oxidative Stress Resistance

Since oxidative stress is a strong contributor to reduced health, and previous studies have revealed opposing results in the ability of NPs to induce or suppress oxidative stress, we subsequently wanted to determine if exposure the differently coated-MNPs could affect the oxidative stress resistance of *C. elegans*. In order to create an oxidative stress environment, *C. elegans* were treated for 24 hrs with a low concentration of the chemical juglone, which has been shown to correlate with an increase in reactive oxygen species and oxidative damage [24]. Worms were exposed to coated-MNPs or control 3-days prior to the juglone treatment and then placed back in the presence of coated-MNPs or control for the remaining 16 days of the assay. The control group had an average survival rate of 17%, the carboxyl-MNPs 14%, the amine-MNPs 24%, and the polyaniline-MNPs 28%. The trend in increased survival rate can be seen throughout the last 5 days of the assay, but is only significant on the last two days (Figure 3A). Surprisingly, the short 24hr low level exposure to oxidative stress resulted in divergent outcomes, as it appears that exposure to amine-MNPs (p=0.01) and polyaniline - MPNs (p=0.001) significantly aided *C. elegans* in resisting temporary oxidative stress, while those exposed to carboxyl-MNPs (p=0.08) had no effect when compared to the control group although the trend was similar to the other two MNPs up to day 19 (Figure 3B).

The increased ability of the *C. elegans* to resist a low level of oxidative stress exposure prompted us to further investigate if they would also respond in this positive manner to higher acute oxidative stress conditions when exposed to the coated-MNPs. For this assay

Figure 2: Coated-MNPs do not correlate with an impact on *C. elegans* longevity. (A) Longevity curves; (B) mean longevity. Each graph is the representative average of 3 different trials, n=100 worms per group. Error bars represent the standard deviation of the average.

Figure 3: The ability of *C. elegans* to resistance temporary oxidative stress is statistically impacted by exposure to coated-MNP exposure. (A) Survival curves; (B) mean survival. Each graph is the representative average of 3 different trials, n=100 worms per group. Error bars represent the standard deviation of the average.
* represents p ≤ 0.01; ** represent p ≤ 0.001, compared with control.

Figure 4: The ability of *C. elegans* to resistance acute oxidative stress is statistically decreased by exposure to coated-MNP. (A) Survival curves; (B) mean survival. Each graph is the representative average of 3 different trials, n=100 worms per group. Error bars represent the standard deviation of the average.
* represents p ≤ 0.01; ** represent p ≤ 0.001, compared with control.

we exposed the worms to coated-MNPs or control starting at the L4 stage until they were 2-day old adults, at which time they were treated to a high concentration of juglone to induce an acute oxidative stress

environment. Interestingly, the findings of this assay demonstrated an inverse correlation with coated-MNP exposure and oxidative stress resistance (Figure 4). It can be clearly seen that throughout the 4 hr exposure period the control group faired better at resisting the high oxidative stress environment which resulted in an overall significant improvement during the last two assessment points of the assay, as compared to the coated-MNP treated groups which showed a significantly decreased ability (Figure 4A). At the end of the assay on average the control group had a survival of 40% compared to the lower amine-MNPs (30%), carboxyl-MNPs (24%), and polyaniline-MNPs (22%) groups (Figure 4B).

Discussion

To our knowledge, this is only the second study ever conducted that investigates the toxicity potential of coated-FeO -MNPs using the *C. elegans* nematode model. The findings of this study suggest that amine-coated, carboxyl-coated, and polyaniline-coated FeO -MNPs can impart toxicity to the living *C. elegans* nematode when administered at a concentration of 100 μg/mL (100 mg/L). In particular, the reduced defecation rate demonstrated in this study suggests that the carboxyl-MNPs and polyaniline-MPNs are having a harmful effect on the *C. elegans*. Interestingly, a 2012 study by Wu et al. investigating the toxicity of DMSA-coated FeO -MNPs on *C. elegans* also found an effect on defecation rate, but unlike our reduction in rate their study showed that the coated-MNPs resulted in an increase in defecation rate [17]. We hypothesize that the reduction achieved in our study might be occurring through the accumulation of coated-MNPs and eventual blockage within the intestinal cavity as has been previously shown by other studies investigating nanotoxicity of NPs [11,31]. Moreover, our assessment of the impact on reproductive health fall in line with similar studies exploring the toxicity of NPs on reproduction health [32-34], as a recent study revealed that while the initial NP exposure was not harmful, the subsequent generations had decreased reproductive health that took as long as the fourth generation to show signs of recovery [35].

The assessment on longevity, although not significant, also displayed a trend towards reduced longevity that can be seen in the amine-MNPs group, which was also unexpected, as these coated-MNPs had not shown any impact with our previous assays. We posit that the 7% difference in longevity between the amine-MNPs group and control did not result in a significant difference, as this difference can be attributed to the seemingly large deviation within worms of all groups, as similar studies have shown [14,31-33]. Lastly, our study also demonstrated that when *C. elegans* are exposed to coated-FeO -MNPs this correlated with a statistical reduction in their ability to resist oxidative stress. This outcome has also been shown in a similar study investigating the nanotoxicity of ceria NPs in *C. elegans*, which also demonstrated a reduced oxidative stress response when challenged with an acute oxidative stress environment induced by juglone administration [36]. Therefore, the outcomes of our temporary oxidative stress assay were unexpected, as past studies have shown either no effect or a reduction in oxidative stress resistance [14,32].

Taken together, our results demonstrating a statistical reduction in defecation rate and acute oxidative stress resistance, along with downward trends in egg production and longevity, indicate that precautions should be taken during the disposal of these NPs. Additionally, future efforts should focus on determining the mechanism through which these coated-MNPs are leading to a reduction in defecation rate, as well as explore the effect that they might have on multigenerational reproductive health. Equally, it would be a great

benefit to explore the impact of multiple nanoparticle exposure, as it is most likely that in a waste environment, these soil nematodes would be in direct contact with more than just one NP type.

Acknowledgement

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