Using *Daphnia magna* as a Test Organism to Study the Toxicity of Metal Ions and Nanoparticles

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Abstract

Wastewater discharged from industrial and commercial sources contain heavy metal ions and nanoparticles. These substances can pose a threat to the environment and our health. Our experiment focused on evaluating the acute toxicity of iron (II) sulfate and zinc oxide nanoparticles and the use of ginger as a treatment to alleviate Fe^{2+} ion- and ZnO nanoparticle-induced oxidative stress. *Daphnia magna* was the test organism used in this experiment. Young *Daphnias* were exposed to varying concentrations of a mixture of iron (II) sulfate with ginger extract, as well as zinc oxide nanoparticles with ginger extract, for 48 hours. The mortality (LC50 48h value) was obtained by probit analysis, and heart rates and behavioural movements of the *Daphnias* were counted through analyses of videos taken in the laboratory. It was found that the LC50 48h value increased in test groups containing a mixture of ginger with $FeSO_4$ as well as ginger with ZnO nanoparticles, indicating a decrease in mortality. Overall, our results show that ginger is effective in reducing mortality and oxidative stress of *Daphnia magna* that are exposed to iron (II) sulfate solution but is less effective in treating *Daphnia magna* that are exposed to zinc oxide nanoparticle suspension.

1 Introduction

1.1 Literature Review

Nanoparticles have been increasingly used in daily products. When such items are discarded, nanoparticles may be released and pollute waters causing health issues (Kumar et al., 2014). Even with the widespread use of nanomaterials in everyday life, knowledge about their benefits, risks and dangers of nanotechnology is still mediocre. As such, more has to be done to study its effects. Nanoparticles could be directly released into the air, water, sediment, and soil media during their manufacturing, use and disposal. Nanoparticles might also unintentionally form from the combustion of fossil fuels.

Zinc oxide nanoparticles have been used in a variety of applications, such as medicine, UV filters and paint (Keller, McFerran, Lazareva, & Suh, 2013). It can be found in sunscreen, cosmetics and personal care products applied on the skin, such products are washed off people's skin into shower

drains or swimming pools and end up in the sewers. According to Chang et al., 2017, wastewater treatment plants are not very effective in removing zinc oxide nanoparticles,this leads to a high risk of zinc oxide nanoparticles contaminating water supplies. Additionally, it also has the potential to be absorbed through the skin. A 2011 study led by researchers from Nanyang Technological University, Singapore (Ng et al., 2011), reported that zinc oxide nanoparticles found in consumer goods can potentially lead to the development of cancer by damaging genes.

Iron overload causes oxidative stress to the heart and is known to trigger cardiomyopathy (Gammella, Recalcati, Rybinska, Buratti, & Cairo, 2015). Cardiomyocytes found in the heart are rich in mitochondria and require large amounts of oxygen; at the same time, they have low levels of antioxidant enzymes (Doroshow, Locker, & Myers, 1980). As such, cardiomyocytes are particularly susceptible to ROS (reactive oxygen species)-mediated damage as when excess iron (II) ions are absorbed into the mitochondria, they catalyse a high rate of ROS formation via the Fenton reaction. Furthermore, iron deposits onto the walls of the heart making it more difficult for it to expand and contract, leading to heart failure eventually. Iron (II) sulfate is added to the test solution to test for toxicity effects of Fe^{2+} ions in this research.

In this study, experiments will be carried out to see if ginger, a commonly used antioxidant, could help to mitigate iron overload induced oxidative stress in *Daphnias*. Ginger (*Zingiber officinale*) contains many antioxidants that help prevent blood vessels from being blocked which may lead to heart attacks and strokes (Lindshield, 2018). Ginger helps prevent these diseases by decreasing the levels of blood lipid and blood pressure. Antioxidants function by stabilizing reactive oxygen species (ROS), which are highly reactive oxygen atoms causing oxidative stress in cells.

Daphnias are bottom level of the freshwater food chain, and they can be found in a wide range of habitats (Herbert, 1978). *Daphnias* have a short life cycle and are relatively easy to culture in the laboratory. They are also sensitive to a broad range of aquatic contaminants, making them suitable and widely used as a test organism for toxicity bioassays. In this study, we will be using *Daphnia magna* to assess the toxicity of iron (II) sulfate and zinc oxide nanoparticles so as to evaluate the effects of these substances, found in our wastewater, on aquatic wildlife. Additionally, we used antioxidants to ameliorate the toxic effects brought about by these substances, with reference to indicators such as the LC50 48h value and changes in *Daphnia* behaviour and movement.

1.2 Objectives

The objectives of this project are to investigate:

1. the effects of iron (II) sulfate and zinc oxide nanoparticles on the test organism, *Daphnia magna*, with reference to its mortality (LC50 48H), heart rate, feeding appendage curling rate, post-abdominal curling rate and hopping frequency over time.
2. the antioxidative potential of ginger extract to mitigate the harmful effects of the zinc oxide nanoparticles and iron (II) ions on *Daphnia magna*.

1.3 Hypotheses

We hypothesise that:

1. zinc oxide nanoparticles and iron (II) ions will adversely affect the heart rate and movements of *Daphnia magna*, and increase its mortality.
2. the above effects can be mitigated by adding ginger extract to the test cultures.

2 Methodology

2.1 Materials and Apparatus

The materials used in our experiment are zinc oxide nanoparticles (ZnO NPs), iron (II) sulfate, ginger extract (*Zingiber officinale*), microalgae suspension, spring water and universal indicator paper. Zinc oxide nanoparticles powder of <50 nm particle size was obtained from Sigma-Aldrich (Catalogue number 677450). Ginger powder was obtained from Organic Veda (Catalogue number 850703004726). *Daphnia magna* was obtained from FishPlay and microalgae suspension was bought from Shopee (link to product: https://shopee.sg/Green-water-daphnia-food-i.98047727.8211410933?position=0).

The apparatus and equipment used in our experiment are as listed: Plastic culture tank, microscope, orbital shaker, thermometer, water hardness test kit, aerator pump, glass beakers, mobile phone camera with a high frame per second (FPS), tripod.

2.2 Culturing *Daphnia magna* (OECD, 2004)

Daphnia magna were cultured in spring water in three separate 3-litre plastic tanks. About 50% of the culture water was replaced weekly to thin the population of *Daphnias* and ensure that the water remains clean (USEPA, 1982; Greene et al., 1988). Two drops of microalgae suspension were added daily into each culture tank. Water temperature was kept at room temperature. The cultures

were aerated with oxygen using an aerator pump. The pH of the water was measured using universal indicator paper to be 7, while water hardness (calcium and magnesium ion concentration in water) was measured using the water hardness test kit to be 120 mg/L, which is within the recommended range of 80 to 250 mg/L (Poirier et al., 1988).

2.3 Toxicity bioassay and Experimental Groups (OECD, 2004)

Adult females bearing embryos in their brood pouches were removed from the stock cultures 24h prior to the start of the test to obtain neonates (young *Daphnias*) that are ≤ 24h old. The adults were transferred to separate tanks containing spring water saturated with oxygen and the same amount of food as that used for culturing. The young that were found in the tanks the following day were used for the toxicity test. The neonates were placed in 1 control and 5 experimental groups as shown in the following table:

Group	Treatment	Data to be collected	Concentrations of test substance(s)
1 (Control)	-	Baseline for normal lifespan of *D. magna*	-
2	Ginger extract added	Effect of ginger on dependent variables	Ginger: 4, 6, 8, 10, 12 mg/L
3	Iron (II) sulfate added	Effect of Fe^{2+} ions on dependent variables	Iron (II) sulfate: 2, 6, 10, 12, 14 mg/L
4	Zinc oxide nanoparticles added	Effect of zinc oxide nanoparticles on dependent variables	Zinc oxide nanoparticles: 1, 1.2, 1.4, 1.6, 1.8 mg/L
5	Iron (II) sulfate and ginger extract added	Effect of ginger in counteracting Fe^{2+} ion induced oxidative stress	Iron (II) sulfate: 2, 6, 10, 12, 14 mg/L Ginger: 6mg/L
6	Zinc oxide nanoparticles and ginger extract added	Effect of ginger in counteracting zinc oxide nanoparticle induced oxidative stress	ZnO oxide nanoparticles: 1, 1.2, 1.4, 1.6, 1.8 mg/L Ginger: 6mg/L

Five neonates are transferred into one 50 ml beaker containing spring water and test substance(s). In groups 4 and 6, the five neonates are transferred into 50 ml centrifuge tubes and placed in the orbital shaker to ensure that the insoluble zinc oxide nanoparticles do not settle onto the bottom of the containers. The beakers are left in the laboratory for 48 hours, with no feeding or aeration throughout the whole duration. At 0-, 24-, and 48-hour intervals, the number of dead *Daphnias* per container was counted. Additionally, one *Daphnia* was retrieved from each container and observed

using a microscope under low power. The *Daphnia* was videoed using our phone cameras and these videos were analysed for 4 variables (Lovern, Strickler, & Klaper, 2007):

1. Heart rate: number of contractions of the heart per minute.
2. Feeding appendage curling rate: number of full rotations of the first thoracic leg per minute.
3. Post-abdominal curling rate: number of times the post-abdominal claw was brought proximally towards the thoracic appendages per minute.
4. Hopping frequency: number of times of downward thrusting of the second antennae below the helmet and then back above, per minute.

In groups 2 to 4, 5 different concentrations of the test substances were added into separate beakers/centrifuge tubes. Three 50 ml beakers/centrifuge tubes containing 5 neonates each were set up for each concentration within the experimental group.

The highest concentration of ginger extract added that resulted in 0% mortality of *Daphnias* (6mg/L) was used in groups 5 and 6, to ensure that the effect of ginger extract in mitigating the toxic effects of iron (II) sulfate and zinc oxide nanoparticles is the greatest, but simultaneously, it would not cause any harm to the *Daphnias*.

Similarly, in groups 5 and 6, 5 different concentrations of iron (II) sulfate and zinc oxide nanoparticles were added respectively into separate beakers/centrifuge tubes, with a fixed concentration of ginger extract. Three 50 ml beakers/centrifuge tubes containing 5 neonates each were also made for each concentration within the experimental group.

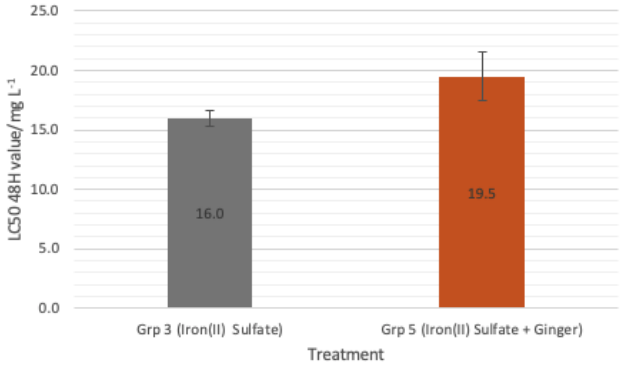
2.4 Data Collection (OECD, 2004; Environment Canada, 2014)

The percentage of *Daphnias* dead at 24/48-hour intervals of the acute toxicity bioassay would be used for calculations of mortality (LC50 24h/ LC50 48h). Probit analysis using Excel was conducted to obtain the LC50 48h value for mortality. Moving-Average Method was used to determine the LC50 48h value when there was no partial mortality in any replicate. Video recording of *Daphnias* using microscope under low power was analysed for heart rate, feeding appendage curling rate, post-abdominal curling rate and hopping frequency.

3 Results

3.1 Mortality

In our assessment of the substance's acute toxicity, the lethal concentration 50 (LC50) of *Daphnia magna* was obtained. Lethal concentration 50 (LC50) refers to the amount of a substance required to kill 50% of test animals during a predetermined observation period. In our test, the predetermined observation for iron (II) sulfate was 48 hours while that of zinc oxide nanoparticle was 24 hours due to limitations and time constraints.

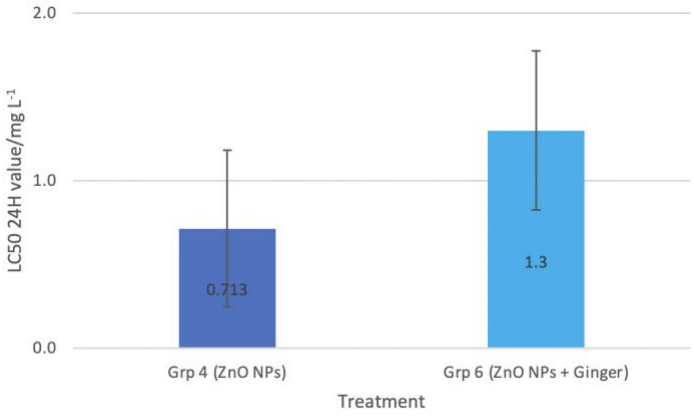


p = 0.0121, n=9

(Mann Whitney-U)

Figure 1. Effect of iron (II) sulfate and a mixture of ginger with iron (II) sulfate on LC50 48h (mortality) of D. magna.

There is a significant difference ($p<0.05$) in LC50 for cultures treated with iron (II) sulfate solution and that treated with the same solution with ginger added (Figure 1). Hence, the addition of ginger reduces the mortality of *Daphnia magna* that is treated with iron (II) sulfate solution, implying that ginger reduces the toxicity of iron (II) sulfate on *Daphnia magna*.



p=0.298, n=9

(Mann Whitney-U)

Figure 2. Effect of ZnO NPs on LC50 24h (mortality) of D. magna in the presence and absence of ginger extract.

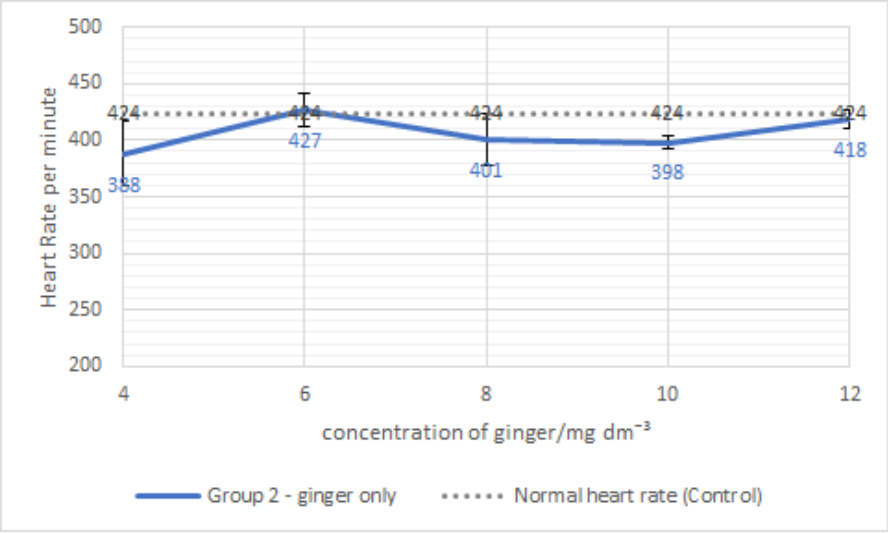
There is no significant difference ($p>0.05$) between the LC50 24h of cultures treated with zinc oxide nanoparticle solution and that treated with the same solution with ginger added (Figure 2).

These observations indicate that ginger is not effective in mitigating the toxicity caused by zinc oxide nanoparticles. Note that for ZnO nanoparticles, LC50 24h was recorded as most *Daphnias* did not survive past 24h.

As seen from Figures 1 and 2, the LC 50 48h of iron (II) sulfate and LC50 24h of ZnO NPs are 16.0 mg dm^{-3} and 0.713 mg dm^{-3} respectively. The addition of ginger to test solutions containing iron (II) sulfate and those containing zinc oxide nanoparticles increases the LC50 48h values (decreases mortality of *Daphnias*) significantly for iron (II) sulfate but not the LC50 24h of zinc oxide nanoparticles, suggesting that ginger increases the neonates' resistance towards the oxidative stress induced by iron (II) sulfate but not ZnO NPs.

3.2 Behavioural Changes

When *Daphnias* were treated with ginger in Group 2, their heart rates remained slightly lower than the original heart rate in the control group at the 48-hour interval, throughout all the concentrations of ginger tested (Figure 3). Hence, it can be concluded that ginger causes minimal harmful effects to the *Daphnia* heart.



p = 0.596, n=9

(Mann-Whitney U test)

Figure 3. Effect of varying concentrations of ginger on the heart rate of D. magna.

There is no significant difference ($p>0.05$) between the *Daphnia* heart rate in varying concentrations of ginger (group 2) and the normal *Daphnia* heart rate (control group 1). This implies that ginger does not significantly cause harmful effects to *Daphnia magna*. Kruskal Wallis test was used to find out if there is any significant difference across the different concentrations of

ginger tested. It showed that a significant difference in *Daphnia* heart rate (p=0.0300, where n=9) exists between varying concentrations (4-12mg/L) of ginger, potentially indicating that higher concentrations of ginger would result in enhanced effects to the *Daphnia* heart. Results from Group 3 show that there is a decrease in heart rate of *Daphnias* due to the addition of iron (II) sulfate into the test solution, when compared with the control group (Figure 4). In Group 5, when ginger is added to the test solution containing iron (II) sulfate, the heart rate of *Daphnias* increase back to the normal range. Firstly, this shows that Fe^{2+} ions were responsible for slowing down the *Daphnia* heartbeat, presumably by inducing oxidative stress to heart muscle cells (Gammella, Recalcati, Rybinska, Buratti, & Cairo, 2015). Ginger was also observed to be effective in mitigating the Fe^{2+} ion-induced oxidative stress since ingestion of ginger helped the heart rate of *Daphnias* to return to the normal range.

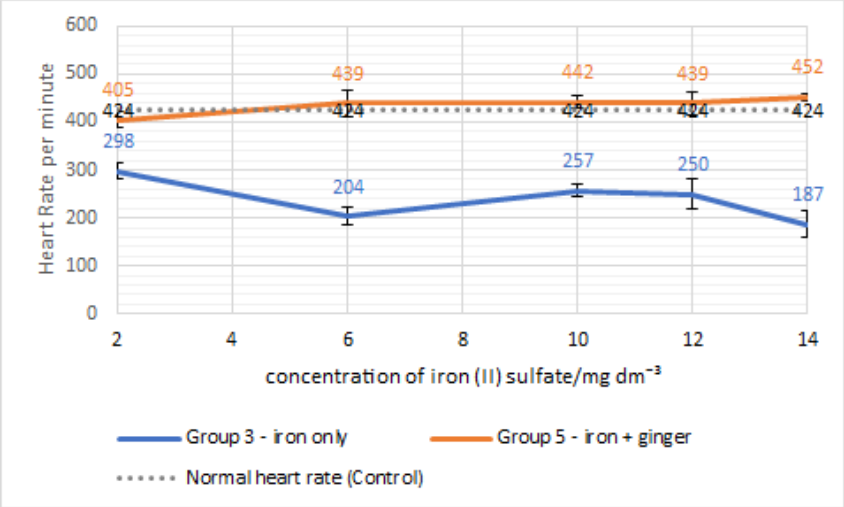


Figure 4. Effect of varying concentrations of iron (II) sulfate on the heart rate of D. magna in the presence and absence of ginger extract.

Solution	p-value	Is there a significant difference in the heart rate?
Iron (II) sulfate vs Control	0.0046 (n=9)*	Yes
Iron (II) sulfate + Ginger vs Control	0.128 (n=9)*	No
Iron (II) sulfate vs Iron (II) sulfate + Ginger	0.00512 (n=9)*	Yes

*Mann-Whitney U test

The p-values in the above table shows that iron (II)sulfate significantly reduces the heart rate of *Daphnias* but this effect is effectively countered by ginger (Figure 4). This may imply that ginger is able to reduce the oxidative stress induced by iron (II) sulfate.

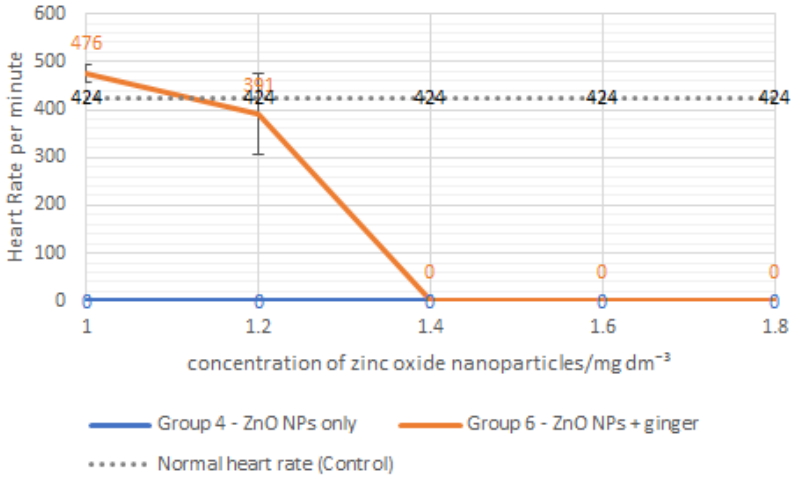


Figure 5. Effect of varying concentrations of zinc oxide nanoparticles on the heart rate of D. magna in the presence and absence of ginger extract.

However, ginger did not exhibit high effectivity in countering the effect of ZnO nanoparticles of *Daphnias* when zinc oxide nanoparticles were added to the culture at concentrations higher than 1.2 mg dm^{-3} (Figure 5). Ginger seems to only be able to mitigate the effect of zinc oxide nanoparticles on the heart rate of *D. magna* at lower concentrations of zinc oxide nanoparticles. This preliminary observation needs to be studied further by using ZnO nanoparticles at concentrations in the lower range but due to time constraints and limited laboratory access, this was not verified. Once this is verified the experiment can be repeated by treating the *Daphnias* with a mixture of ZnO nanoparticles and ginger to study the mitigating effect of ginger on ZnO nanoparticles. At 48 hours, all of the *Daphnias* in both groups 4 and 6 showed no contraction of the heart, although they could still move other parts of their body. However, at 24h, results imply, to some extent, that ginger was effective in keeping the heart rate in the normal range at lower concentrations of zinc oxide nanoparticles. As most *Daphnia magna* died at the 24-hour mark, it is not possible for us to carry out Mann Whitney-U test on groups 4 (*D. magna* treated with zinc oxide nanoparticles) and group 6 (treated with zinc oxide nanoparticles in the presence of ginger) to determine if the observations mentioned were significant.

4 Conclusion

From our data, we can conclude that ginger helps to reduce mortality of *D. magna* in both the iron (II) sulfate and zinc oxide nanoparticle set-up, as seen in Figures 1 and 2 where the LC50 of both substances are higher in the presence of ginger. Ginger appeared to have a significant effect on reducing mortality caused by iron (II) sulfate. However, preliminary observation showed that the effect it had on reducing mortality caused by zinc oxide nanoparticles was not significant. We believe this could be due to the fact that when the *Daphnia magna* was exposed to zinc oxide nanoparticles, they exhibited severe oxidative stress which is most likely due to an excessive generation of reactive oxygen species (ROS), causing many of them to die in 24h. This is evident where the hearts of most *Daphnia magna* stopped beating after 24 hours.

Our findings showed that ginger has the potential to mitigate oxidative stress in invertebrates. As the *Daphnia* heart is structurally different from that of vertebrates, studies need to be carried out in vertebrates to verify its effect in them.

5 Limitations and Future Extensions

Due to the COVID-19 pandemic, we did not have access to the laboratories during the June Holidays, and hence we were unable to consistently care for our *Daphnia magna* cultures, resulting in several culture tanks being wiped out in our absence. We lacked the time to carry out zinc oxide nanoparticle treatments at lower concentrations, this resulted in *Daphnia* magna being completely wiped out in certain test setups. Time constraints made it impossible for us to use 5 replicates for each group at each time frame, and we were unable to collect data at the 2-hour time point due to laboratory opening hours. Hence, this could have potentially impacted the accuracy of our results. Additionally, we did not have time to process the data for *Daphnias'* behavioural movements (feeding appendage & post-abdominal curling rate, hopping frequency).

Our study can be extended by repeating our experiments with more replicates so that ANOVA can be done with data collected to enable more statistically sound conclusions to be made. More investigations can be conducted on the oxidative stress induced by certain compounds, such as anticancer drugs and antioxidants such as Vitamins C and E. We could also use other methods to directly measure oxidative stress such as by measuring the damage to proteins or DNA within cells, or the activity of catalase enzymes.

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