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Article

The effect of microplastics on the speed, mortality rate, and swimming patterns of *Daphnia Magna*

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SUMMARY

Microplastics are a danger to the environment. They are polluting ecosystems, threatening the survival of many species and being ingested by humans. Two common microplastic polymers, polystyrene and polyethylene, contaminate fresh and saltwater ecosystems, thereby affecting life processes of marine organisms, such as *Daphnia magna*. While the effects of these plastics have been investigated individually, both plastic polymers have not yet been compared in one study. We sought to compare the effect of both polystyrene and polyethylene microplastics on the speed, mortality rate, and swimming patterns of *D. magna*. *D. magna* were divided into three groups which were fed either polystyrene microplastics and algae, polyethylene microplastics and algae, or only algae. We recorded the number of living *D. magna* and performed phototactic response tests to determine the effect of each condition on *D. magna* speed and swimming patterns. Overall, we found that microplastic consumption did not affect *D. magna* speed or mortality rate. However, microplastic consumption significantly increased "spinning" swimming patterns, which, based on prior research, suggests that microplastics may have a toxic effect on *D. magna*.

INTRODUCTION

Plastic is a material composed of synthetic polymers (1). Although plastic products are very common in society, they pose a danger for the environment and survival of many species (2). Most plastics are made from fossil fuels and are non-biodegradable contributing to their harmful impact on the environment (3). Furthermore, plastics often end up in landfills, where they take several years to break down and may instead blow away or enter water sources, such as the ocean, via water runoff, not being recycled and not breaking down for many years (1, 4). Over time, exposure to UV rays will cause plastics to degrade (5). Degraded forms of plastic consumer products less than 5 mm are known as microplastics (6).

Recently, the accumulation of microplastics in oceans has been discovered to be a threat to the survival of marine species and the health of humans (2). For example, microalgae are a common aquatic food source that form aggregates which collect and transport microplastics to the ocean floor (7). Through a process known as biomagnification, microplastics make their way up the food chain, increasing in concentration and toxicity (8).

Two major microplastic polymers that make up everyday items are polyethylene and polystyrene. Polyethylene is a compound that consists of ethylene monomers bonded together and is used in a majority of popular plastic consumer products, such as plastic water bottles, plastic bags, and toys (9). Polystyrene, on the other hand, consists of styrene monomers bonded together (10). Common polystyrene products include plastic cups, toys, and foam packaging (10). The main difference between polyethylene and polystyrene is that a carbon is bonded to a phenyl group in styrene instead of a hydrogen molecule in ethylene (**Figure 1**) (9, 10).

Daphnia magna is a freshwater organism also known as the water flea (11). Its size ranges from between 0.5 mm to 1 cm, and it can be found in a diverse range of aquatic environments, such as lakes and ponds (11). *D. magna* is commonly used as a model organism in biological assays because they are a compliant organism with a transparent exoskeleton, a visible internal structure, and sensitivity to many environmental changes (11, 12).

Current research on the effects of plastic polymers on *D. magna* shows varied results (13). While polyethylene microplastics ranging in size from 63–75 μm had no significant effect on the mortality of *D. magna*, those that ingested 1 μm polyethylene microplastics became immobilized (14, 15). Additionally, the ingestion of 1 μm or 10 μm polystyrene microplastics did not affect the mortality of *D. magna*, but *D. magna* that ingested 53 nm and 2 μm microplastics had an increased mortality rate (16-18). Therefore, results regarding the effect of the ingestion of polystyrene and polyethylene microplastics on the mortality rate of *D. magna* are inconsistent.

It has also been observed that *D. magna* increased in speed after ingesting polystyrene microplastics (16). We measured speed during phototactic response tests, which

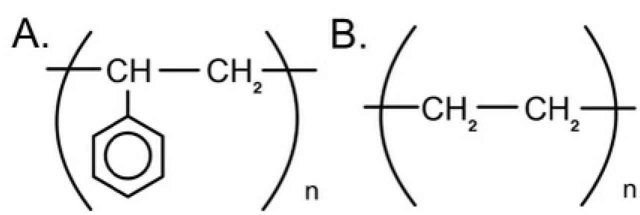

Figure 1: Chemical composition of microplastics. A) Polystyrene consists of multiple styrene molecules bonded together. A styrene molecule is made from a phenyl group, which has a hexagonal shape, bonded to a CH molecule, and to a CH_2 molecule. B) Polyethylene consists of multiple ethylene molecules bonded together. Ethylene, which has the molecular formula C_2H_4, is composed of two carbon atoms bonded together by a double bond, with each carbon also bonded to two hydrogen atoms. As the hydrocarbon chain grows, the double bond between the carbon atoms becomes a single bond.

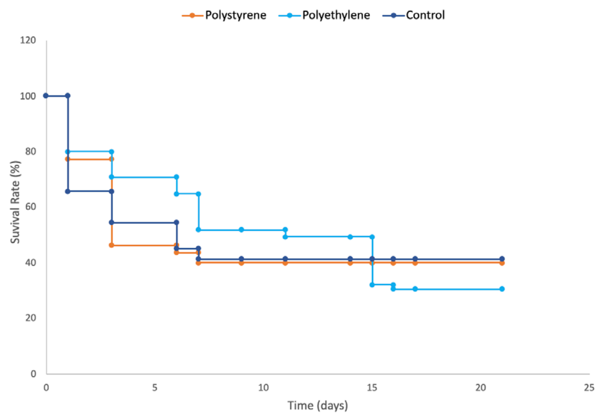

Figure 2: *D. magna* survival curve for a 21-day period. A Kaplan-Meier survival of *D. magna* fed either polystyrene (orange), polyethylene (turquoise), or only algae (control, navy blue) over a 21-day period (n = 35, starting number of *D. magna* in each group). No statistical significance was observed (one-way ANOVA, $p = 0.774$).

are tests to observe vertical swimming behavior in response to light (16). *D. magna* have a positive phototactic response, meaning that they respond to visible light by swimming toward it (19). Phototactic response tests can also be used to indicate *D. magna* exposure to a variety of toxic substances, or to assess the toxicity in an environment (20-23).

Studies have separately tested the effects of microplastic consumption on a majority of *D. magna*'s life processes; however, researchers have not yet compared the effects of polystyrene and polyethylene microplastics in a single study. Given that microplastic ingestion can lead to negative effects in *D. magna*, and these effects may vary based on different types of polymers of microplastics, this study may lead to a better understanding of the effects of microplastics on ecosystems around the globe.

We aimed to compare the effects that polyethylene and polystyrene microplastics have on *D. magna's* speed and mortality rate. This study seeks to clarify the effect of the ingestion of polystyrene and polyethylene microplastics on the mortality rate of *D. magna* given the mixed results from previous studies. We hypothesized that *D. magna* that consume polystyrene microplastics and polyethylene microplastics will have a greater mortality rate than those in the control group. We also hypothesized that *D. magna* that consume polystyrene microplastics and polyethylene microplastics will swim at a greater speed than those in the control group. Ultimately, we found no statistical significance between microplastic consumption and the mortality of *D. magna* or microplastic consumption and the speed of *D. magna*. However, a statistically significant spinning swimming behavior was observed in the experimental groups. These conclusions help further our understanding of the potential dangers of microplastic ingestion for *D. magna*, other aquatic organisms, and even humans.

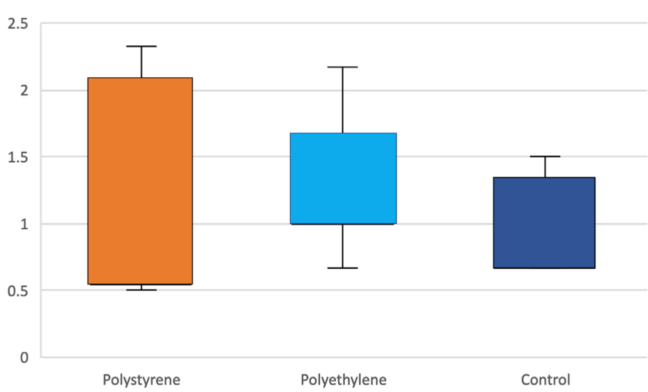

Figure 3: Ingestion of microplastics did not significantly affect *D. magna* swimming speed. *D. magna* in the control group had the greatest speed (0.92 ± 0.40 cm/s), followed by those fed polystyrene microplastics (1.21 ± 0.83 cm/s), and lastly those fed polyethylene microplastics (1.24 ± 0.51 cm/s). No statistical significance was observed (one-way ANOVA, $p = 0.667$) (n = 21).

RESULTS

We observed the effects of the ingestion of polyethylene and polystyrene microplastics on *D. magna* by comparing the mortality rate in each group (polyethylene, polystyrene, and control), as well as the speed and swimming behavior of the *D. magna* from phototactic response tests. We looked at the survival rate of *D. magna* over the course of a 3-week trial period. The survival curve demonstrates that groups fed polyethylene and polystyrene microplastics experienced a higher mortality rate at the end of this experiment than the control group (**Figure 2**). By day 21 of the trial, 41.25% of the *D. magna* in the control group remained living (N = 35), while only 39.90% of the *D. magna* in the polystyrene group remained living (N = 35), and 30.40% of the *D. magna* in the polyethylene group remained living (N = 35). A one-way ANOVA testing between microplastic type and *D. magna* mortality rate was conducted and showed a lack of statistical significance ($p = 0.774$).

In addition, we performed phototactic response tests to compare the speeds of *D. magna* in each group. We administered a phototactic response test for one individual from each of the 7 jars for each group on days 9 and 21 of the study. We found that *D. magna* fed polyethylene microplastics had the greatest speed (1.24 ± 0.51 cm/s). *D. magna* fed polystyrene microplastics had a speed of 1.21 ± 0.83 cm/s, and those in the control group had a speed of 0.92 ± 0.40 cm/s. (**Figure 3**). A one-way ANOVA testing between microplastic type and *D. magna* speed showed that these results were not statistically significant ($p = 0.667$).

During the phototactic response tests, we observed a spinning swimming behavior within all experimental groups, but not in any of the control groups of *D. magna* (**Figure 4**). We used the TrackMate plug-in from ImageJ to reveal the swimming path of *D. magna* (24). Those fed microplastics swam in a spinning swimming pattern. On the other hand, *D. magna* in control groups swam directly downward. For the remainder of the study, swimming patterns were tracked as a component of the phototactic response test. All *D. magna* in both the polystyrene and polyethylene microplastic groups exhibited this swimming pattern, while none of *D. magna* in the control showed this behavior. A chi-squared test between the observed swimming pattern of the *D. magna* (yes or no) and the test group (polyethylene or polystyrene or control) demonstrated a statistically significant difference ($p < 0.001$).

DISCUSSION

We compared the effects of the ingestion of polyethylene

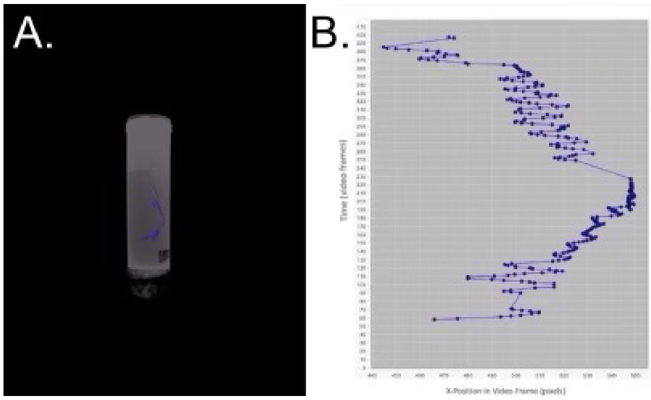

Figure 4: TrackMate image and graphic representations of *D. magna* swimming patterns. A) This swimming path is from a speed test of one *D. magna* from the third polyethylene experimental group. We used the TrackMate plugin from ImageJ to generate these lines, which show the track and the *D. magna*'s swimming patterns for this trial. B) The position (pixels) of *D. magna* was recorded over time (video frames) and graphed using a plugin (TrackMate (24)) for all three experimental groups. The swimming pattern for one *D. magna* from the polyethylene group is shown. A chi-squared test found statistical significance between the observed swimming pattern (yes or no) and the test group (polyethylene or polystyrene or control) ($p < 0.001$).

and polystyrene microplastics on the speed, mortality rate, and swimming patterns of *D. magna*. Mortality rate tests revealed that the *D. magna* within the control group experienced a high mortality rate at the beginning of the experiment (**Figure 2**). We hoped to clarify the effect of the ingestion of microplastics on the mortality rate of *D. magna*. Similar to prior studies, we observed that the ingestion of microplastic particles did not significantly affect mortality of *D. magna* (14, 16). The high mortality rate of the control group could have resulted from the location, as we performed this study in a school setting without constant control over ambient factors like temperature, and the control group was located closest to a heater in the classroom. Temperature fluctuation has been found to cause increased mortality rate for *D. magna* (25). Therefore, this may have also caused the high mortality rate of *D. magna* in the control group at the beginning of this study. Also, we did not collect data every day due to the school setting of our experiment. The lifespan of *D. magna* is about 8 weeks, but we only observed changes in mortality rate of a 21-day period, so a longer time span could have shown a statistically significant effect on mortality rate (11). These factors may have affected the results by skewing the data and causing the lack of statistical significance for mortality rate. Although there was no statistical significance, the survival curve indicated that the polystyrene and polyethylene groups had higher mortality rates than the control group by the end of this study (**Figure 2**).

D. magna in the polystyrene group exhibited the fastest speed, followed by those in the polyethylene microplastic group and the control group (**Figure 3**). The more the *D. magna* would spin, the faster they traveled to the bottom of the graduated cylinder. However, these differences in speed were not statistically significant, so the results failed to support the hypothesis. The lack of statistical significance is most likely due to the small sample size, which is a result of the high mortality experienced at the beginning of the study. Furthermore, although we made the room as dark as possible to prevent *D. magna* from being attracted to other light sources while running the phototactic response tests, other light sources, such as sunlight, could have affected the phototactic response of *D. magna* (19).

During the phototactic response tests, we observed a spinning swimming behavior in polyethylene and polystyrene groups. All *D. magna* in both the polystyrene and polyethylene microplastic groups experienced this swimming behavior, while none of the *D. magna* in the control group experienced it (**Figure 4**). The results suggest that microplastics may have a toxic effect on the swimming behavior of *D. magna*. This is consistent with research examining other toxic substances. Previous research demonstrated that *D. magna* developed erratic swimming behaviors after being exposed to a variety of toxins (23). For example, both titanium dioxide nanoparticles and sublethal levels of silver, NaCl and Ag^+ were found to cause a decrease in *D. magna* swimming speed when ingested — evidence of toxic changes to swimming behavior (20, 26, 27). The swimming behavior adopted by the *D. magna* after ingesting titanium dioxide nanoparticles was "hop and sink," contributing to a lower speed (27, 28). We observed a spinning swimming behavior in this study, which contributed to a faster speed. The spinning swimming behavior is not uncommon, though, as it was also observed after *Daphnia pulex*, an organism in the same genus as *D. magna* and another model organism, were exposed to the pesticide and toxin Carbaryl (29). These studies demonstrate that the ingestion of toxic particles may lead to erratic swimming patterns similar to the one observed in this study (27, 29). This suggests that the spinning swimming behavior observed by *D. magna* after the ingestion of polyethylene and polystyrene microplastic particles is a symptom of a toxic effect on the species. A plausible explanation for this swimming behavior could be either an escape response or loss of body-balance control in *D. magna* (23, 29). Spinning swimming patterns may harm *D. magna* by exhausting them quickly, depleting them of their resources, and causing them to be viewed as prey (29).

Microplastic consumption has been suspected to be toxic when ingested, and our study supports that idea. Future studies could expand on this concept by addressing the limitations described in this paper. For instance, a professional laboratory setting would allow for greater control over conditions like temperature, which would ensure better animal survival. Also, to confirm that *D. magna* ingested microplastics, an epi-fluorescent microscope could be used to view microplastics in their digestive tract (18). Future studies could create a fourth experimental group combining microplastic polymers into one environment to observe the combined impact on *D. magna*. This model with both microplastic polymers would better represent aquatic ecosystems as there are multiple microplastic polymers contaminating waters. In addition, changes in swimming behavior of *D. magna* after the ingestion of microplastics may suggest changes in behavior of other species as well. A future study could seek to observe how microplastics affect behaviors observed in other marine organisms, such as flatworms, shrimp, or isopods. One could also observe how microplastic polymers, such as polypropylene, affect other behaviors of *D. magna*, such as reproduction or heart rate. Finally, one could study the effect of microplastics on *D. magna* within an environment in which the toxins had previously been present. Most importantly, future studies should investigate further into possible relationships between microplastics and behaviors of various species to determine whether microplastics act as a toxin. Discovering the impact of microplastics on various species can help expand the understanding of the relationship between plastic pollution and the environment.

MATERIALS AND METHODS

Maintenance of *D. magna* and Microplastics

We observed the effects of two microplastic polymers on the speed and mortality rate of *D. magna* in this study. The type of microplastics included clear polystyrene polymer spheres that measured 0.95±0.025 mm in diameter with a density of 1.05 g/mL and clear polyethylene microspheres that measured 850-1000 µm with a density of 0.96 g/mL. These microplastics were purchased from Cospheric. A total of 35 *D. magna* were divided into seven 30 mL jars that each contained 5 *D. magna* and 25 mL of fresh pond water. Containers either received 25 polystyrene microplastics and 1 mL of algae per week separately, 25 polyethylene microplastics and 1 mL of algae per week separately, or 1 mL of algae per week (the control). The algae, *D. magna*, and fresh pond water were purchased from Carolina Biological Supply Company. In addition, the pond water and excess microplastics not consumed by the *D. magna* in each container were replaced weekly. We used tweezers to temporarily remove the microplastics from the container, and a pipette to temporarily take the *D. magna* out of the container. We emptied the pond water, cleaned the container, and added new water. The *D. magna* and microplastics were added back to the container to resume the assay.

Mortality Rate Test

To measure mortality rate, the number of living *D. magna* per container were counted every other weekday (Monday, Wednesday, Friday) for 21 days. *D. magna* were considered dead macroscopically if they sank to the bottom of the container and failed to move after a light stir of the container. We created a Kaplan-Meier curve in Excel to analyze the trend of *D. magna* mortality rate.

Speed Test

To measure speed, we administered a phototactic response test on one *D. magna* from each container. To do this, we placed one *D. magna* into a 30 mL graduated cylinder filled with 25 mL of pond water. A flashlight was held above the graduated cylinder (**Figure 5**). The *D. magna* were recorded on a Canon Eos Rebel T6 camera swimming from the top of the graduated cylinder toward the light at the bottom of it. We considered the video recording to be complete when the *D. magna* reached a piece of blue tape indicating a 6 cm distance from the top of the water. We ran phototactic response tests twice throughout the experiment (on days 9 and 21).

To analyze the results, we uploaded the video onto ImageJ software. First, we converted the video to grayscale, as required by TrackMate. Next, we adjusted the brightness and contrast to increase the visibility of the *D. magna*. Once visible, we determined the first and final frames of the 6 cm path of *D. magna*. Each frame was matched with its corresponding time to assess the speed. We confirmed the 6 cm distance and divided by the time in seconds. We created a scatter plot in Excel to demonstrate these results (**Figure 3**).

Figure 5: Phototactic response test setup. This is a picture of the setup for the phototactic response test. We held a flashlight above the graduated cylinder to shine light towards the bottom. Then, we used a blue tape marking to identify a 6 cm distance from the top of the water. The *D. magna* were then dropped into the graduated cylinder and recorded swimming towards the light, and we stopped the recording once they passed the blue marker.

Swimming Behavior Test

After viewing the video footage of the speed tests, it became obvious that *D. magna* in experimental groups exhibited spinning swimming patterns. While this was not an initial variable in our experiment, we felt that it was too profound to ignore, and added it to our study. This was not a blinded analysis; however, it was readily apparent that there was a striking difference in swimming behavior.

To examine the swimming behavior of *D. magna*, we analyzed the videos from the speed tests with ImageJ software. Specifically, we used footage to track swimming patterns of *D. magna* with the TrackMate plugin (24). We uploaded the video using FFMPEG to extract video frames as images, converted it to grayscale, and adjusted it in brightness and contrast so the shape of the *D. magna* was visible in the graduated cylinder. We utilized TrackMate for a 30-pixel-sized moving figure in the video. Once circles that predicted spots of movement were displayed on the video, we adjusted the variables to remove the spots that did not represent the specific track of the *D. magna*. The software used the final spots to determine the swimming path of the *D. magna*. We illustrated The swimming path of the *D. magna* in the graduated cylinder generated by the program on a line graph (**Figure 4**).

Statistical Analyses

In the statistical program SPSS, we ran a one-way ANOVA to analyze mortality rate and the results from phototactic response tests.

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