Entry to the Stockholm Junior Water Prize 2022:

Plankton Wars: An Innovative Analysis of *Daphnia* Genotype Biomanipulation for Algae Bloom Prevention

Annabelle M. Rayson

Ontario, Canada

2. Preliminary Matters

2a. Abstract

Harmful algae blooms plague aquatic ecosystems around the world. They impact water quality and ecosystem diversity, cause dead zones, and cost the fishing and tourism industries millions of dollars. From past research, *Daphnia magna* was discovered to be the best species of freshwater zooplankton to biomanipulate to treat and prevent algae blooms. However, very little is known about the species' distinct genotypes which could allow for more effective and sustainable biomanipulation for algae bloom treatment and prevention. In this experiment, the abilities of four genotypes of *D. magna* to consume algae were compared and then the most effective genotype was tested in different environmental conditions of pond mud (aquatic microbes), nutrient pollution, microplastics, and calcium carbonate to discover its success in the ever-changing Great Lakes. It was discovered that genotype 4 is the ideal genotype of *D. magna* to biomanipulate to treat and prevent harmful algae blooms, can effectively do this in nutrient and plastic polluted environments, and can have their health and success improved through calcium carbonate and naturally occurring aquatic microbes.

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2c. Key Words

biomanipulation · harmful algae blooms · eutrophication · freshwater · aquatic ecosystems · dead zones · hypoxia · genotype · nutrient pollution · plastic pollution · microplastics · decalcification · liming · *Daphnia magna* · algae · aquatic microbes · keystone species · filter feeders · toxicity · gut microbiota · climate change · biodiversity · cloning · neonate · Great Lakes

2d. Abbreviations and Acronyms

D. magna (*Daphnia magna*) · ANOVA (Analysis of variance) · *C. fusca* (*Chlorella fusca*) · *M. aeruginosa* (*Microcystis aeruginosa*) · LED (Light-emitting diode)

2e. Acknowledgements

I would like to express my deepest appreciation to all those who provided me with the possibility to complete my project. I would like to thank Kurtis Tamming from Lambton College who provided me with a hemocytometer; Mrs. Diane Yurkewich from St. Patrick's High School who provided me with a microscope, calcium carbonate, stock solution calculation assistance, and encouragement and support; Carol D'Andrea from Nautilus Environmental who provided me with *C. fusca* and encouragement and support; Dr. Shahmohamadloo and Dr. Rudman from the University of Guelph and Washington State University who provided me with the *D. magna* genotypes, *M. aeruginosa*, test tubes, test tube racks, the LED light fixture and timer, a light table, ImageJ instructions, feedback, and advice; and most importantly, my family for their encouragement and support and allowing me to turn our basement into a limnology research lab.

2f. Biography

Annabelle Rayson is a grade elven student at St. Patrick's High School who is passionate about improving the world around her, helping people and solving problems. She enjoys math, science, geography, history, writing and politics. She is a competitive curler, volunteer youth curling coach and member of her school's curling team. She plays the violin, viola and alto saxophone and performs in her school's concert band. She has been the Chair of the Sarnia Shoebox Project, a non-profit charity that provides holiday gifts for homeless women, for four years and has raised over $60,000 to date for this cause and was awarded a place on the Sarnia Mayor's Honour List for her work. Annabelle is the Co-Founder and Co-President of her school's Social Justice Club where she has worked to raise awareness towards water injustices within Canada and the Global South. Annabelle is a member of her local Member of Parliament's Non-Partisan Youth Advisory Board and the Ontario Youth Environment Council. Annabelle is a SHAD alumna as well as a Hugh O'Brian Youth Leadership Western Ontario and World Leadership Conference alumna and is the 2022 Student Trustee for her school. Growing up in Southwestern Ontario, the Great Lakes have been a large part of her life and she is incredibly

passionate about freshwater protection and conservation. Annabelle has been participating in the Lambton County Science Fair focusing on projects for environmental protection and sustainability since the fourth grade and has participated in the Canada Wide Science Fair three times medalling bronze in 2019, silver in 2021, and gold in 2022. Furthermore, with her 2022 project, she won *The Beaty Centre for Species Discovery Award*, *The Canadian Meterological and Oceanographic Society and the Weather Network Award*, *The Environment and Climate Change Challenge Award*, the *Platinum Award for Best Senior Discovery Project*, and the *Crystal Award for Best Discovery Project*. Annabelle was inspired to create her 2022 project after reading about the impacts of harmful algae blooms on Lake Erie and the communities that surround it.

3. Introduction

Over 300 harmful algae blooms were reported around the world in 2018 [1]. Harmful algae blooms destroy water quality and when they decompose, they absorb excessive amounts of oxygen causing hypoxia and dead zones in aquatic environments. In fact, algae blooms are one of the main causes of fish kills [2] and their harmful toxins make water bodies unsafe for recreational use. In communities around Lake Erie, a Great Lake significantly impacted by harmful algae blooms, these two factors contribute to an estimated $272 million loss to the Lake Erie economy over a 30-year period if nothing is done to treat and prevent algae blooms [3]. Algae blooms also contaminate and raise costs of drinking water treatment; destroy aquatic ecosystems by blocking out sunlight, causing dead zones, and releasing toxins; and cause severe illness and possibly death in humans. With climate change increasing precipitation rates, more agricultural runoff and fertilizer will enter aquatic environments causing increased eutrophication and algae blooms [4]. Harmful algae blooms are not going away anytime soon and a method to treat and prevent them is desperately needed. From past research, *Daphnia magna,* a keystone filter-feeding species of freshwater zooplankton, was discovered to be the best species of freshwater zooplankton to biomanipulate to treat and prevent harmful algae blooms. Biomanipulation is when an ecosystem is manipulated to create a desired effect; it "is a type of biological engineering in which manipulations of biota are used to reduce objectionable algal *types* and biomass in addition to, or to supplant, reductions of nutrient loading" [5]. However, very little is known about the species' distinct genotypes which could allow for more effective, successful, and sustainable biomanipulation of the species for algae bloom treatment and prevention. In addition, the Great Lakes and freshwater ecosystems are very dynamic and there are a variety of environmental factors such as the presence of

microplastics and aquatic microbes, decalcification, algae toxicity, and nutrient pollution which in turn could help or hinder the ability of *D. magna* to effectively treat and prevent harmful algae blooms.

4. Purpose

The purpose of the first experiment was to test the effectiveness of four genotypes of *D. magna* independently on two different types of algae, *Chlorella fusca* (non-toxic) and *Microcystis aeruginosa* (toxic), in three different algae combinations (100% *C. fusca*, 75% *C. fusca* and 25% *M. aeruginosa*, and 50% *C. fusca* and 50% *M. aeruginosa*) to test for toxicity. The purpose of the following experiments was to compare and test the health implications, biomanipulative success, and effectiveness of the selected *D. magna* genotype at algae bloom treatment and prevention when exposed to pond mud (aquatic microbes), microplastics, nutrient pollution, and calcium carbonate in the three different algae combinations and levels of toxicity.

5. Hypothesis

All genotypes should result in significant algae decreases and should be able to consume the toxic *M. aeruginosa*, but less effectively than the non-toxic *C. fusca*. The genotype exposed to pond mud should be better able consume the toxic algae due to exposure to microbes and an improved gut microbiota. They should have a better tolerance to the toxic algae and have low mortality as well as increased reproduction. The clones exposed to microplastics should consume the least algae because they will consume the microplastics which will clog their digestive tracts and make them feel full. These clones should also have low reproduction and high mortality rates, especially in the more toxic algae concentrations. The clones exposed to nutrient pollution should be less effective at algae bloom treatment and prevention as the nutrient pollution will increase the number of algae and algae growth making it more difficult to be entirely consumed. The clones exposed to calcium carbonate should have increased reproduction and body length growth as well as low mortality rates and significant algae decreases and have better success in the more toxic concentrations of algae.

6. Materials and Procedure

6a. Experiment One

A table and hanging LED light fixture with a timer were set up with the light timer set for 16 hours of light and 8 hours off. *D. magna* genotypes 4, 9, 11, and 27 were cultured and cloned. 75 test tubes were cleaned and labelled numbers 1-75. 25 test tubes were filled with 12 mL of *C. fusca* using a pipette. Another 25 test tubes were filled with 9 mL of *C. fusca* and 3 mL of *M. aeruginosa* using a pipette. The remaining 25 test tubes were filled with 6 mL of *C. fusca* and 6 mL of *M. aeruginosa* using a pipette.

Four genotype 4 *D. magna* neonates were placed in a petri dish on a light table with a ruler, a piece of white paper, and a label to match the number of their corresponding test tube and their picture was taken. The four *D. magna* were then placed into their corresponding test tube. This was repeated for every test tube containing *D. magna* in the experiment. The pictures of the *D. magna* were uploaded to a laptop and the body lengths of the organisms in each petri dish were measured and recorded using ImageJ software. Four genotype 4 *D. magna* neonates were added to each of test tubes 16-30. Four genotype 9 *D. magna* neonates were added to each of test tubes 31-45. Four genotype 11 *D. magna* neonates were added to each of test tubes 46-60. Four genotype 27 *D. magna* neonates were added to each of test tubes 60-75. All the test tubes were placed in test tube racks under the light fixture on the table and left for a two-week period in a room with a temperature of 18 ºC. Initial algae cell counts were performed using a microscope and hemocytometer for the three different algae combinations. The algae cells in the four corners of the hemocytometer grid were counted. The hemocytometer was rinsed and dried after each test. To calculate the algae cells per mL of liquid from each test, the total from the four corners were averaged and multiplied by 10,000 and then multiplied by the concentration of algae. At the end of the two-week period, a microscope and hemocytometer were used to calculate the final algae cells per mL in each test tube. The contents of each test tube were poured into a petri dish on a light table set up with a ruler, white piece of paper, and appropriate label and a picture was taken. These pictures were uploaded to a laptop and the body lengths of the organisms were measured using ImageJ software. The number of organisms alive and dead in each test tube was recorded. Neonate reproduction numbers were calculated for each test tube. Excel was used to record data and perform calculations.

6b. Experiment Two

Genotype 4 was cultured and cloned. Mud was collected from a local pond. 30 test tubes were cleaned and labelled numbers 1-30. 10 test tubes were filled with 12 mL of *C. fusca* using a pipette. Another 10 test tubes were filled with 9 mL of *C. fusca* and 3mL of *M. aeruginosa* using a pipette. The final 10 test tubes were filled with 6 mL of *C. fusca* and 6 mL of *M. aeruginosa* using a pipette. 1 mL of pond mud was added to test tubes 16-30. Four genotype 4 neonates were placed in a petri dish on a light table with a ruler, a piece of white paper, and a label matching their corresponding test tube number and their picture was taken. The four genotype 4 neonates were then placed into test tube 1. This was repeated for all 30 test tubes. The pictures were uploaded to a laptop and the body lengths of the organisms were measured using ImageJ software. The test tubes were placed in test tube racks on the same table and light fixture set up as Experiment One with the same light settings and temperature. All Experiment One

procedures for initial algae cell counts were repeated. At the end of the two-week period, all Experiment One procedures for final algae cell counts and *D. magna* data recording were repeated.

6c. Experiment Three

Experiment Two was repeated using 0.1 g of crushed polyethylene plastic pellets instead of pond mud.

6d. Experiment Four

Experiment Two was repeated using 1mL of 0.02 ppm of acidic high nitrate industrial grade fertilizer stock solution instead of pond mud.

6e. Experiment Five

Experiment Two was repeated using 1mL of 10 mg/L of calcium carbonate stock solution instead of pond mud.

7. Results and Observations

7a. Experiment One

Genotype 4 demonstrated the highest algae decrease in all 3 algae combinations with an overall average algae decrease of 97%. Genotype 4 had the most consistent average algae decreases with the lowest standard deviation range of 1-3%. Genotype 4 had the most neonates produced with a total of 75 neonates but was second in neonate growth to genotype 11. Genotype 4 had the most *D. magna* alive at the end on average in each sample.

7b. Experiments Two to Five

Genotype 4 with pond mud (aquatic microbes) had a higher algae decrease than the control in all 3 algae conditions with an overall average algae decrease of 96%. It produced more than twice as many neonates and had more *D. magna* alive on average per sample than the control. Genotype 4 with pond mud resulted in low standard deviation values of 1-7%. Genotype 4 with microplastics demonstrated less algae decrease than the control in all 3 algae conditions and had an overall average algae decrease of 70% in comparison to the controls which had an overall average algae decrease of 90%. However, the genotype 4 exposed to microplastics had an equal average final amount of *D. magna* alive to the controls and reproduced less neonates. Genotype 4 in nutrient pollution demonstrated an overall equal algae decrease to the control with an overall average algae decrease of 94% with low standard deviations. The genotype 4 exposed to nutrient pollution did have slightly less *D. magna* alive on average at the end and reproduced less neonates than the controls. Genotype 4 in calcium demonstrated an overall higher algae decrease than the control in all 3 algae conditions with an overall average algae

decrease of 91% with low standard deviation. It produced significantly more neonates than the control and all other environmental conditions with a total of 65 neonates.

Experiment 1: Genotype Comparison								
	Overall Algae Decrease	100% CF Algae Decrease	75% CF 25% MA Algae Decrease	50% CF 50% MA Algae Decrease	Neonates Reproduced	Neonate Growth	Alive	Dead
Control averages	-51%	-8%	-85%	-61%				
Genotype 4 averages	97%	99%	97%	94%	75	85%	4	4
Genotype 9 averages	75%	95%	65%	66%	29	78%	3	3
Genotype 11 averages	59%	97%	55%	27%	30	113%	3	3
Genotype 27 averages	78%	98%	67%	69%	28	76%	2	4
Experiment 2-5: Environmental Conditions								
Pond mud replicate averages								
Control averages	89%	98%	85%	84%	14	40%	2	3
Average of 3 algae combinations	96%	97%	96%	94%	38	32%	3	4
Microplastics replicate averages								
Control averages	90%	95%	90%	84%	21	49%	2	4
Average of 3 algae combinations	70%	89%	79%	43%	12	101%	2	3
Nutrient Pollution replicate averages								
Control averages	94%	99%	96%	88%	27	45%	2	4
Average of 3 algae combinations	94%	98%	99%	85%	10	33%	1	4
Calcium replicate averages								
Control averages	86%	99%	68%	93%	24	-10%	0	5
Average of 3 algae combinations	91%	97%	90%	85%	65	0%	1	7

Figure 1 shows quantitative data (algae decrease, neonates reproduced, neonate growth, neonates alive, and neonates dead) for Experiments One to Five.

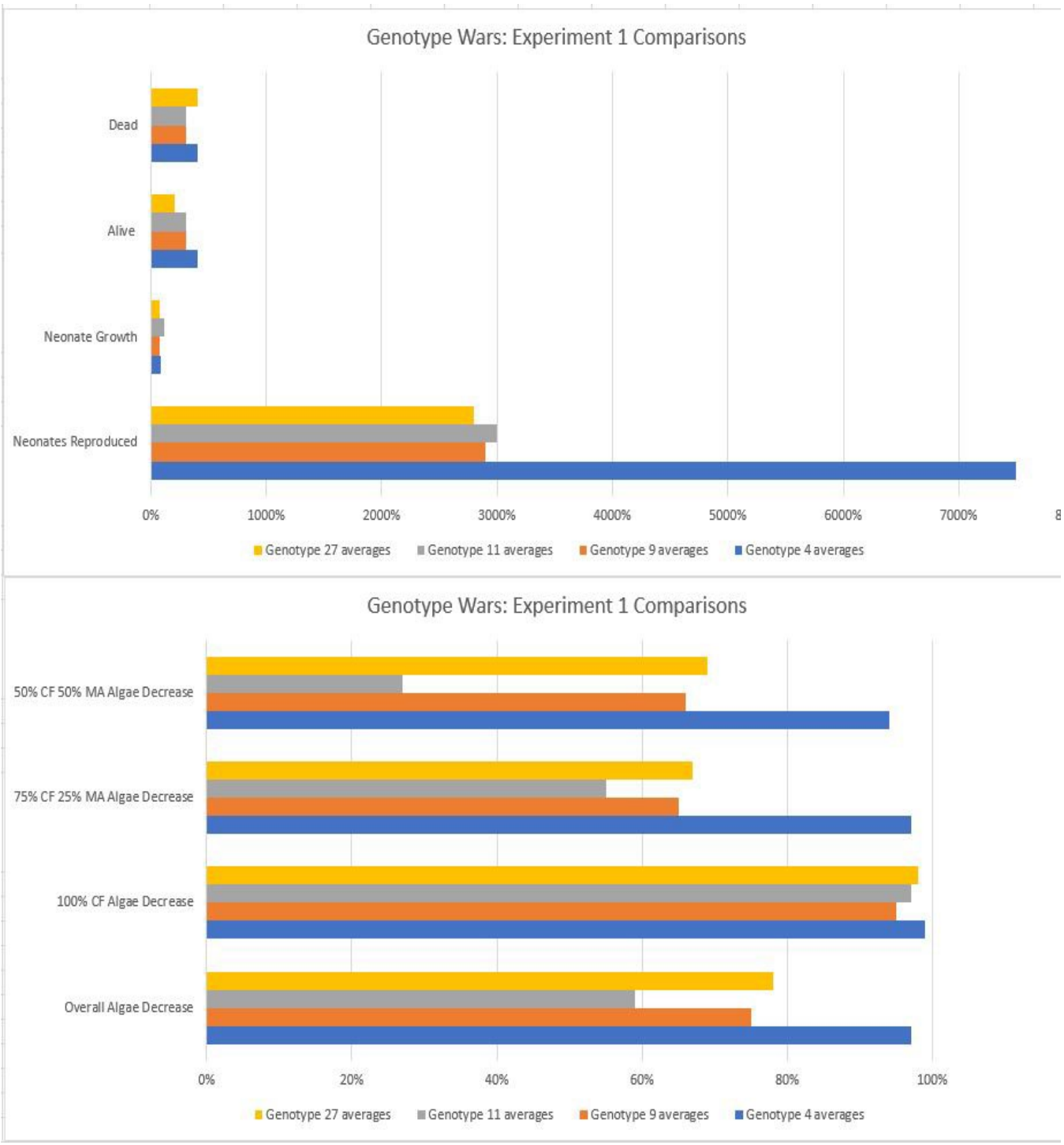

Figure 2 displays comparisons of *D. magna* genotype health metrics and algae reduction during Experiment One.

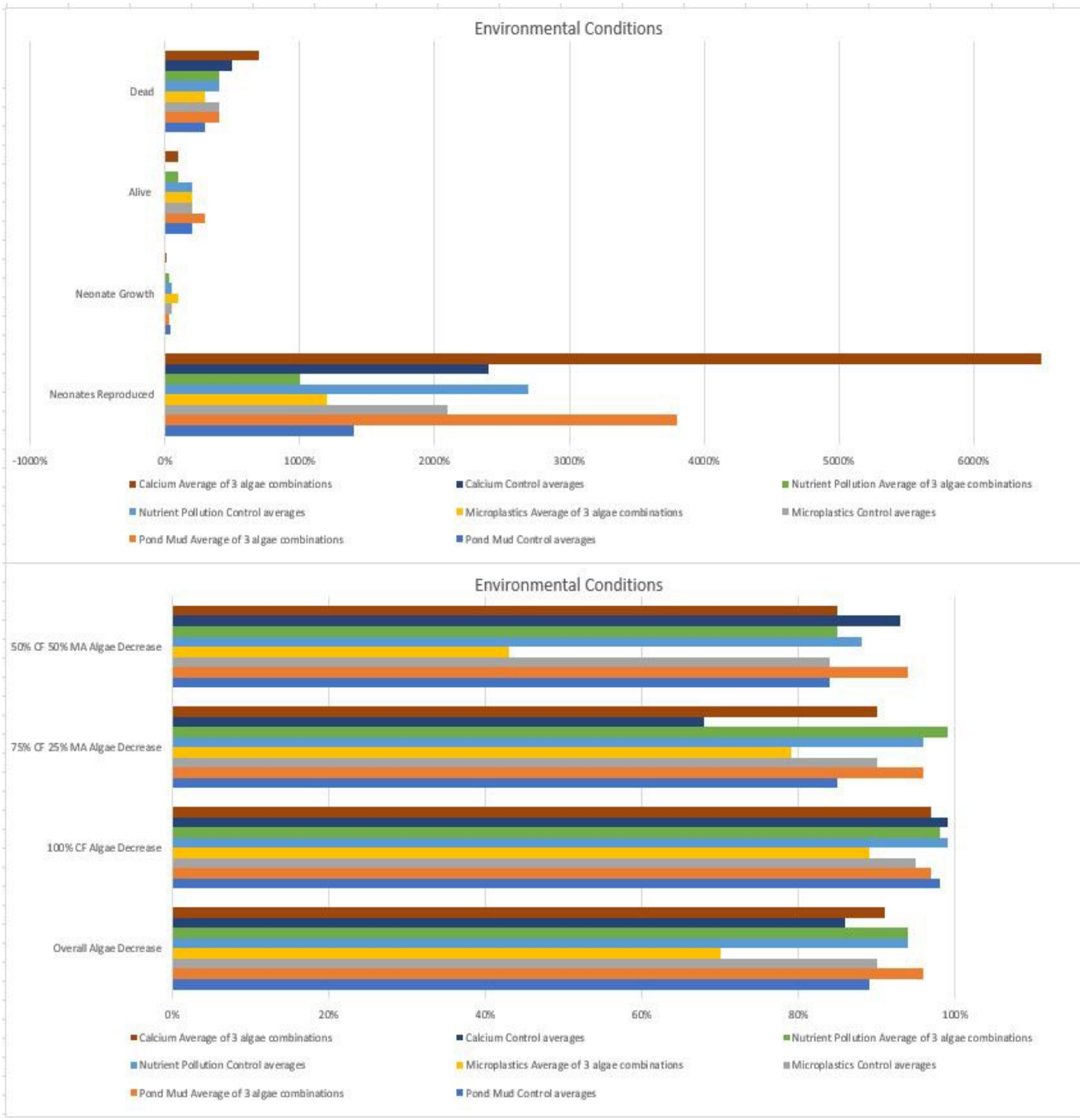

Figure 3 displays genotype 4 health metrics and algae reduction when exposed to the different environmental conditions in Experiements Two to Five.

7c. Statistical Analysis

Statistical analysis was performed and the majority of experiments were found to have normal distribution. For all statistical analysis performed, results were considered significant if below a p value

of 0.05, giving the experiment a confidence level of 95%. For Experiment One, four Two-Way ANOVAs were performed for the dependent variables of percent algae decrease, *D. magna* mortality, neonate reproduction, and *D. magna* body length growth comparing the independent variables of *D. magna* genotype and algae combination and toxicity level independently and in interaction with each other. It was found that genotype, algae combination and toxicity level independently and in interaction had statistically significant effects on all the dependent variables with a few exceptions. For Experiments Two to Five, four Two-Way ANOVAs were performed for the dependent variables of percent algae decrease, *D. magna* mortality, neonate reproduction, and *D. magna* body length growth comparing the independent variables of the added environmental condition and algae combination and toxicity level independently and in interaction with each other. It was found that algae combination and toxicity level and the added environmental condition independently and in interaction had statistically significant effects on all the dependent variables with a few exceptions.

7d. Sources of Error

Due to larger standard deviations in some tests, it would have been better to have even more replicates. Many of the *D. magna* throughout all the experiments died by the mid to end of the second week due to running out of algae to eat. For some of the *D. magna* that did die, their bodies started to decompose which made it difficult to measure their body lengths using ImageJ. Despite using neonates, the *D. magnas'* reproductive cycles could not be controlled which led to inconsistent neonate reproduction results. The pond mud used had some tiny particles and debris that made it a bit of a challenge to count the algae cells in those tests. Due to the pandemic, there was no way to access a lab to test and identify the microbes in the sample of pond mud that was used. For ImageJ, there was no way to mark the *D. magna* before and after counting them to ensure that the same one was being measured each time. Instead, the averages of the *D. magnas'* body lengths in each sample were calculated and compared before and after. This meant that where there was more reproduction, there was a larger number to divide by and more neonates which are smaller in size which could have skewed these numbers.

8. Conclusions

Genotype 4 was proven to be the ideal genotype of *D. magna* to biomanipulate to treat and prevent algae blooms as it had the largest overall average algae decrease, reproduced the most neonates, had the second most average body length growth overall, and had the most *D. magna* alive at the end in comparison to the other genotypes tested. Genotype 4 was also able to produce significant algae decreases in all of the different algae combinations. Pond mud / aquatic microbes was proven to be the

environmental condition that resulted in the largest average algae decreases overall and most organisms alive on average in comparison to the other environmental condition experiments. This environmental condition also resulted in the second best numbers of reproduced neonates of the environmental condition experiments. This shows that the exposure to naturally occurring microbes and having better developed gut-microbiota improved the clone's ability to digest, consume, and tolerate the toxic and non-toxic algae. This was also closer to a more natural environment and field test in comparison to a lab environment and helps prove that the biomanipulation of *D. magna* will be successful in a real-world ecosystem. Clones exposed to microplastics had the worst algae decrease of all environmental condition experiments; however, an overall average algae decrease of 70% is still a moderate decrease that can help freshwater ecosystems. The *D. magna* were even still able to reproduce and live to some extent demonstrating that the *D. magna* can still treat and prevent harmful algae blooms in lakes with plastic pollution such as Lake Erie which has the second largest amount of plastic particles out of all the Great Lakes [6]. Clones exposed to nutrient pollution had equal average algae decreases to the controls, which were significant average algae decreases of 94%. These clones were able to grow and reproduce as well. This proves that biomanipulation of *D. magna* can and will work in real-life eutrophic lakes as they are able to produce significant algae decreases and live in eutrophic conditions. Clones exposed to calcium carbonate had the best reproduction numbers, significant algae reduction, and body length growth. This highlights that biomanipulation will be best and easiest in lakes with high calcium content or that the technique of liming could be used to aid in the biomanipulation of the *D. magna.* In short, genotype 4 is the ideal genotype of *D. magna* to biomanipulate to treat and prevent harmful algae blooms, can effectively do this in nutrient and plastic polluted environments, and can have their health and success improved through calcium carbonate and naturally occurring aquatic microbes.

9. Discussion

Biomanipulation of *D. magna* to increase their population to clean up and prevent algae blooms can be done in multiple ways: first, by culturing *D. magna* native to freshwater environments at hatcheries and adding cultured *D. magna* to the freshwater environments; or second, by increasing pike populations and other predators to consume fish that pray on *D. magna* and decreasing or removing predators to *D. magna*. Additionally, freshwater is a vital, yet finite, resource that must be protected and thus society must do everything in its power to ensure that the initiatives and solutions to protecting freshwater environments are as effective as possible. By discovering the ideal genotype of *D. magna* to biomanipulate to treat and prevent harmful algae blooms, the success of biomanipulation and algae

bloom treatment and prevention can be maximized. Cloning and culturing *D. magna* is also a simple, straight forward, and well known and used lab procedure that can easily be initiated for large scale use. By cloning genetically identical *D. magna* genotypes this solution will be even easier to implement on a large scale and in the real world. Furthermore, by studying the different genotypes and further analyzing their performance and health metrics, limnologists can gain a better understanding of the genetic variation, fitness, evolution, and adaptation of a keystone freshwater species as freshwater ecosystems evolve which can allow for a better understanding of ecosystem health. Additionally, according to the United States Geological Survey, there are 112,000 particles of microplastics per square mile of Great Lakes water [7] and 22 million pounds of plastic enter the Great Lakes each year from Canada and the United States [8]. These microplastics then can be digested by aquatic organisms, blocking their gastrointestinal tracts and tricking the organisms into thinking that they are full so they do not eat and thus die of starvation; microplastics can absorb toxins and when consumed expose the organism to those toxins and then can bioaccumulate up the food chain [9]. Ingestion of microplastics can also cause a species to experience problems such as disruption in their reproductive system, stunted growth, a lack of an appetite, tissue inflammation and liver damage [9] which poses a risk to *D. magna* biomanipulation. However, the *D. magna* proved that they were still able to moderately reduce algae, survive, and reproduce when exposed to microplastics proving that they can still be successful in lakes with plastic pollution. But, by preventing, limiting, and cleaning up plastic pollution in freshwater ecosystems, genotype 4 will be better able to reproduce, thrive, and decrease harmful algae blooms. By discovering the positive health effects and improved success of genotype 4 biomanipulation and algae decrease when exposed to naturally occurring aquatic microbes in pond mud, this proves that there are already elements in place in the natural environment which will allow for more successful *D. magna* biomanipulation and harmful algae bloom reduction. *D. magna* require plenty of calcium, but due to acid rain, the invasion of zebra mussels, climate change, and tree logging, which prevents a tree's calcium from returning to its environment, calcium levels in freshwater environments are decreasing and *D. magna* populations along with it. In fact, many freshwater lakes in Canada and around the world have calcium levels below 1.5 mg/L which is the threshold of calcium for *D. magna* survival [10]. The experiment clearly shows that *D. magna* biomanipulation and ability to treat and prevent harmful algae blooms can be improved in environments with adequate amounts of calcium. This problem of freshwater decalcification can be solved by adding calcium to lakes through the process of liming or using calcium based road salts and road dust suppressants. The experiment showed that *D. magna* can survive, grow, and reproduce and

decrease harmful algae blooms when exposed to average eutrophic lake conditions of 20 µg/L of phosphorus [11] equally as well as the ones without nutrient pollution. This means that this method of harmful algae bloom treatment and prevention will be successful in eutrophic lakes with harmful algae blooms. As the population of humans grows and food demands increase the use of agriculture, nutrient use and pollution will increase as well which along with climate change will increase eutrophication. This means that it is vital for algae bloom treatment and prevention to be able to succeed in eutrophic conditions with nutrient pollution which is exactly what *D. magna* biomanipulation can do. Furthermore, the experiment illustrated that genotype 4 can successfully decrease algae blooms in a variety of toxicities while still thriving as a species and experiencing limited negative health effects. This is important because within an algae bloom the toxicity of the algae and types of toxic algae varies and it is important that the genotype 4 *D. magna* can thrive and clean up the algae bloom. Genotype 4 biomanipulation in freshwater ecosystems is also a more sustainable and safer method of algae bloom treatment and prevention in comparison to the existing method of algaecide use since the *D. magna* are able to eat the algae bloom before it can decompose and cause hypoxia whereas algaecides result in the algae bloom decomposing and causing hypoxic conditions. Possible next steps for continued research of this project include testing invasive species to see their impact on algae reduction and *D. magna* biomanipulation; testing different types of nutrient pollution to see how *D. magna* are impacted by a variety of nutrient pollution; testing water and algae collected from a natural algae bloom to test the effectiveness of biomanipulation on a naturally occurring bloom; testing environmental conditions in combinations to see how they interact to impact *D. magna* biomanipulation and algae decreasing capabilities; testing in a larger facility with a larger and more accurate microcosm; and receiving permissions to test at an experimental pond or lake site. By discovering the ideal genotype of *D. magna* to biomanipulate to treat and prevent harmful algae blooms, that it is still successful in plastic and nutrient polluted environments, and that *D. magna* health and success can be improved through calcium carbonate and naturally occurring aquatic microbes, the future of algae bloom treatment and prevention is much clearer.

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12. Annex I. Visual Observations

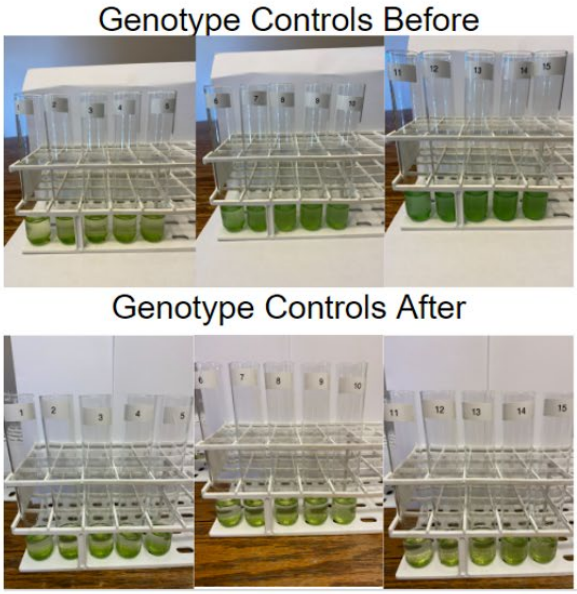

Figure 4 Experiment One control before and after.

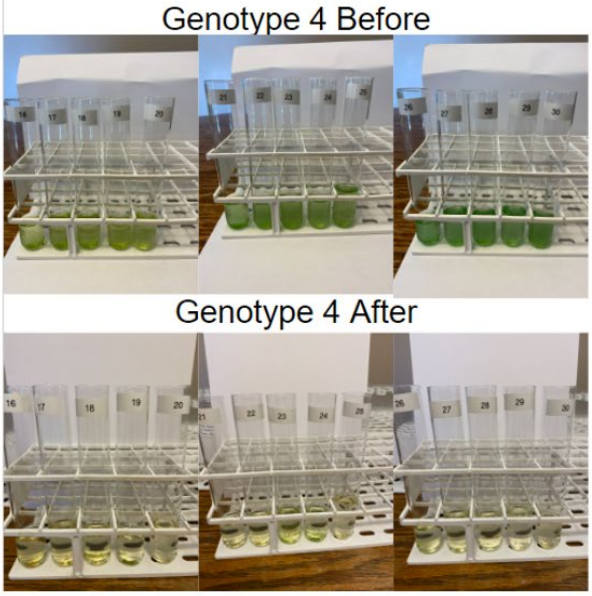

Figure 5 Experiment One genotype 4 before and after.

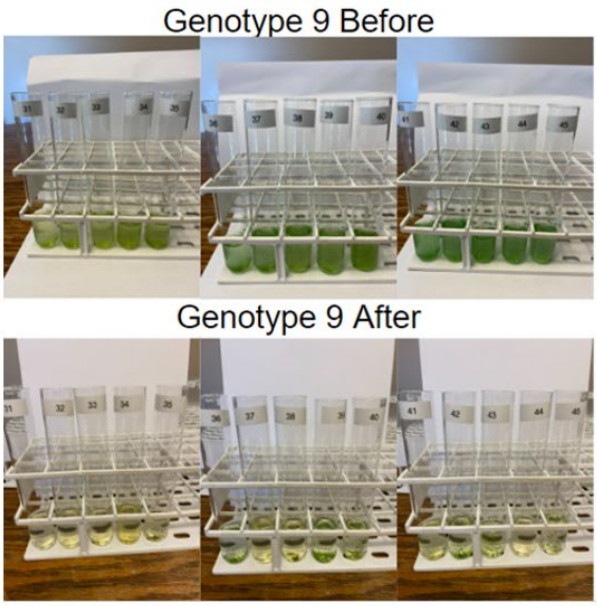

Figure 6 Experiment One genotype 9 before and after.

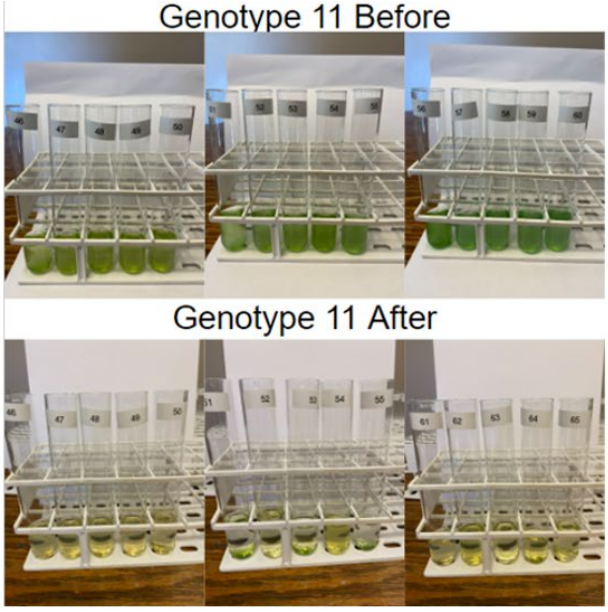

Figure 7 Experiment One genotype 11 before and after.

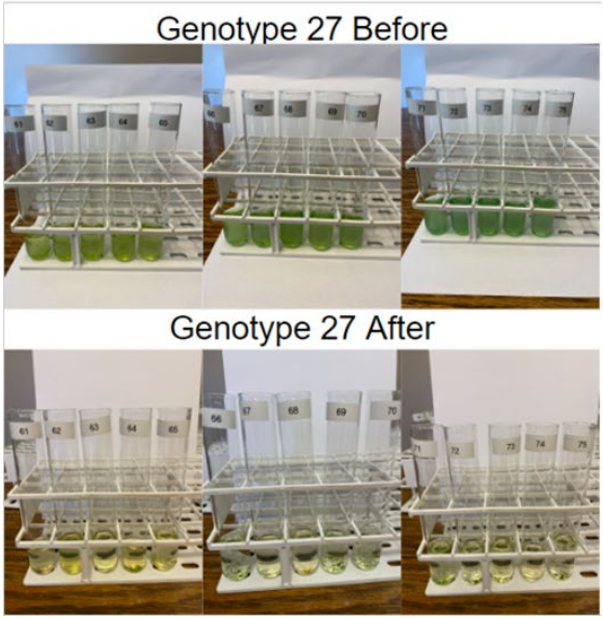

Figure 8 Experiment One genotype 27 before and after.

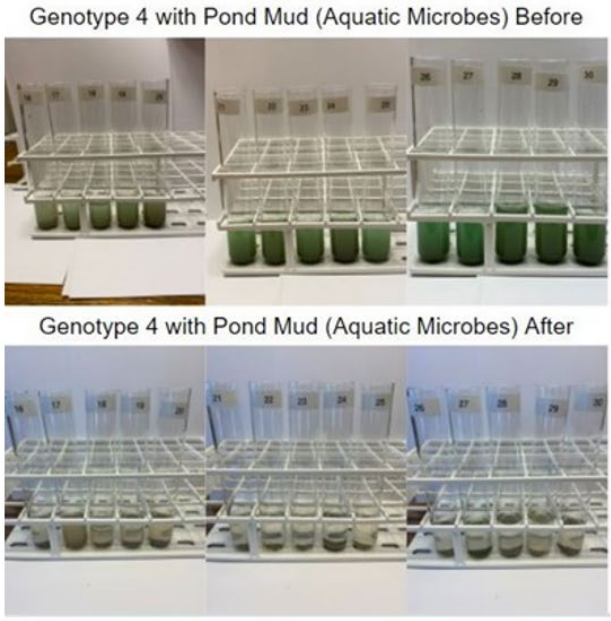

Figure 9 Experiment Two genotype 4 with pond mud (aquatic microbes) before and after.

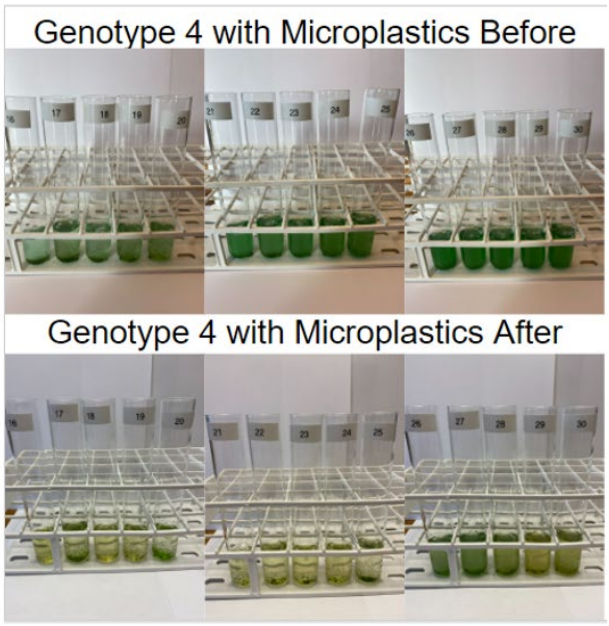

Figure 10 Experiment Three genotype 4 with microplastics before and after.

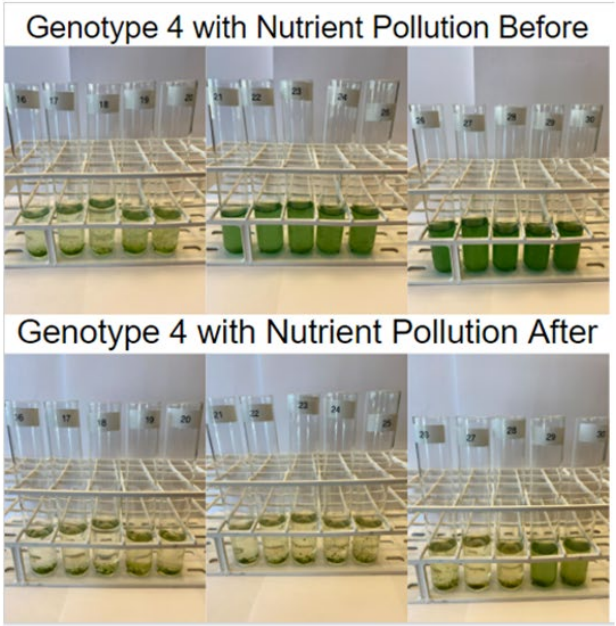

Figure 11 Experiment Four genotype 4 with nutrient pollution before and after pictures.

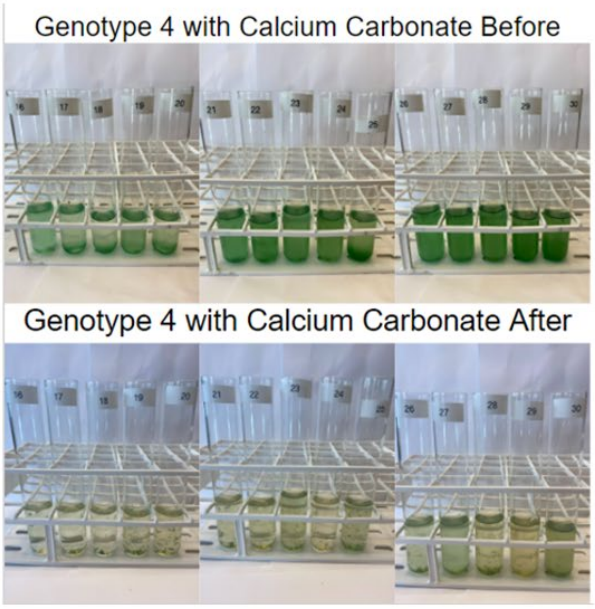

Figure 12 Experiment Three genotype 4 with microplastics before and after.

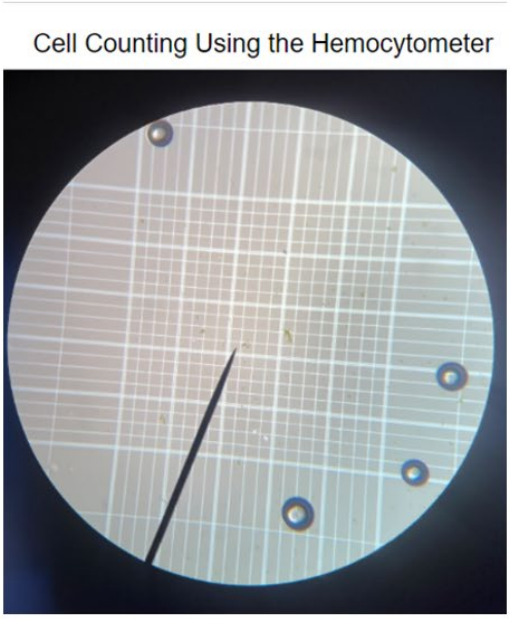

Figure 13 An algae cell count using a hemocytometer and microscope.