9-27-2018

Sensitivity of Marine Cynobacteria and Green Microlage to Nano and Bulk Zinc Oxides

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SENSITIVITY OF MARINE CYNOBACTERIA AND GREEN MICROALAGE TO NANO AND BULK ZINC OXIDES

A thesis submitted in partial fulfillment of

the requirements for the degree of

MASTER OF SCIENCE

in

ENVIRONMENTAL STUDIES

by

Jennifer Gil

2018

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This thesis, written by Jennifer Gil and entitled Sensitivity of Marine Cyanobacteria and Green Microalgae to Nano and Bulk Zinc Oxides, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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Date of Defense: September 27,2018

The thesis of Jennifer Gil is approved.

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Dean Michael R. Heithaus
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Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2018

DEDICATION

I dedicate this thesis to microalgae because without them we would not have this planet and I will not have a thesis topic. I always say I did not find microalgae, microalgae found me when I was at my lowest. My thesis topic had to change because I became sick and then one day I just looked it up in one of my books and the word microalgae was there. I was able to study microalgae without getting sick and started even taking microalgae-based supplements for my sickness. Microalgae have taken me places I could only dream of. Thanks to them I will be able to write for my favorite magazine, National Geographic, and I will be collecting them throughout the Panama Canal area. I want to thank God for creating the microalgae and bringing them to my attention. By the grace of God and microalgae my sickness got better, and I was able to continue to keep loving life and having hope. Finally, I dedicate my thesis to Dr. Eve Hershberger and my mentors (Dr. Kateel G. Shetty, Dr. Krishnaswamy Jayachandran, and Miroslav Gantar) for believing in me, helping me, and listening to me when I felt hopeless.

ACKNOWLEDGMENTS

The support that I have had during my master's program is more than I can put into words. I want to thank my mentor Dr. Kateel G. Shetty, Dr. Krishnaswamy Jayachandran, and Miroslav Gantar for their constant support and for believing in me even when I did not. I want to thank them for not giving up on me and seeing my potential. Especially Dr. Shetty for his great guidance and encoring words. Also, I want to thank everyone from FIU's Agroecology Department: Eric, Ariel, Mary, Shagufta, Ganesh, Meghan, Jessica, Claudia, Nick, Daphne, Cristina, Jazmin, Emily and Will. I would also like to thank the undergraduate lab assistant, Myles, for helping and repeating some of my experiments. The students, Maria Jose, for editing my microscope videos. I would like to thank my undergraduate student Ana for helping me in the lab especially for collecting samples with me and giving me inspiration. Special thanks to Dr. Gantar, David Berthold, and Javier Broche for sharing their algal cultures and their lab facilities. I would also like to acknowledge FIU's Student Health Center for taking care of my health and encouraging me to keep working on my master's. I would like to acknowledge FIU's Writing Center for helping correct my proposal especially Corey, Tricia, and Dr. Glenn and the staff members from the Department Earth and Environment especially Gail. Also, I will like to thank my friend Dominic Lomando for his willingness to help me with my writing. My boyfriend Osvaldo for not letting me give up achieving my goals. My family specially mom, dad and my brother for always keeping me on track and encourage me too keep perusing my dream.

ABSTRACT OF THE THESIS

SENSITIVITY OF MARINE CYNOBACTERIA AND GREEN MICROALAGE TO NANO AND BULK ZINC OXIDES

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Nanoparticles are particles with sizes between 1 and 100 nanometers (nm). Owing to their unique chemical, electrical, mechanical, optical, and piezoelectric properties, zinc oxide nanoparticles (ZnO-NPs) are finding widespread use in numerous applications with yearly production over 550 tons per year. Increasing use of ZnO NPs, and NPs in wastewater discharges from domestic and industrial sources will have significant potential for adverse impacts on aquatic phototrophic organisms. Comparative studies on microalgae species response to ZnO NPs and variation in tolerance among species is still mostly unexplored. The proposed research aims to evaluate interspecies' variation in tolerance to ZnO NPs among marine and freshwater microalgae. Multi-well culture plate and flask culture screening methods were utilized for assessing microalgae species' tolerance to various levels of ZnO NPs. Microalgae cell morphology changes in response to nano ZnO exposure were explored using both the Optical Coherence Microscope (OCM) and SEM. Availability of Nano ZnO tolerant microalgae species may provide an impetus for future studies to understand the mechanism of tolerance and potential applications in NPs bioremediation in aquatic systems.

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ABBREVIATIONS AND ACRONYMS

A. coffeaeformis	*Amphora coffeaeformis*
A.*flosaquae*	Aphanizomenon flos-aquae
cyno 7	*Apistonema* sp.
B.C.E.	Before Common Era
green 2	*Chlamydomonas* sp.
green 3	*Chlorella* sp.
green 1	*Chlorococcum* sp.
DI water	Deionized Water
EDS	Energy Dispersive X-Ray Spectroscopy
E.gracilis	*Euglena gracilis*
E. paludosa	*Eurybia paludosa*
EPS	Exopolysaccharides
cyno 2	*Leptolyngbya* sp.
cyno 1	*Limnothrix* sp.
MAA	Mycosporine-like Amino Acids
NPs	Nanoparticles
cyno 3	*Neolyngbya* sp.
OCM	Optical Coherence Microscope
cyno 4	*Oscillatoria* sp.
ppm	Parts Per Million
cyno 5	*Porphoridium* sp.

Section 1: INTRODUCTION

1.1 Significance of Research

Nanotechnology may seem like a new futuristic concept. The reality is that nanotechnology has always existed, even when humans were not aware of it. Nanoparticles (NPs), which are found in nature, have been used as far back as 1,000 B.C.E. to change the color of pottery (Binns, 2010). It was in 1959 that the American physicist Richard Feynman defined the concept of nanotechnology in his talk, "There's Plenty of Room at the Bottom" (Feynman, 1959). Nanotechnology is one of the fastest growing markets in the world; worldwide investments estimate to be close to a quarter of a trillion USD (Adeel Irfan, 2014). Due to their fast-growing and enhancing properties, nanoparticles are being used in many commercially available products such as paint, sunblock, foods, and cosmetics. Some of these products may come in direct contact with aquatic ecosystems, for example, when we put on sunblock and go into the ocean. (Colvin, 2004; Kahru & Dubourguier, 2010).

The third highest used nanoparticle is ZnO NPs. These NPs are being used in products (cosmetics and sunblock) that have a direct contact with the aquatic ecosystem. There have been several studies on the effects of ZnO NPs on marine and freshwater life, especially on smaller organisms like microalgae (Aravantinou, Tsarpali, Dailianis, & Manariotis, 2015; Wu, Torres-Duarte, Cole, & Cherr, 2015). Microalgae produces more than half of the oxygen humans breathe, demonstrating the direct and powerful impact they have on human oxygen consumption (Hall & Benemann, 2011). Microalgae has a future potential for renewable energy, food, fertilizer and ecofriendly sunblock. However, the research has been inconsistent regarding the toxicity of ZnO NPs on microalgae.

As the microalgae growth conditions and the size of ZnO NPs used is commonly different for each study, it is not possible to make reliable conclusions. There are only a few reports involving more than two microalga species showing variation in response to ZnO NPs (Manzo, Miglietta, Rametta, Buono, & Francia, 2013). Finding marine microalgae species sensitive to ZnO NPs could help improve the ecotoxicology studies, and it can help regulate nanoparticles in products that have a direct contact with aquatic systems. On the other hand, finding tolerant microalgae species may provide an impetus for future studies to understand the mechanism of tolerance and potential applications for NPs bioremediation in aquatic systems.

1.2 Statement of Research

Through the increasing use and application of ZnO NPs, the potential for negative environmental impacts on aquatic phototrophic organisms is quite considerable. It is known that microalgae species differ in their response to various abiotic and biotic sources of stress. However, serious research on variation in tolerance among species to NPs is very limited. Therefore, it is predicted that by screening microalgae species for tolerance to ZnO NPs, it is possible to identify tolerant and sensitive species. Among the potential prospects, a greater understanding of the mechanisms involved will improve bioremediation of nanoparticle pollution using tolerant species. It would, therefore, be interesting to investigate the variation in tolerance to ZnO NPs among different marine microalgae species.

1.3 Objectives

1. To assess the variation in growth response to various levels of ZnO NPs amongst marine microalgae species by comparison to bulk ZnO.

2. To evaluate tolerance of marine microalgae species to ZnO NPs by screening microalgae species for growth response to higher levels of ZnO NPs.

3. To determine whether the growth response of selected ZnO NPs tolerant marine microalgae species will remain unchanged when exposed to ZnO NPs of different sizes.

4. To characterize the variation in cell morphology of ZnO NPs tolerant and sensitive marine microalgae species in response to exposure to ZnO NPs.

1.4 Hypotheses

1- Some species of marine microalgae are more tolerant to ZnO NP exposure than other species.

2- The cell morphology of various species of marine microalgae will differ.

Section 2: LITERATURE REVIEW

2.1 Coastal systems

Marine aquatic systems are vulnerable to anthropogenic effects due to human impacts both directly, through fishing, boating and sunscreens, and indirectly such as through events like chemical runoff (Hazeem, et al., 2015). Coastal environments that are the most vulnerable to pollution are those that are most visited by tourists. Miami's beaches in Florida are some of the most popular beaches in the United States of America, which makes them extremely vulnerable to direct and indirect pollution (U.S. Census Bureau). There have been many routine studies done on the quality of the coastal environment along Miami beaches, but the concentration of Zinc and other metals has not yet been measured. In addition, there is limited research on pollution caused by nanoparticles (NPs). NPs can leech into the coastal environment both directly and indirectly through products like boat paints, bathing suits and sunscreen. ZnO and ZnO NPs and its ecotoxicity was one of the focuses of this study.

2.2 Nanoparticles (NPs)

NPs are particles that are from 1 to 100 nm in size. Due to their minute size, they exhibit unique properties that affect physical, chemical, and biological behavior (Brauer, 2016). Some NPs can be found naturally in the environment and are a product of natural physical and biogeochemical processes. These NPs can be found in volcanic ash, ocean spray, fine sand, dust, and in biological matter. Some organisms, like microalgae, may also be able to produce them. There are also human made NPs, which can be synthesized intentionally and unintentionally. Unintentional NPs can be formed as a byproduct of

anthropogenic activities, such as running diesel engines, large-scale mining operations, and other processes (Binns, 2010). The intentionally made NPs are synthesized for various commercial and research purposes such as cosmetics.

Some of the properties that NPs have are: UV-absorbance, transparency, high-strength, and water and strain repulsion (Nagarajan, 2008; Woomer, et al., 2015). The increased use of purposely synthesized NPs is increasing exponentially, which may be dangerous for the aquatic ecosystem, especially since NPs have a direct impact on the costal environment.

2.2.1 Zinc Oxide (ZnO) NPs

Zinc is one of the most abundant elements found in the earth's crust. Although ZnO is found in nature (Zincite), synthetic ZnO is the variety used most. Macroscale or bulk ZnO is an inorganic compound, in the form of a white colored powder, that is insoluble in water but soluble in acid or alkaline solutions (Moezzi, et al., 2012). ZnO is popularly used in sunblock, other cosmetics, pharmaceuticals, and other products due to its high absorption along the entire UV spectrum and its good photo stability (Klingshirn, Waag, Hoffmann, & Geurts, 2013).

NPs do not exhibit the same properties as macro (bulk) particles. NPs have a large surface area-to-volume ratio and varying surface charges that give them unique characteristics (Auffan, et al., 2009). This principle can be seen in the difference between ZnO nanoparticles and

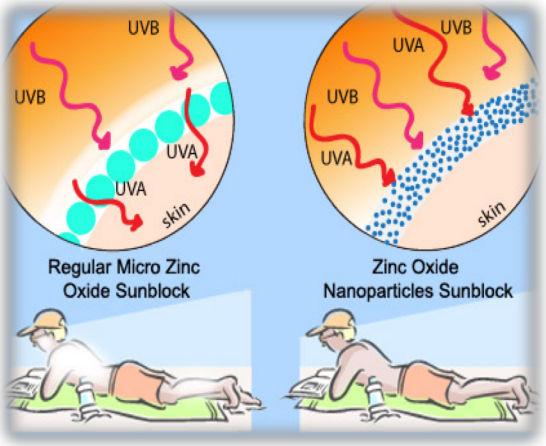


Figure 1: Compress the regular micro ZnO sunblock to ZnO NPs sunblock.

bulk ZnO (Figure 1). Zinc Oxide NPs are used in sunblock because the particles scatter the ultraviolet light in sunlight, assuring a broad-band UV protection.

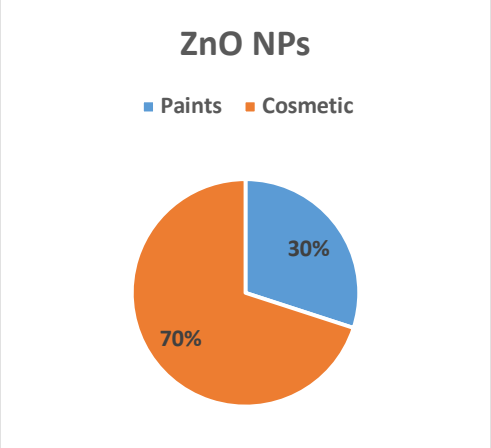


Figure 2: The percentage of ZnO NPs usage

ZnO NPs are one of the most popular heavy metals, and are the third highest heavy metal used in annual production volume with 550 tons estimated per year (Piccinno, Gottschalk, Seeger, & Nowack, 2012). Even though ZnO NPs have uses other than sunscreen, like for example paint, sunblock is the most popular with 70% of ZnO NPs being used in cosmetics (including sunscreens). In comparison, only 30% of ZnO NPs are being used in paints (Figure 2) (Piccinno, Gottschalk, Seeger, & Nowack, 2012).

The excessive use of ZnO NPs in sunblock is alarming because of its direct exposure to the aquatic system. Even though these NPs have some benefits to human health, they may also have a negative impact on the environment. When the sunblock washes off the skin during recreational activities, it can reach the aquatic environment directly and can potentially affect marine organisms. In fact, 25% of sunscreens will be washed off upon immersion (Danovaro et al. 2008) and 250 tons of sunscreen-originated NPs enter the marine environment each year (Wong et al. 2010). As the impact of NPs becomes a growing concern, studies on sunblock suggest that caution should be used as new sunscreens are being developed and more detailed research should be done on NPs

stabilization, chronic exposures, and reduction of NPs free-radical production (Smijs, T., & P., 2011).

2.3 Ecotoxicology

Many recent studies have proven that ZnO NPs, and even bulk ZnO, may be toxic to many organisms, especially aquatic ones. Bondarenko, et al., (2013) compares levels of toxicity within numerous aquatic organisms. Their research indicated that crustaceans, *Daphnia sp*., showed a 100% toxicity at 100 mg/L of ZnO NPs (Blinova et al., 2010, Akhil & S., 2016); algae, *Pseudokirchneriella subcapitata,* showed a comparable toxicity for NPs ZnO, bulk ZnO, and ZnCl2, with a 72-h IC50 value near 60 µg Zn/L, attributable solely to dissolved zinc (Linkous et al., 2000; Franklin et al., 2007); and fish, zebrafish, showed acute toxicity during 96-h LC(50) at 4.92 mg/L ZnO NPs, at 3.31 mg/L bulk ZnO suspension, and at 8.06 mg/L Zn(2+) solution (Zhu et al. 2008, Xiong et al., 2011).

In addition, research also indicates that although the survivability of nematodes, specifically *C. elegans,* was not significantly affected by the exposure of 50 nm and 100 nm ZnO NPs, their reproduction was affected in a low concentration as compared to their survivability. Nematode exposure to 10 nm of ZnO NPs had shown even distribution extended nearly the entire length of the body, which means that the intestine is the major target tissues for NP toxicity (Gupta, Kushwah, Vishwakarma, & Yadav, 2016).

Several studies demonstrated high toxicity among bacteria. Damage within the *E. coli* cells was shown in the gram-negative triple membrane disorganization (Brayner, et al., 2006), while research using *Streptococcus agalactiae* and *Staphylococcus aureus* demonstrated both gram-negative membrane and gram-positive membrane disorganization when exposed to ZnO NPs. The ZnO NPs do a surface modification which causes an increase in membrane permeability of these NPs (Huang, et al., 2008).

Some research has had different results that proves that *E. coli* can have an adaptive resistance to the ZnO NPs in long periods of time (Zhang, et al., 2018).

Testing of yeast/fungi, *Aspergillus Fumigatus* Fungus and *Candida Albicans* Yeast, with a ZnO NPs concentration of 0, 3, 6 and 12 mmll-1 showed ZnO NPs have considerable antifungal activity (Jasim, 2015), while mammalian cells *In Vitro* were exposed to ZnO Macro and Nano particles with various concentrations (5–500 μg/mL) for 24 h. Zinc Oxide particles showed concentration-dependent cytotoxicity, but nano

ZnO showed more toxicity than its macro size (Sahu, Kannan, Tailang, & Vijayaraghavan, 2016). In the Heinlaan, Ivask, Blinova, Dubourguier, and Kahru (2008) study, vibrio fishes were found to be very toxic for all three (bulk ZnO, nano ZnO and ZnSO4.7H2O) at low concentrations (1.8, 1.9, and 1.1). Finally, protozoa**,** *Tetrahymena thermophila,* show toxicity for bulk and nano ZnO while Zn (2+) had of similar toxicity to ZnO NPs and salts (Mortimer, Kasemets, & Kahru, 2010) (Figure 3).

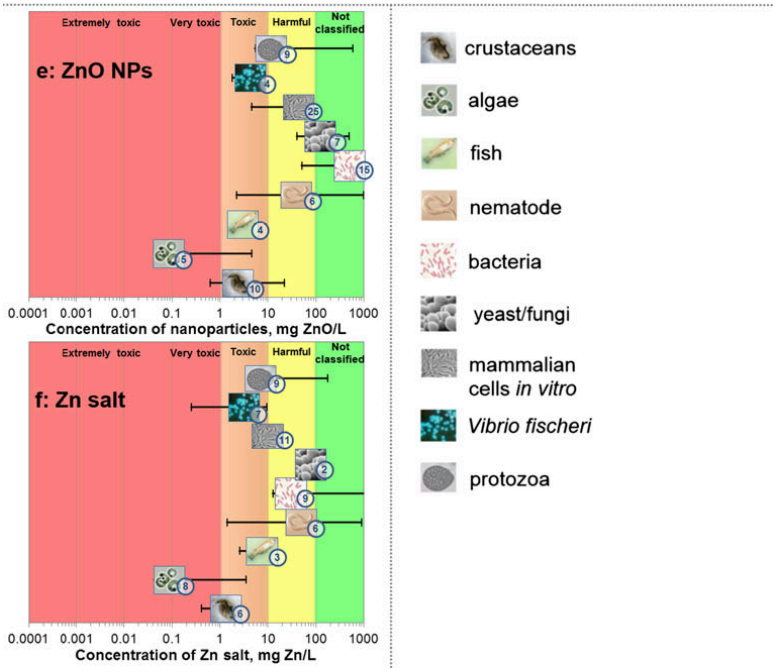


Figure 3: The toxicity effects of ZnO NPs and Zn salts (Bondarenko, et al., 2013).

For this research, we will be focusing on the results for ZnO NPs and salts. The more sensitive organism to ZnO NPs and salts is the aquatic organism including: Crustaceans, algae and fish (Table 1). Out of these aquatic organisms, algae are the most sensitive to ZnO NPs and salts showing toxicity at only 5mg/l NPs and 8mg/L of salts. The ZnO NPs and Zn salts are classified as extremely toxic (Sanderson, 2003; Blaise, Gagné, Férard, & Eullaffroy, 2008).

Other aquatic organisms affected by ZnO NPs are water fleas, crustaceans, mussels, and sea urchins. The ZnO NPs have shown unusual patterns of development in marine life. The nanoparticles make the embryos more susceptible to damage from other toxic compounds. For example, some urchin embryos exposed to nano ZnO in sunscreen never grew to become larvae or appeared to grow normally but were unable to eat and

died (Wu, Torres-Duarte, Cole, & Cherr, 2015). Finally, many studies have proven that NPs are toxic to marine algae. Nanoparticles of ZnO affects the algal growth rate starting from lower tested concentrations (Manzo, Miglietta, Rametta, Buono, & Francia, 2013).

Table 1 Median L(E)C50 values for all organisms except bacteria and median MIC for bacteria for Ag, CuO and ZnO nanoparticles (NPs) and the respective metal salts

Group of organisms	Median L(E)C50 or MIC, on compound basis, mg/L (number of data)*			Median L(E)C50 or MIC, on metal basis, mg metal/L (number of data)*		
	Ag NPs	CuO NPs	ZnO NPs	Ag salt	Cu salt	Zn salt
Crustaceans	0.01 (17)	2.1 (8)	2.3 (10)	0.00085 (8)	0.024 (8)	1.3 (6)
Algae	0.36 (17)	2.8 (5)	0.08 (5)	0.0076 (10)	0.07 (20)	0.09 (8)
Fish	1.36 (17)	100 (1)	3.0 (4)	0.058 (4)	0.28 (19)	7.5 (3)
Nematodes	3.34 (21)	Not found (0)	39 (6)	4.8 (4)	19.4 (6)	49 (6)
Bacteria	7.10 (46)	200 (13)	500 (15)	3.3 (27)	32 (13)	30 (9)
Yeast	7.90 (14)	17 (4)	121 (7)	2.16 (5)	11.1 (4)	78 (2)
Mammalian cells in vitro	11.3 (25)	25 (21)	43 (25)	2 (18)	53 (10)	9.8 (11)
V. fischeri[a]	32 (2)	73.6 (4)	4.3 (4)	5.7 (2)	0.78 (7)	3.2 (7)
Protozoa	38 (7)	124 (6)	11.7 (9)	1.5 (3)	0.43 (14)	7 (9)
Lowest L(E)C50, MIC	0.01	2.1	0.08	0.00085	0.024	0.09
Most sensitive organisms	Crustaceans	Crustaceans	Algae	Crustaceans	Crustaceans	Algae
Classification	Very toxic	Toxic	Very toxic	Very toxic	Very toxic	Very toxic
(EU-Directive 93/67/EEC (CEC 1996)[b]						
Classification (Sanderson et al. 2003; Blaise et al. 2008)[c]	Extremely toxic	Toxic	Extremely toxic	Extremely toxic	Extremely toxic	Extremely toxic

* In the brackets next to the median value, the number of data used to derive the median value is presented

2.4 Marine Photosynthetic Microorganism

Photosynthetic microorganism (photoautotrophs) are organisms that are capable of to do photosynthesis. All of this organism contain chlorophyll, which is a pigment able to absorbs light energy. In this research two types of marine photosynthetic organism were used green microalgae and cyanobacteria. Even do these two organisms are photosynthetic, the have key major differences. Cyanobacteria are prokaryotes while green microalgae are eukaryotic.

2.4.1 Cyanobacteria

Cyanobacteria are prokaryotic gram-negative oxygenic photosynthetic autotrophic organisms (Schopf, 2000). Cyanobacteria have various morphologies: unicellular, colonial, filamentous, and branched filamentous forms. Cyanobacteria are important in the biogeochemical cycles of nitrogen, carbon, and oxygen.

Cyanobacteria can also be used for food and has biologically active compounds that are valuable for medicinal purposes (Sharma, Rai, & Stal, 2014). Even though cyanobacteria have many beneficial properties, they can also be toxic; the most common toxic algae are blue-green algae (cyanobacteria).

Cyanobacteria have metal requirements different from other bacteria: copper in thylakoidal plastocyanin, zinc in carboxysomal carbonic anhydrase, cobalt in cobalamin, magnesium in chlorophyll, molybdenum in heterocystous nitrogenase, manganese in thylakoidal water-splitting oxygen-evolving complex (Cavet, Borrelly, & Robinson, 2003). The zinc stress response in *Cyanobacterium Synechococcus* sp. was toxic when exposed to 50 mg/L ZnCl2 (Newby, Lee, Perez, Tao, & Chu, 2017).

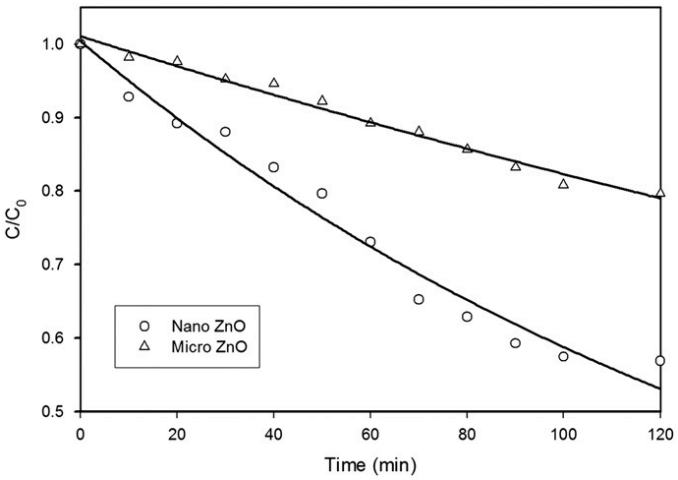


Figure 4: Show the results of Nano ZnO and Micro ZnO 50 ppm.

An ecotoxicity study using *A.flosaquae* and *E.gracilis* showed a decrease of photosynthetic activity for both in the first 10 days when exposed to ZnO NPs (Brayner, 2010). In another study, using the microalgae A.*flosaquae* was compared to the control,

micro and nano ZnO. The control showed an increment in algal growth. The micro and nano ZnO showed a lowered growth than the control. The research also showed (Figure 4) how nano ZnO have a lower growth than the micro ZnO (Nandi, et al., 2012).

Djearamane, Lim, Wong, and Lee (2018) conducted a study of cyanobacterium *Spirulina (Arthrospira) platensis* using different concentrations of ZnO NPs (10–200 mg/L) from 6 to 96 h to explore the dose- and time-dependent cytotoxic effects. They discovered that ZnO NPs triggered substantial cytotoxicity within the *S. platensis* and caused cell death. The maximum cell death occurred at 96 h cell death of 44.3±4% for 10 mg/L, 69.7±2.1% for 50 mg/L, 83.8±0.9% for 100 mg/L, 86.7±1.2% for 150 mg/L, and 87.3 ±1% for 200mg/L of ZnO NPs. The cyanobacteria used in this study included: *Limnothrix* sp., *Leptolyngbya* sp., *Lyngbya 1* sp., *Lyngbya* 2 sp., *Porphyridium* sp., *Roseofilum* sp. and *Apistonema* sp.

2.4.2 Green Microalgae (Chlorophytes and Charophytes)

Green microalgae are one of the most common types of algae and have chlorophylls a and b. Green algae are the only algae that store their photosynthates within the chloroplast (Chapman, 2013). The green microalgal cells are negatively charged, providing a set of binding sites for metal cations like Zn^{+2}, which have the potential to lower toxicity in the microalgae (Monteiro, Fonseca, Castro, & Malcata, 2010). Despite this, there has been research showing Zn toxicity. In a study where both *S. obliquus* and *D. pleiomorphus* were exposed to Zn concentrations, researchers discovered levels of toxicity; although, *S. obliquus* can tolerate higher Zn concentrations than *D. pleiomorphus* (Monteiro, Fonseca, Castro, & Malcata, 2010). Surprisingly,

Raphidocelis subcapitata and *Chlorella vulgaris* are proven to have a tolerance to Zinc within a 72-hour exposure (Muyssen & Janssen, 2001).

There have been inconsistent results with the toxicity of ZnO NPs among some green microalgae. For example, the green microalgae *Dunaliella tertiolecta* of the order of *Chlamydomonadales* demonstrated different results in three different studies. In the first study, there was no conclusion as a consequence to the low estimate samples (Miller, 2010). When the experiment was repeated, it showed an inhibition growth at 96 h with the concentration of 133 mg/L ZnO NPs (Miglietta, et al., 2011). Finally, when the experiment was last conducted, there was also an inhibition growth at 96 h, but in a smaller concentration of 2.42 mg/L ZnO NPs (Manzo, Miglietta, Rametta, Buono, & Francia, 2013).

Results of other green microalgae that show inhibition at low and high concentrations are classified in tables 2 and 3. For the *Tetraselmis sucica*, there was no effect in a high concentration of 100 mg/L ZnO NPs (Castro-Bugallo, González-Fernández, Guisande, & Barreiro, 2014). While for *Scenedemus rubescens* there was a 50% inhabitation rate at 14.27 mg/L ZnO NPs (Aravantinou, Tsarpali, Dailianis, & Manariotis, 2015). With *Chlorella vulgaris* there was a 35% cell viability in a high concentration of 200 mg/L ZnO NPs (Suman, Rajasree, & Kirubagaran, 2015). *Pseudokirchneriella subcapitata* had a high inhibition of 80% with a very low concentration of 0.1 mg/L ZnO NPs (Lee & An, 2013). All of these experiments included: *Chlorococcum*, *Chlamydomonas*, and *Chlorella*.

Table 2
Nano ZnO effects upon microalgae.

Algae	Endpoint/s	ZnO effect concentration ($mg\ L^{-1}$)	Reference
Marine species			
T. pseudonana	Growth rate inhibition	0.5	Miller et al.
S. marinoi	(LOEC[a])	1	(2010)
D. tertiolecta		NA	
I. galbana		NA	
T. pseudonana	Fv/Fm	IC50 range	Miao et al.
	growth rate	(as uM Zn^{2+})	(2010)
	Chl a	0.39–6.94	
T. pseudonana	Growth rate inhibition	10	Peng et al.
C. gracilis	(EC100)		(2011)
P. tricornutum			
S. costatum	96 h growth inhibition	2.36 (2.11–2.64)	Wong et al.
T. pseudonana	(EC50)	4.56 (4.06–5.11)	(2010)
D. tertiolecta	96 h growth inhibition (EC50)	2.42 (0.97–2.87)	Present work
D. tertiolecta	96 h growth inhibition[b]	133	Miglietta et al. (2011)
Freshwater species			
P. subcapitata	72 h growth inhibition (IC50)	about 0.04 (as Zn^{2+})	Aruoja et al. (2009)
P. subcapitata	72 h growth inhibition test (IC50)	0.06 (as Zn^{2+})	Franklin et al. (2007)
Chlorella sp.	6 d growth inhibition (EC30)	20	Ji et al. (2011)
C. reinhardtii	12 d growth inhibition (LOEC[a])	1	Luo (2007)

NA: not available.
[a] Lowest observed effect concentration.
[b] 100% effect at single limit test concentration.

Table 3: NPs ZnO effects upon microalgae (Miazek, Iwanek, Remacle, Richel, & Goffin, 2015).

Metal	Microalgae Strain	Cultivation Time	Concentration	Effect on Growth	Ref.
	Phaeodactylum tricornutum		100 mg/L	80% inhibition	
ZnO-NPs	*Alexandrium minutum*	–	100 mg/L	80% inhibition	[51]
	Tetraselmis suecica		100 mg/L	No effect	
ZnO-NPs	*Scenedesmus rubescens*	96 h	14.27 mg/L or >810 mg/L [CM]	50% inhibition	[53]
ZnO-NPs	*Chlorella vulgaris*	72 h	200 mg/L	35% cell viability	[50]
ZnO-NPs	*Dunaliella tertiolecta*	96 h	2.4 mg/L	50% inhibition	[56]
ZnO-NPs	*Pseudokirchneriella subcapitata*	72 h	0.1 mg/L	80% inhibition	[52]

2.4.3 Diatoms

Diatoms are the only organisms with cell walls composed of transparent, opaline silica. This silica comprises their cell wall, making them useful in the manufacturing of pool filters. Diatoms are a type of plankton called phytoplankton, which store carbon in the form of natural oil or as a polymer of carbohydrate (Chapman, 2013). Diatoms are unicellular organisms that exist as solitary cells or in colonies which form various shapes like ribbons, fans, zigzags, and stars (Tomas & Hasle, 1997).

There have also been studies conducted with the toxicity Zn to different types of diatoms. Zinc (Zn) ions can be chelated by exopolysaccharides as in *Skeletonema costatum* or in the cytoplasm by phytochelatins, which are cysteine-rich pseudopeptides (Imber, Robinson, Ortega, & Burton, 1985). After comparing four marine diatoms (*Amphora acutiuscula, Nitzschia palea, Amphora coffeaeformis and Entomoneis paludosa*) some show tolerance and others sensitivity to Zn. *E. paludosa* was found to be the most sensitive to Zn since growth is drastically decreased (figure 5), while *A. coffeaeformis* is the most tolerant species (Nguyen-Deroche, et al., 2012).

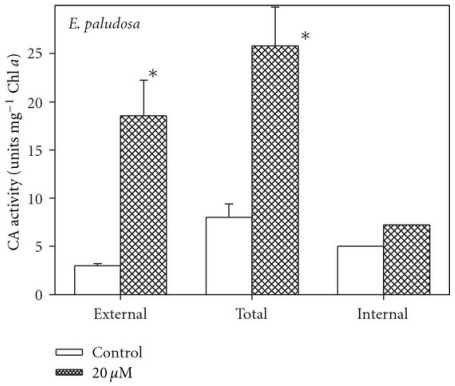


Figure 5: *E. paludosa* grown in (control) or in the presence of 20 µM Zn.

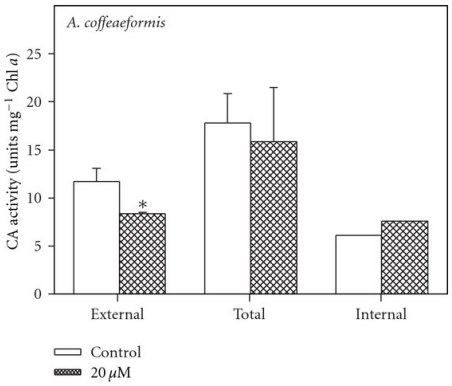


Figure 6: *A. coffeaeformis* grown in (control) or in the presence of 20 µM Zn.

Another study used the diatoms, *T. pseudonana* and *S. marinoi* which show inhibition in low concentrations of ZnO NPs at 0.5 and 1 mg/L (Miller, 2010), while another study shows growth rate inhibition in a much higher concentration for *T. pseudonana* at 10 mg/L ZnO NPs (Peng, Palma, Fisher, & Wong, 2011). Finally, the results of a 2014 study of the same microalgae shows 80% inhibition, but in a high concentration of 100 0.5 and 1 mg/L (Castro-Bugallo, González-Fernández, Guisande, & Barreiro, 2014). The marine diatom *Thalassiosira pseudonana* was exposed to ZnO NPs with a higher temperature and salinity. ZnO NPs exposed to greater temperature formed larger aggregations and released less zinc ions (Zn2+). The toxicity of ZnO NPs to *T. pseudonana* was less at 25 °C than at 10 °C, but the toxicity was significantly greater at 30 °C (Yung, Kwok, Djurišić, Giesy, & Leung, 2017).

2.5 Toxicity Mechanism

Information about the toxicity mechanism of NPs has been lacking, but there are many suggestions. The top four most suggested mechanisms are: reactive oxygen species (ROS) generation, metal ion release, nanomaterials accumulation on member surface, and internalization of nanomaterials (Figure 7). It is important to note that these are toxic mechanisms to bacteria and not to the actual microalgae (Djurišić, et al., 2014).

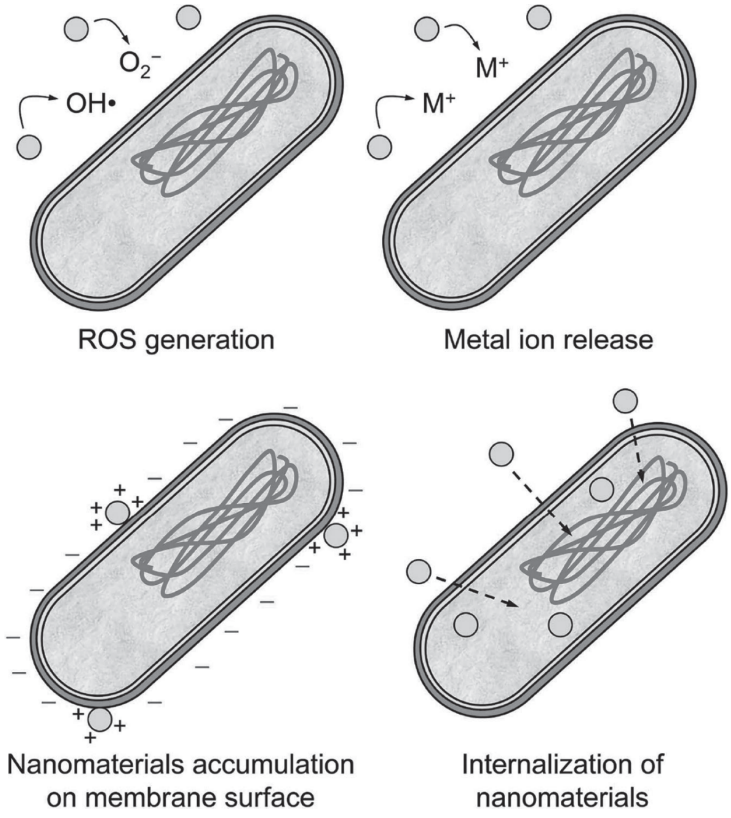


Figure 7: Possible toxicity mechanisms against bacteria cells.

2.5.1 ROS Induce Damage

Reactive oxygen species (ROS) is one of the most studied toxicity mechanisms. Reactive oxygen species (ROS) are species containing oxygen that are chemically reactive. Reactive oxygen species (ROS) levels can increase dramatically when there is environmental stress that could result in damage to cell structures. Nnaoparticles (NPs) induce ROS damage because of the unique factors NPs have: NPs internalization, particle chemistry, and physical properties. The NPs unique physicochemical properties, like their size, have the potential to generate ROS (Ray, Yu, & Fu, 2009).

Charge transfer of photogenerated charge between NPs and the bacteria can cause cell membrane damage. Nanoparticles (NPs) can trigger increased expression of pro-inflammatory, fibrotic cytokines and activation of inflammatory cells, which can influence the enhanced generation of ROS. Some metal oxide NPs can incite free-radical-facilitated toxicity via Fenton-type reactions (Abdal, et al. 2017).

2.5.2 Metal Ion Toxicity

Metal toxicity is the toxic effect of certain metals in certain forms. Some metals are toxic when they form lethal soluble compounds. The toxicity of NPs which are moderately soluble is often attributed to the metal ion release (Tyupa, 2016).

2.5.3 NPs accumulation on membrane surface

The integration between the membrane surface and NPs may be another mechanism. In research by Applerot, et al. (2009), the NPs remained firmly attached to the bacteria membrane even after washing the bacteria.

2.5.4 Internalization of NPs

When the NPs are attached on the cell surface, redox reactions on the surface of adsorbed NPs can result in oxidative stress to the bacteria and cytotoxicity (Handy, Owen, & Valsami-Jones, 2008). Another mechanism involves NPs adhesion to the membrane in combination with ROS generation, resulting in a change in membrane permeability and cell death. The adsorption of NPs on the cell membrane can affect the membrane viscosity and the transport exchanges (Guarnieri, et al., 2014).

2.6 Toxic mechanism of ZnO NPs

Since ZnO NPs are the particles studied in this research, their toxicity mechanism must be discussed. Wang, Gao, Lin, Yao, & Zhang (2014) found that mixtures of different types of NPs and surfactants revealed aggressive and additive effects, with the highest toxicity of ZnO was mainly the results of dissolved Zn ions. Another reason for ZnO NPs toxicity may be environmental factors such as temperature or organic acid. The mechanism is composed of the dissolution of ZnO and release of Zn^{2+}. They increase temperature, decrease dissolution, and increase aggregation (Majedi, Kelly, & Lee, 2014).

2.6.1 Toxic mechanism of ZnO NPs on microalgae

Chlamydomonas reinhardtii was exposed to various coated ZnO NPs showed the highest toxicity was from bare ZnO NPs, suggesting possible mechanisms such as particle solubilization, metal complexation, and metal bio-uptake (Merdzan, Domingos, Monteiro, Hadioui, & Wilkinson, 2014). Research using *T. pseudonana, Skeletonema marinoi and Dunaliella tertiolecta* indicated that the reason for the ecotoxicity is the competition between ZnO NPs and nutrients, thereby inhibiting growth and nutrient uptake (Matranga & Corsi, 2012; Peng, Palma, Fisher, & Wong, 2011).

2.7 Bioremediations

Bioremediation is the process that uses organisms, such as microorganisms, to degrade and reduce or detoxify waste products and pollutants (Prasad, M. V., 2016). Microalgae have the potential to serve as a method of bioremediation for the toxicity of ZnO NPs for several reasons. One of the reasons discussed in the research of Hazeem et al. (2015) is the marine microalgae's (*Picochlorum* sp.*)* ability to adapt to long term

exposure of ZnO NPs. In this study, the toxicity of NPs in marine microalgae was reduced by aggregation and sedimentation.

2.7.1 Biosorbent

Biosorption, for these purposes, is the removal of metal or metalloid species, compounds, and particulates from a solution by a biological organism (Wang & Chen, 2009). An ideal biosorbent should possess features like availability, non-toxicity, high metal binding capacity, large-scale usability, and regeneration/re-usability (Wang & Chen, 2009). Microalgae are considered a low-cost biosorbent, and the cell wall characteristics give them a high metal ion binding capacity (Anastopoulos & Kyzas, 2015; Gong, et al. 2005; Tien 2002). A non-living algal mass can be an even better biosorbent since it has a higher metal ion sorption capacity at a higher rate, and it does not require nutrients grown in a medium. Furthermore, a dead algal mass can be removed using de-ionized water (Zeraatkar, Ahmadzadeh, Talebi, Moheimani, & Mchenry, 2016).

Many algae have been found to be good biosorbents. For example, brown algae have good biosorption capacity as consequences of the presence of alginates in their cell walls (Demey, Vincent, & Guibal, 2018). The microalgae *Chlorella miniata* (Tam et al., 2001), and *Chlamydomonas acidophila* (Nishikawa and Tominaga 2001) have demonstrated a high resistance to toxic metals present in their surrounding habitat. *P. lanceolatum* algae can remove zinc metal up to 118.66 mg g^{-1} (Sbihi, K., Cherifi, O., El Gharmali, A., Oudra, B., Aziz, F., 2012).

2.7.2 Sunscreens

Another way microalga can be used as bioremediation is with sunscreen. Mycosporine-like amino acids (MAA) extracted from cyanobacteria, absorbing maximally at 335 nm, are encapsulated into liposomes where they have been shown to be photostable and photoprotective in vitro (Schmid, Schurch, and Zulli, 2006). Another study showed that MAA are biocompatible, photoresistant, and thermoresistant, and exhibit a highly efficient absorption of both UV-A and UV-B radiations (Fernandes, et al., 2015).

2.7.3 Microalgae Synthesis NPs

Research published by Singh et al. (2014) discusses the synthesis of ZnO NPs using the cell extract of the cyanobacterium, *Anabaena* strain. The natural synthesis of ZnO NPs serves as bioremediation for the NPs toxicity. The aim of this research was not only to study the toxicity of the NPs, but the potential bioremediation that microalgae can potentially have.

Diatoms that produce valves could be evolved for cultivation in chemostat cultures to mass-produce nanoscale components. Diatoms can also synthesis micro- and nano-scale structures which may be of use in a range of devices, including: optical systems, semiconductor nanolithography, and even vehicles for drug delivery (Bradbury, 2004). In addition, diatoms can substitute photosensitive titanium dioxide for the silicon dioxide component of solar cells (Johnson, R.C., 2009).

Even though ZnO NPs can be potentially harmful to microalgae, eco-NPs can be synthesized from microalgae, and these microorganisms can even be used for sunblock, which is a product of major concern.

Section 3: Materials and Methods

3.1 Marine Algae Culture

3.1.1 Algal Species

Ten South Florida marine microalgae strains, three green microalgae: *Chlorococcum* sp. (146-2-6), *Chlamydomonas* sp. (146-2-10), and *Chlorella* sp. (146-2-16) (Figure 8), and seven cyanobacteria *Limnothrix* sp. (173-10-1), *Leptolyngbya* (146-5-2), *Lyngbya* 1 sp. (EK17-3A), *Lyngbya* 2 sp. (Bpm 173F), *Porphoridium* sp. (173-10-2), *Roseofilum* sp. (101-1), *Nannochloris* sp. (146-2-11) were used in this project. All of them were obtained from Dr. Miroslav Gantar's culture collection. These marine strains were isolated from the South Florida region.

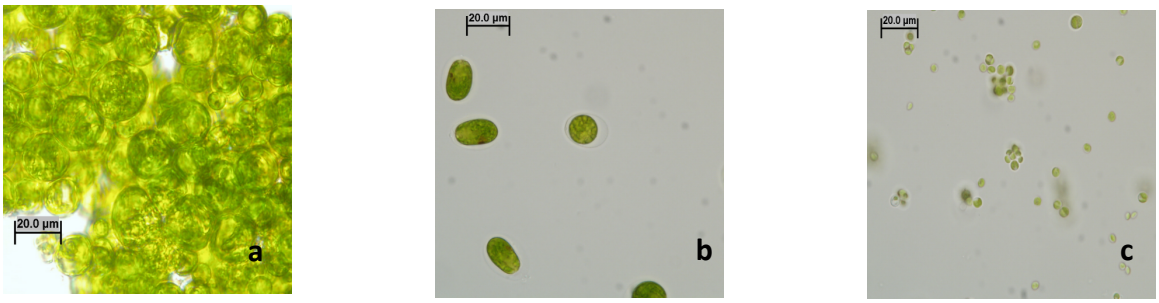


Fig 8. Green Microalgae strains used in this research: a) 146-2-6 *Chlorococcum* sp. *b*) 146-2-10 *Chlamydomonas* sp. and *c*)146-2-16 *Chlorella* sp. 100X magnification, scale bar represents 20µm.

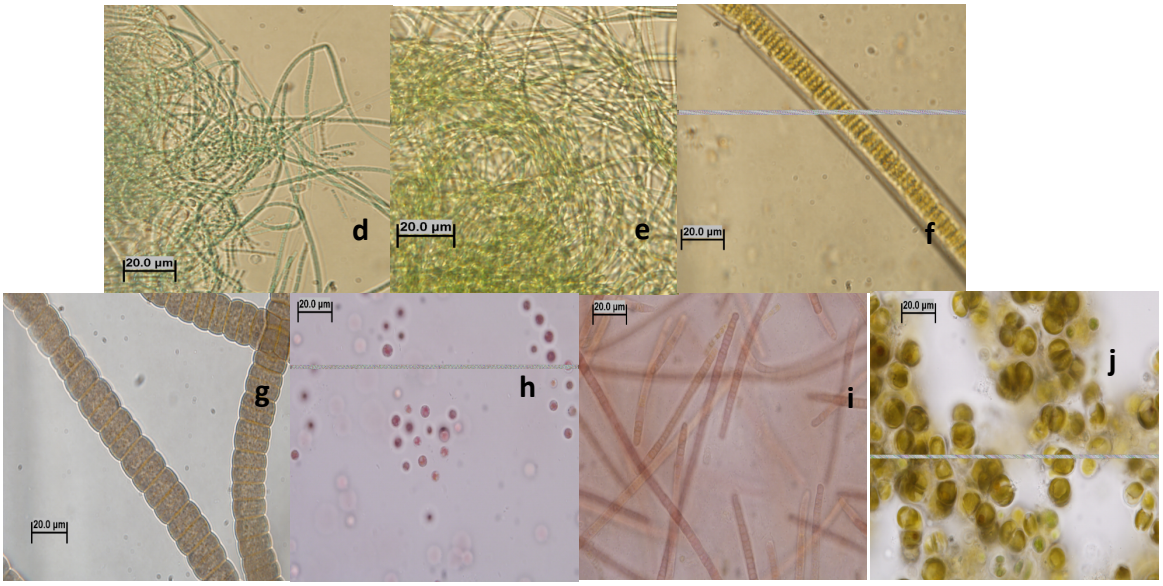


Figure 9. Cynobacteria strains used in this research: d) *Limnothrix sp.* (173-10-1), e) *Leptolyngbya sp.* (146-5-2), f) *Lyngbya* 1 sp. (EK17-3A), *g) Lyngbya* 2 sp. (Bpm 173F), h) *Porphoridium sp. (*173-10-2), i) *Roseofilum sp.* (101-1), j) *Apistonema sp.* 146-2-11 sp. 100X magnification, scale bar represents 20 µm.

3.1.2 Microalgae Culture Conditions

All of the microalgae were cultivated in flasks with marine BG-11 Medium. The marine BG-11 Medium was prepared with one-liter distilled water and six stock solutions (Tasic, Rios, Santos, Filipini, & Maciel 2016). The six stock solutions were diluted with 500 mL: Dipotassium phosphate (K_2HPO_4) – 15.25 g, Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)- 37.5 g, Sodium carbonate (Na_2CO_3- 10 g), Fe-Chelate (Fe(III) Citrate- 3 g, Citric Acid 3g and Na_2 EDTA 0.5g) and Micro-elements (H_3BO_3- 1.43g, $MnCl_2 \cdot 4 H_2O$- 0.90g, $ZnSO_4 \cdot 7 H_2O$- 0.111g, $Na_2MoO_4 \cdot 2 H_2O$- 0.195 g, $CuSO_4 \cdot 5 H_2O$- 0.040g, $Co(NO_3)_2 \cdot 6 H_2O$- 0.025 g). After adding the six stock solutions and 1 mL of Deionized water (DI water), 35 g of marine salt water and 1.5 g of sodium nitrate

($NaNO_3$) were added. This solution was then stirred to make it homogenous. After verifying the marine medium was at 8 pH, the medium was autoclaved.

All of the medium was transferred into 125 mL flasks. Isolate culture was then added into every flask. The microalgae were incubated with New Brunswick Scientific Excella E24 Incubator Shaker Series at 25°C degrees under continuous lighting of 50µmol of photons $m^{-2}s^{-1}$for with and continuous shaking of 155 rpm for a week or more (Figure 10).

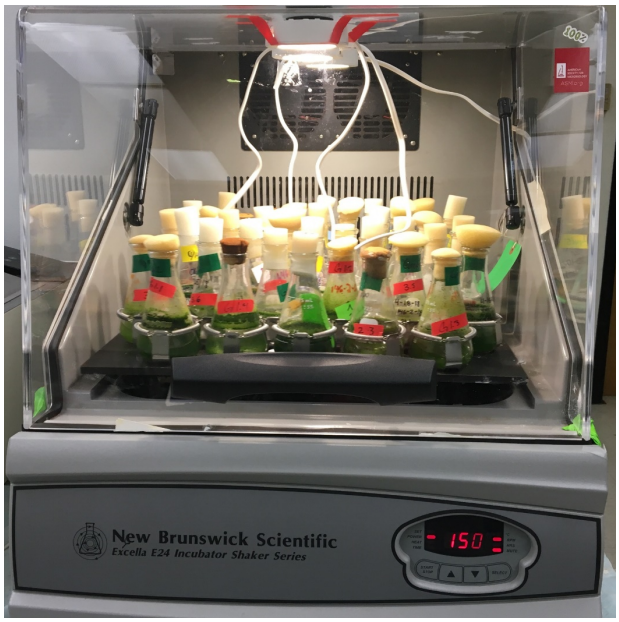


Figure 10: The incubator used to grow the microalgae.

3.2 Microplate Experiment

3.2.1 Stock solution

ZnO Bulk (Sigma-Aldrich Zinc Oxide) and ZnO Nano Zinc oxide (Sigma-Aldrich nanoparticles, <100 nm particle size), stock solutions were used in the experiments. For the Bulk stock solution, the concentrations were: 0.125 ppm, 0.25 ppm, 0.5 ppm, 1 ppm, 2.5 ppm, 5 ppm, 7.5 ppm ,10 ppm, and 20ppm.

For the preparation of bulk zinc oxide stock solution for low Zn concentration treatments (0.125 ppm, 0.25 ppm, 0.5 ppm, and 1 ppm), a solution of 4.669 mg Bulk ZnO and 20mL DI water was made. Then, 3mL of this solution was combined with 27 mL of BG11 medium without zinc ($ZnSO_4 \cdot 7\ H_2O$- 0.111g) to create 0.0155 mg Zn/mL. Using a pipette 1 mL of the previous solution and 99mL BG-11 Media without zinc were combined, creating the 0.125 ppm bulk Zn stock solution. For the 0.25 ppm Zn bulk solution, 2 mL of the of solution of 4.669 mg Bulk ZnO and 20 mL DI solution was pipetted and combined with 98 mL BG-11 Media without zinc. For the 0.5 ppm bulk Zn stock solution, 4 mL of the pervious solutions was pipetted and combined with 96 mL BG-11 Media without zinc. Finally, for the bulk zinc oxide stock solution of 1 ppm, 8 mL Zn was pipetted and combined with 92 mL BG-11 Media without zinc. All of the stock solutions were then put into individual flasks and autoclaved. For the stock solution of 2.5 ppm Zn, 5 ppm Zn, 7.5 ppm Zn, and 10 ppm Zn, another solution was created. First, a solution of 3.11 mg ZnO/mL 62.20 mg bulk ZnO was added to 20 mL DI Water. Using a pipette, 2 mL of the previous solution and 18 mL DI Water was combined to create a stock solution of 0.311 mg ZnO/mL. Next, four flasks were used for the 2.5 ppm Zn, 5 ppm Zn, 7.5 ppm Zn, and10 ppm Zn bulk stock solutions. For the 2.5 ppm Zn, 1 mL of the previous solution was pipetted and combined with 99 mL BG-11 Media without zinc. For the 5 ppm Zn bulk solution, 2 mL of the pervious solution was pipetted and combined with add 98 mL BG-11 Media without zinc. For the 7.5 ppm Zn bulk solution, 3 mL of the previous solution was pipetted and combined with 97 mL BG-11 Media without zinc. For the 10 ppm Zn bulk solution, 4 mL of the previous solution was pipetted and combined with 96 mL BG-11 Media without zinc. Finally, for the 20 ppm

Zn bulk solution, 8 mL of the previous solution was pipetted and combined with 92 mL BG-11 Media without zinc. All of the flasks were then autoclaved.

For nano stock solution, similar steps were followed except for the two additional steps, the use of a surfactant Igepal CA630 and sonication to prevent aggregation of nano particles. To prepare the Igepal solution, .734 mL Igepal was pipetted into 1L DI water. This solution is called IgM solution. From the IgM solution two other solutions were made: the IgA was done by pipetting 1 mL of IgM and adding 9 mL DI Water and the IgB was done by pipetting 1 mL of IgM and adding 99 mL DI Water. After creating the IgM, IgB and IgA solutions, the nano stock solution made. First, 4.669 mg Nano size 10nm ZnO was mixed with 18 mL DI Water to create a stock solution. This solution was sonicated for 30 minutes to keep the nanoparticles separated. After the solution was sonicated, 2 mL of IgA solution was added. Using a pipette, 2 mL of the sonicate solution was added to 18mL IgB solution (0.155mg ZnO/mL). Out of this solution, four stock solutions were made: .125 ppm Zn, 0.25 ppm Zn, 0.5 ppm Zn, and 1 ppm. For the 125 ppm Zn nano solution, 1mL of the previous solution was pipetted and combined with 98 BG-11 Media without zinc and 1mL IgM solution. For the 0.25 ppm Zn nano solution, 2 mL of the previous solution was pipetted and combined with 97 BG-11 Media without zinc and 1mL IgM solution. For the 0.5 ppm Zn nano solution, 4 mL of the previous solution was pipetted and combined with 95 Medium BG-11 Media Zinc and 1 mL IgM. Finally, for the 1 ppm Zn nano solution, 8 mL of the previous solution was pipetted and combined with 91 mL Medium BG-11 Media Zinc and 1 mL IgM. All of the stock solutions were then autoclaved. The next set of nano stock solutions were 2.5 ppm Zn, 5 ppm Zn, 7.5 ppm Zn, 10 ppm Zn, and 20ppm Zn. First, 62.20 mg Nano ZnO was mixed in 18 mL DI Water. This solution was sonicating for 30 minutes to keep the

nanoparticles separated. After the solution was sonicated 2 mL of IgA solution was added. Using a pipette, 2 mL of the pervious solution was combined with 18mL IgB solution to create 0.155mg ZnO/mL. For the 2.5 ppm Zn nano solution, 1 mL of the previous solution was pipetted and combined with 98 BG-11 Media without zinc and 1mL IgM solution. For the 5 ppm Zn nano solution, 2 mL of the previous solution was pipetted and combined with 97 BG-11 Media without zinc and 1mL IgM solution. For the 7 ppm Zn nano solution, 3 mL of the previous solution was pipetted and combined with 96 Medium BG-11 Media Zinc and 1mL IgM. For the 10 ppm Zn nano solution, 4 mL of the previous solution was pipetted and combined with 95 mL Medium BG-11 Media Zinc and 1mL IgM. Finally, for the 20 ppm Zn nano solution, 8 mL of the previous solution was pipetted and combined with 91 mL Medium BG-11 Media Zinc and 1mL IgM. All of the stock solutions were then autoclaved.

The last stock solution was high concentration of zinc (ZnSO4 x 7 H2O). Measure 65.97 mg ZnSO4 x 7 H2O and add 10 mL DW. Pipette 1mL and add 9 mL DW (6.597 mg ZnSO4 x 7 H2O /mL). Out of this pippete 1mL and add 99 mL BG-11 no Zn (0.6597 mg ZnSO4 x 7 H2O /mL). Finally autoclave.

All of the stock solutions were used for both the microplate experiment and flask experiments.

3.2.2 Exposure to Low Zn Concentrations

The microplate reader Synergy™ HT was use with the Falcon™ Polystyrene Microplates (Figure 3) of 24 wells was used for all of the bulk microplate experiments (Figure 4); the same format was used in all experiments. The lower concentrations (0.125 ppm Zn, 0.25 ppm Zn, 0.5 ppm Zn, and 1 ppm) for bulk and NPs were arranged as shown in Figure 3 (Figure 3). The blank concentration contained 3 mL of BG-11 media

with micro-element Zn. The control contained 2 mL of BG-11 media with micro-element Zn and 1 mL of the selected algae. The lower concentrations were then prepared as follows: 1) 2 mL stock solution 0.125 ppm Zn (NPs or bulk particle) was combined with 1 mL of the selected algae, 2) 2 mL stock solution 0.25 ppm Zn (NPs or bulk particle) was combined with 1 mL of the selected algae, 3) 2 mL stock solution 0.5 ppm Zn (NPs or bulk particle) was combined with 1 mL of the selected algae, 4) 2 mL stock solution 1 ppm Zn (NPs or bulk particle) was combined with 1 mL of the selected algae. The vertical lines are the different algae used, from green micro algae to cyanobacteria. Five 24-well plates were used for bulk particles and five 24-well plates for NPs. The plates were then incubated at 25°C degrees under continuous lighting of 50 µmol of photons $m^{-2}s^{-1}$f for one week. Algal growth was assessed by using a microplate reader at 600 on days 0, 1, 2, 3, 4, 5, 6, and 7. To avoid contamination of the original 24-well plate, a minimal quantity of culture samples was transferred to a 24-well plate under sterile conditions during each observation period and the OD recording was made using Biotek™ plate reader (Figure 11).

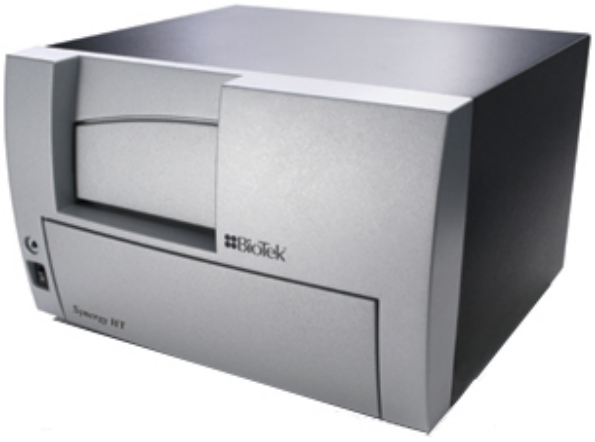


Figure 11: The Biotek™ microplate reader.

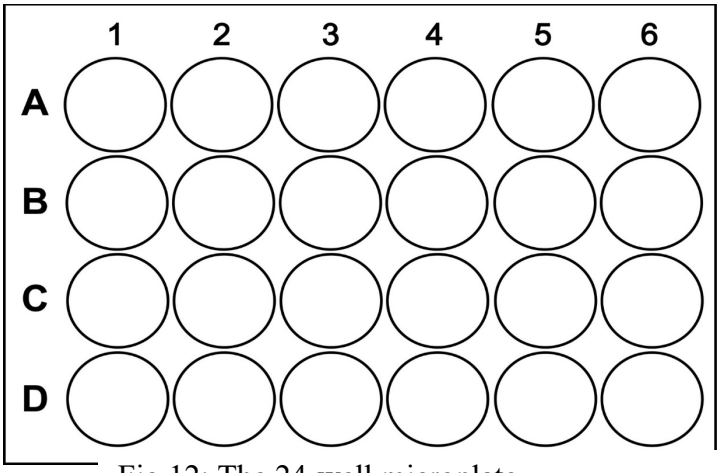


Fig 12: The 24-well microplate

3.2.3 Exposure to High Zn Concentrations

The higher concentrations (2.5 ppm Zn, 5 ppm Zn, 7.5 ppm Zn, and 10 ppm Zn) for bulk and NPs were also arranged as shown in Figure 3. The blank concentration contained 3 mL of BG-11 media with micro-element Zn. The control contained 2 mL of BG-11 media with micro-element Zn and 1 mL of the selected algae. The higher concentrations were then prepared as follows: 1) 2 ml stock solution 2.5 ppm Zn (NPs or bulk particle) and 1 mL of the selected algae, 2) 2 ml stock solution 5 ppm Zn (NPs or bulk particle) was combined with 1 mL of the selected algae, 3) 2ml stock solution 7.5 ppm Zn (NPs or bulk particle) was combined with 1 mL of the selected algae, 4) 2ml stock solution 1 ppm Zn (NPs or bulk particle) was combined with 1 mL of the selected algae. The vertical lines are the different algae used, from green micro algae to cyanobacteria. Five 24-well plates (Figure4) were used for each experiment representing the 5 replicas. The plates were then incubated at 25°C degrees under continuous lighting of 50 μmol of photons $m^{-2}s^{-1}$f for one week. Algal growth was assessed by using a microplate reader at 600 on days 0, 1, 2, 3, 4, 5, 6, and 7. To avoid contamination of the original 24-well plate, a minimal quantity of culture samples was transferred to a 24-well

plate under sterile conditions during each observation period and the OD recording was made using Biotek plate reader.

A final higher concentration was done for the green microalgae with 10 ppm Zn and 20 ppm Zn (NPs and bulk particles). The selected algae for the higher concentration were: Algae 1: *Chlorococcum* sp. 146-2-6, Algae 2: *Chlamydomonas* sp. 146-2-10, Algae 3: *Chlorella* sp. 146-2-16, and Algae 4: *Nannochloris* sp. 146-2. The blank concentration contained 3 mL of BG-11 media with micro-element Zn. The control contained 2 mL of BG-11 media with micro-element Zn and 1 mL of the selected algae. The higher concentrations for the green microalgae were then prepared as follows: 1) 2ml stock solution 10 ppm Zn bulk particle and 1 mL of the selected algae, 2) 2 mL stock solution 20 ppm Zn bulk particle and 1 mL of the selected algae, 3) 2 mL stock solution 10 ppm Zn NPs and 1 mL of the selected algae, 4) 2 mL stock solution 20 ppm Zn NPs and 1 mL of the selected algae. Five 24-well plates were used for each experiment representing the 5 replicas. The plates will then be incubated at 25°C degrees under continuous lighting of 50 µmol of photons $m^{-2}s^{-1}$f for one week. Algal growth will be assessed by using a microplate reader at 600 on days 0, 1, 2, 3, 4, 5, 6, and 7. To avoid contamination of the original 24-well plate, a minimal quantity of culture samples was transferred to a 24-well plate under sterile conditions during each observation period and the OD recording was made using Biotek plate reader.

3.2.4 Exposure to Low Zn Concentrations with Additional Zn Source

This experiment was similar to the low concentration but with an extra well containing ZnSO4 (ZnSO4, 0.125 ppm Zn, 0.25 ppm Zn, 0.5 ppm Zn, and 1 ppm Zn) in addition to bulk and NPs were arranged as shown in Figure 3. It is important to note that this experiment was done with only four cynobacteria (*Limnothrix* sp., *Leptolyngbya* sp.,

Lyngbya 1 sp., and *Lyngbya* 2 sp.). The control contained 2 mL of BG-11 media with micro-element Zn and 1 mL of the selected algae. The extra zinc contained 2 mL stock solution ZnSO4. The 0.125 ppm Zn contained 2 mL stock solution (NPs or bulk particle) was combined with 1 mL of the selected algae. The 0.25 ppm Zn contained 2 mL stock solution (NPs or bulk particle) was combined with 1 mL of the selected algae. The 0.5 ppm Zn contained 2 mL stock solution (NPs or bulk particle) was combined with 1 mL of the selected algae. The 1 ppm Zn contained 2 mL stock solution (NPs or bulk particle) was combined with 1 mL of the selected algae. The vertical lines are the different algae used, from green micro algae to cyanobacteria. Five 24-well plates were used for bulk particles and five 24-well plates for NPs. The plates were then incubated at 25°C degrees under continuous lighting of 50 μmol of photons $m^{-2}s^{-1}$f for one week. Algal growth was assessed by using a microplate reader at 600 on days 0, 1, 2, 3, 4, 5, 6, and 7. To avoid contamination of the original 24-well plate, a minimal quantity of culture samples was transferred to a 24-well plate under sterile conditions during each observation period and the OD recording was made using Biotek™ plate reader.

3.2.5 Optical icroscope

Figure 13: Olympus™ BX51 research microscope.

Light microscope observations of algal samples under different magnifications (20x – 100x) were carried out using an Olympus BX51 microscope equipped with Olympus DP70 digital camera and associated software (DP Controller and DP Manager).

3.2.6 Statistical Analysis

The data collected will be tested for statistical significance using two-way mixed (ANOVA), taking the level of $P< 0.05$ as significance according to Tukey multiple comparison test. The within factor being the days (0, 2, 5, 7) and the between-subjects factors the treatments. The codification for low concentration experiment treatments s was as follow: B1: 0.125ppm Bulk ZnO, B2: 0.25 ppm Bulk ZnO, B3 0.5 ppm: Bulk ZnO, B4: 1 ppm Bulk ZnO, N1: 0.125ppm NPs ZnO, N2: 0.25 ppm NPs ZnO, N3: 0.5 ppm NPs ZnO, N4: 1 ppm NPs ZnO and control. Cyno 1: *Limnothrix* sp.*;* Cyno 2: *Leptolyngbya* sp.*,* Cyno 3*: Lyngbya* 1 sp., Cyno 4: *Lyngbya* 2 sp. (Bpm 173F), Cyno 4: *Oscillatoria* sp. Cyno5: *Porphoridium* sp. Cyno 6: *Roseofilum* sp. Cyno 7: *Nannochloris* sp.*;* Green 1: *Chlorococcum* sp., Green 2: *Chlamydomonas* and Green 3: *Chlorella sp.* The codification for higher concentration experiment treatments s was as follow: B1: 2.5 ppm Bulk ZnO, B2: 5 ppm Bulk ZnO, B3 7 ppm: Bulk ZnO, B4: 10 ppm Bulk ZnO, N1: 2.5ppm NPs ZnO , N2: 5 ppm NPs ZnO, N3: 7 ppm NPs ZnO, N4: 10 ppm NPs ZnO and control. Finally the codification for the higher Zn Concentration was:

B1: 0.125ppm Bulk ZnO, B2: 0.25 ppm Bulk ZnO, B3 0.5 ppm: Bulk ZnO, B4: 1 ppm Bulk ZnO, N1: 0.125ppm NPs ZnO , N2: 0.25 ppm NPs ZnO, N3: 0.5 ppm NPs ZnO , N4: 1 ppm NPs ZnO , control, C2: Higher Zn concentration.

Before running the ANOVA eight assumption had to be met to be able to run the test. Assumption #1: one dependent variable that is measured at the continuous level. Assumption #2: one between-subjects' factor that is categorical with two or more categories. Assumption #3: one within-subjects' factor that is categorical with two or more categories. Assumption #4: There should be no significant outliers in any cell of the design (Boxplots). Assumption #5: dependent variable should be approximately normally distributed for each cell of the design (Test normality). Assumption #6: The variance of dependent variable should be equal between the groups of the between-subjects' factor (Levene's test for equality of variances). Assumption #7: There should be homogeneity of covariance (Box's test of equality of covariance matrices). Assumption #8: The variance of the differences between groups should be equal (Mauchly's test of sphericity). After the assumption was met, procedure for a significant interaction can be done and finally reporting. All statistical analyses were performed using the 22.0 SPSS software package for Windows (SPSS Inc., IL, USA).

3.3 Flask Experiment

3.3.1 Stock Solution

For the bulk stock solution, 62.20 mg ZnO bulk particles were added to 20 mL DI water (3.11 mg ZnO/mL). Using a pipette, 2 mL of the previous solution was combined

with 18 mL DI water (0.311 mg ZnO/mL). Three flasks where then filled with 4 mL of the previous solution and 96 mL BG-11 media (10 ppm Zn) bulk particles. Each of the three flasks where then divided in half to create a total of 6 flask with 50 mL of solution (Figure 14).

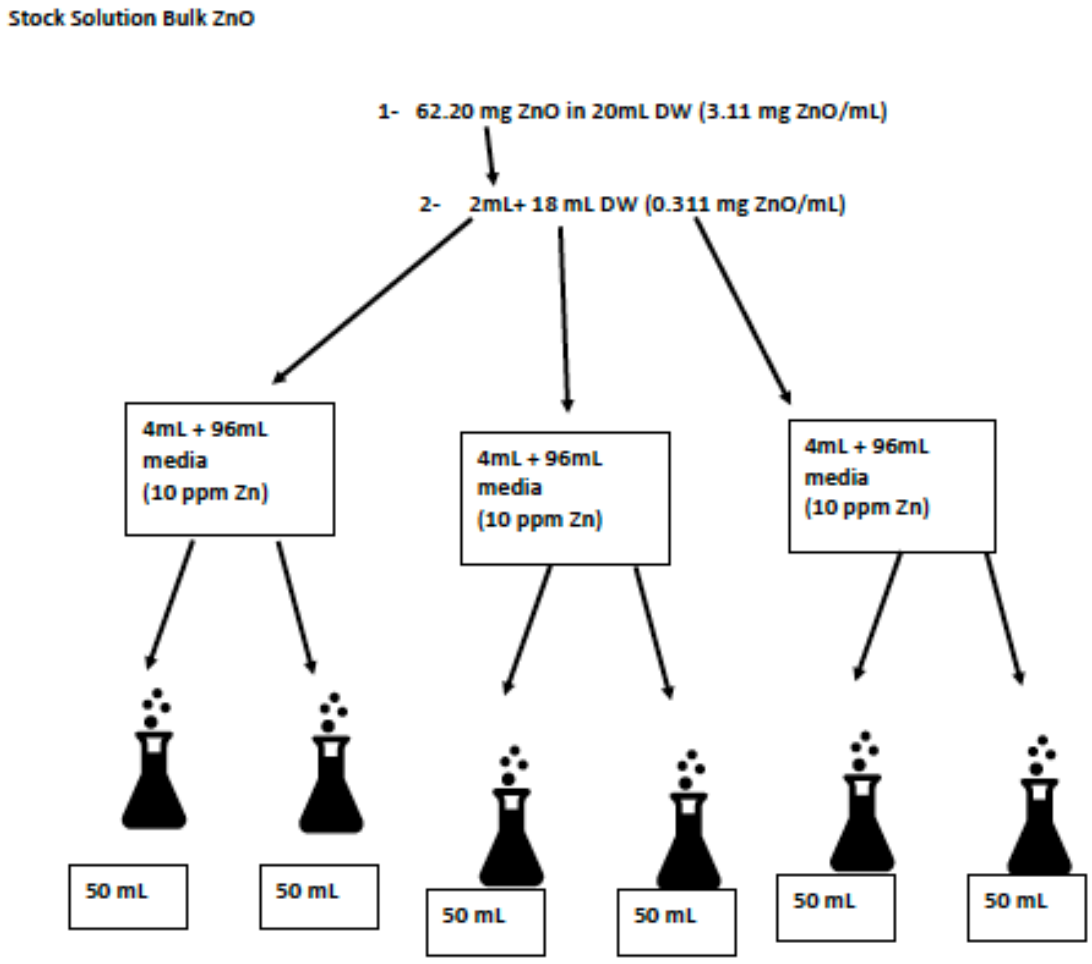


Figure 14: Bulk stock solution.

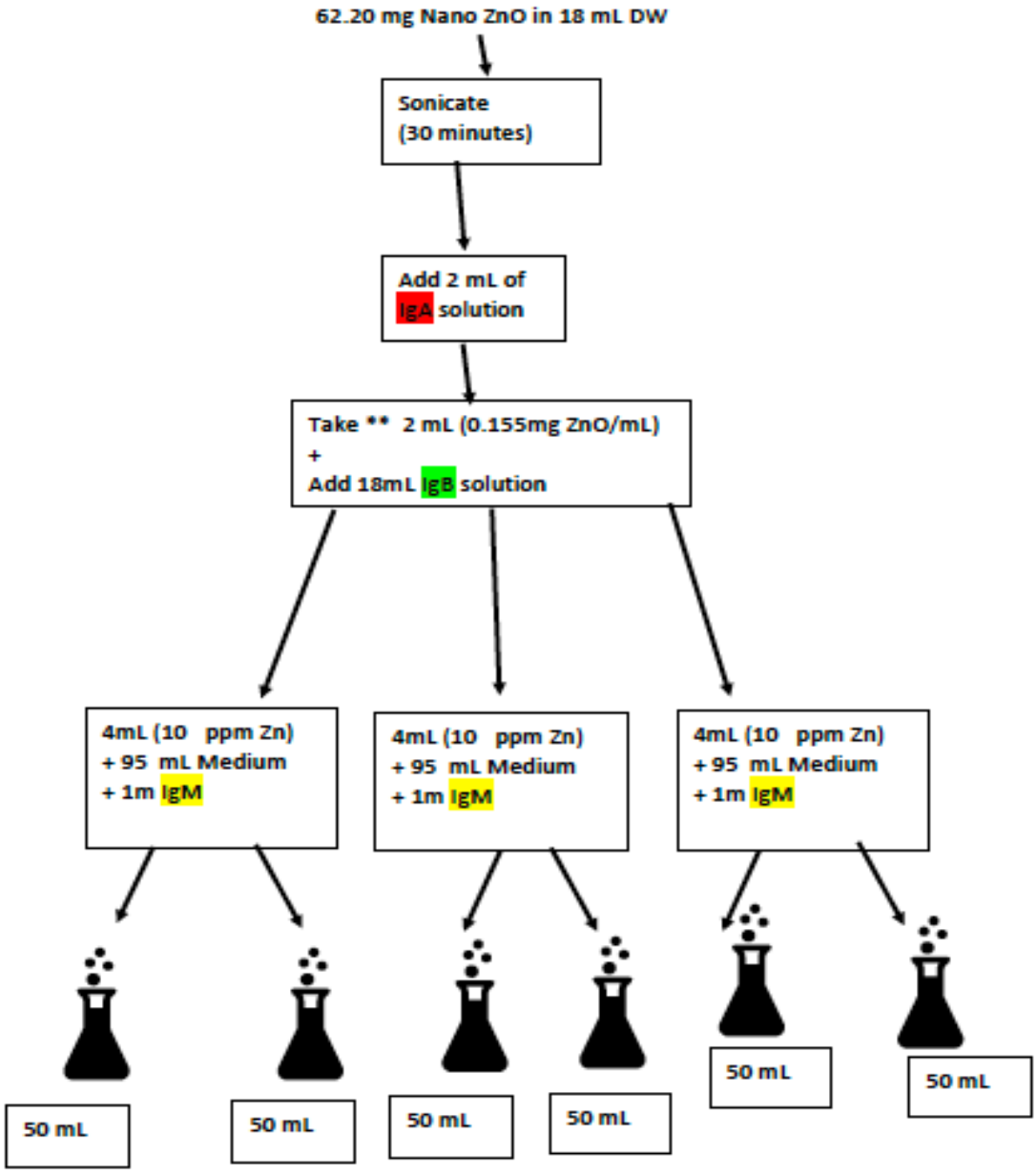


Figure 15: Nano stock solution.

For the nano stock solution, 62.20 mg ZnO nano articles were added to 18 mL DI water. Sonicate the sample for 30 minutes and add 2 mL of IgA solution. Using a pipette, 2 mL of the previous solution was combined with 18 mL IgB solution. Three flasks where then filled with 4 mL of the previous solution and 95 mL BG-11 media with no Zn (10 ppm Zn) bulk particles and add 1mL of IgM. Each of the three flasks where then divided in half to create a total of 6 flasks with 50 mL of solution (Figure15).

3.3.2 Cell Counting Chamber

Before preparing the algal inoculum, cell count in each culture was measured using the cell counting chamber (Neubauer Hemocytometer). The algae used were three green microalgae *Chlorococcum* sp., *Chlamydomonas* sp. and *Chlorella* sp. The glass hemocytometer was cleaned with alcohol before use. The samples were prepared by pipetting 1 mL of algae to an Eppendorf™ then exposing it to the vortex for a minute. One mL of algae was gently added to fill both chambers underneath the coverslip, allowing cell suspension to be drawn out by capillary action. A microscope was used to focus on the grid lines of the hemocytometer with a 10X objective. The number of algae cells were counted in all four outer squares and divided by four (the mean number of cells/square). The number of cells per square x 10^4 = the number of cells/ml of suspension (Figure 16). After, the hemocytometer and cover slip were sprayed with 70% ethanol to kill the cells. Both were then washed with deionized water, wiped dry with a Kimwipe™, wrapped in a clean Kimwipe™ and returned to the storage box (Privalsky, 2018).

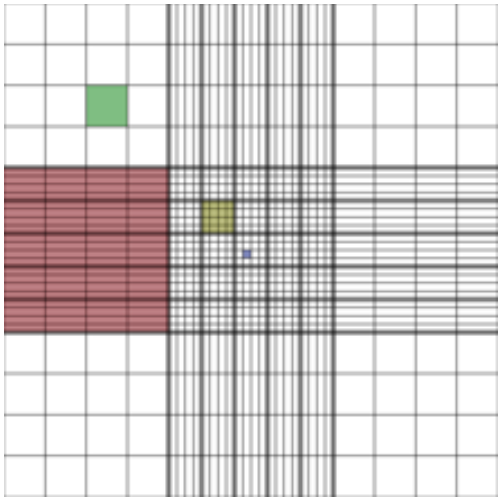


Figure 16: Hemocytometer.

3.3.3 Chlorophyll Measurements

After 7 days, the chlorophyll was measured. Using a pipette, 1 mL of the algae sample was transferred to the Eppendorf™. The sample was then centrifuged, and the excess water removed. Then, 1 mL of 95% methanol was added, and this solution was stored for 24 hours with no light. After 24 hours, the solution was transferred to a cuvette. The absorbance read 600 nm/652.4 (chlorophyll a) / 665.2 (chlorophyll b) (Ritchie, 2006; Lichtenthaler & Buschmann, 2001).

3.3.4 Absorbance Measurements

After 7 days, the relative amount of biomass cased on chlorophyll was measure. By pipetting 3 mL of the samples were pipetted into the 24-well plates and placed in the microplate reader at 600 nm (Ritchie, 2006).

3.3.5 Optical Microscope

The two green microalgae that were and were not exposed to the bulk and nano treatments were observe under the Olympus BX51™ research microscope under different

magnifications (20x-100x). This was done by doing slides of each of the algae with and without treatments.

3.3.6 Scanning Electron Microscope (SEM)

Throughout this process, pictures were taken under a light microscope. The Scanning Electron Microcopies (SEM) was also used with the cell fixation procedure, dehydration using increasing concentration of ethanol, critical point drying, mounting on the stub, sputter coating and finally processing the sample (Figure 9) (Hughes, 2011).

Preparation of the round slides. Pipetted 1 mL of polylysine on to microscope glass cover slides, wait until it dries. The cell fixation procedure began with the centrifuge of the samples. Then, add 2% glutaraldehyde in 0.1% Na cacodylate. After an hour, the sample was centrifuged again. After this add 1 mL medium non-Zn for bulk and Nano and medium with Zn for the control. After this, it was shaken well and centrifuged again.

Mount the sample with medium to the round slide wait 15 minutes. Then, start dehydration using increasing concentration of ethanol was used (40 %, 50 %, 80%, and 100% ethanol). After applying the 100% ethanol put at the fridge.

The critical point drying was done with Samdri-PVT-3D™. This method is used to preserve the morphology of the algae using CO_2 at very high pressure (Ruwin, 2012). The first step using the Samdri-PVT-3D™: Turn on the power and then wait 3 minutes for the equipment to warm up. Make sure all metering valves are close. Second step open the main LCO_2 tank valve. The third step pour high purity alcohol in the chamber to completely cover the samples before transfer. Transfer your samples into the chamber. For the fourth step open the cool calve to 0.50 positing and turn it off when temperature 0 ℃. The fifth step Fill Valve adjust by opening the fill metering valve to 0.50 and keep it

open. The sixth step of Purge-vent valve adjust: slowly open the purge vent and place Erlenmeyer Flask at the end of the clear purge to collect waste alcohol. Close it when all the ethanol is exhausts. The seventh step close the fill metering valve when the meniscuses travel across the viewing window. The eighth step close all the valves and turn on the heat. Wait until it hit critical point at critical pressure for CO_2 (1,072 psi) and critical temperature 31℃. Then wait 4 minutes. The ninth steps start after the 4 minutes pass, turn the bleed flow rate from 8-10 SCFH and close the bleed metering valve at 400 psi. The tenth step open the purge vent metering valve to 0.50 to reduce the chamber at 0 psi, the close. Finally, the eleventh step when the pressure reaches 0 psi, loosen 3 chamber nuts and remove your sample (Tousimis, 2018).

After the sample is critical dried, a sputter coating of gold (Au) was applied to the sample. (Sputter coating is the process of applying an ultra-thin coating of electrically-conducting metal onto a non-conducting or poorly conducting specimen.) This technique prevents charging of the algae, which would otherwise occur because of the accumulation of static electric fields. It also increases the number of secondary electrons that can be detected from the surface of the specimen in the SEM and, therefore increases the signal to noise ratio.

Sputtered films for SEM typically have a thickness range of 2–20 nm ("Brief Introduction to Coating Technology for Electron Microscopy", 2013).

For the Sputter Coating Procedure: First, Place samples in the chamber, make sure the chamber valve is closed and close the lid. The second step turn the power on and wait for it to pump down. The third step is to open the valves on the argon gas and Pump to below 2 Torr. Slowly bleed in the argon using the gas leak valve until the pump gets noisy then Wait 1 minute, fourth step back off the argon until 2 Torr is reached. Fifth step

Press the test button and Press the start button. Then continually adjust the argon using the gas leak valve to keep the gauge at 18 mill amperes. The sixth step turn the power off, close the gas leak valve and open the chamber leak valve slightly until air can be heard entering the chamber. Remember to close the argon valve ("Electron Microscopy Sciences", 2018).

Finally, the sample was processed by introducing it to the SEM, capturing pictures and using Energy Dispersive X-Ray Spectroscopy (EDS) analysis. The SEM Loading Procedure starts with venting the instrument so that the door can be opened. Make sure the stage is at a lower position (30 mm) so that there is less risk of hitting the pole piece or backscatter detector. Once the instrument beeps, open the door and slide the sample holder into place. Slowly close the door to the SEM. Then, evacuate the chamber wait for the beep and for the "HT Ready" button to turn on. And click on "HT Ready" to turn on the beam. Then SEM is ready to be navigating (Robbins, 2015). For the EDS procedure just turn on the program and run the chemical analysis.

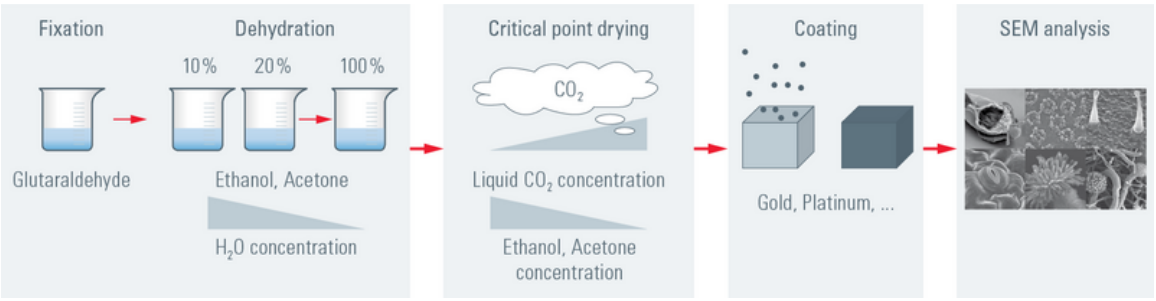


Figure 17: Preparation of the algae sample.

3.3.7 Statistical Analysis

The data collected was tested for statistical significance using two-way mixed (ANOVA), taking the level of $p < 0.05$ as significance according to Tukey multiple

comparison test. With the two-way mixed ANOVA is used to understand if there is an interaction between the days and treatments. The between-subjects factor are the treatments of different concentration of bulk and nano particles (B1, B2, B3, B4, N1, N2, N3, N4 and control) and one within-subjects factor being days (0 and 7). To analyze the data using a two-way mixed ANOVA we had check that the data can be analyze using this test.

The two-way mixed ANOVA has eight assumptions that you have to consider. Assumption #1: Have one dependent variable that is measured at the continuous level (i.e. the interval or ratio level). Assumption #2: Have one between-subjects factor that is categorical with two or more categories. Assumption #3: Have one within-subjects factor that is categorical with two or more categories. Assumption #4: There should be no significant outliers in any cell of the design. Assumption #5: Dependent variable should be approximately normally distributed for each cell of the design. Assumption #6: The variance of dependent variable should be equal between the groups of the between-subjects factor. Assumption #7: There should be homogeneity of covariances. Finally, Assumption #8: The variance of the differences between groups should be equal ("We make statistics easy. The ultimate IBM® SPSS® Statistics guides."). All statistical analyses were performed using the 22.0 SPSS software package for Windows (SPSS Inc., IL, USA).

3.4 Enrichment and Isolation of Microalgae Tolerant to High Nano-ZnO Concentration

3.4.1 Location

Ten sea water samples were collected at locations along the South Point Miami Beach, Florida (25.7635° N, 80.1297° W). This site was selected because is one of

the most popular beaches in Miami Beach, FL and it also next to the pier. This point is vulnerable to anthropogenic effect.

3.4.2 Flask experiment

For the 100ppm ZnO NPs Flask experiment 6.223 mg ZnO NPs, 0.1 mL K_2HPO_4 and 75 mg $NaNO_3$ added to the 50 mL of the sample collected. The Erlenmeyer™ flask left in the incubator for a week with 25°C degrees under continuous lighting of 50µmol of photons $m^{-2}s^{-1}$for with and continuous shaking of 155 rpm. After a week optical microscope picture were collected.

3.4.3 Optical Microscope

Light microscope observations of algal samples were done using Olympus BX51 microscope.

Section 4: Results

4.1 Cyanobacteria – Exposure to Low Zn Concentrations

The multi-well plate experimental results from low-level concentrations treatments (0.125 ppm, 0.25 ppm, 0.5 ppm, and 1 ppm Zn) are presented for four cyanobacteria: Limnothrix sp. (173-10-1), Leptolyngbya (146-5-2), Neolyngbya sp. (EK17-3A), and Oscillatoria sp. (bpm 173F). Results from studies on three additional cyanobacteria: Porphoridium sp. (173-10-2), i), Roseofilum sp. (101-1), j), and Nannochloris sp. (146-2-11) are also included.

4.1.1 Optical Microscope

After seven days of testing, the four cyanobacteria were observed in 100x magnification. For the *Limnothrix* sp., there was a considerable morphology change. The control was noted at retaining their bright green color. The bulk at 1 ppm Zn remained green, however was not observed as having as bright a coloring. The nano at 1pm had no pigmentation (Figure 18). In the trial, two *Limnothrix* sp. had similar results as for the first trial.

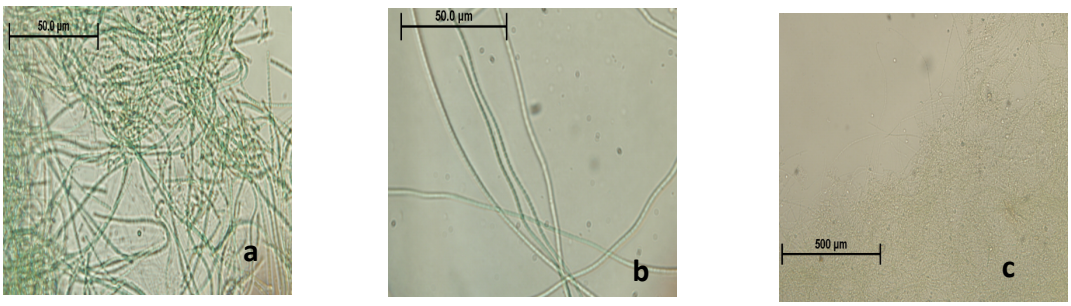


Figure 18: *Limnothrix* sp., in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 1ppm c) Nano ZnO 1ppm.

For the *Leptolyngbya* sp., there was a notable morphology change from control to nano, but not from control to bulk. The control retained their bright green color; the bulk at 1 ppm Zn was still green and the nano at 1 ppm displayed no pigmentation (Figure 19). In trial 2, *Leptolyngbya* sp. had similar results as for trial 1.

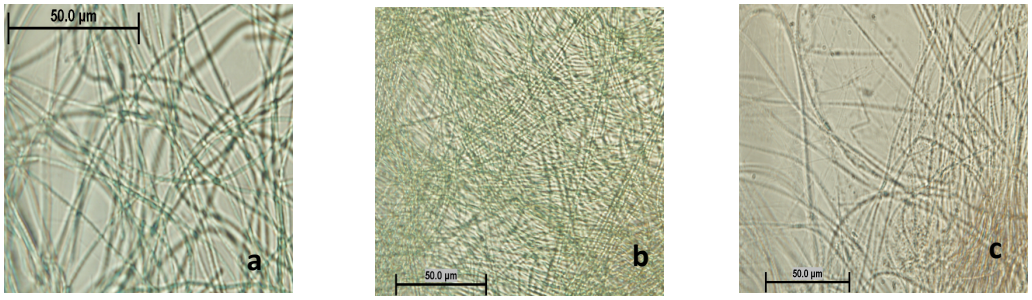


Figure 19: *Leptolyngbya* sp., in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 1ppm c) Nano ZnO 1ppm.

For the *Lyngbya* 1 sp., there was a noted morphology change in color and shape. The control had a light green color; the bulk at 1 ppm Zn was still green, but darker while the nano at 1 ppm had change in color to brown. In trial 2, the *Lyngbya* 1 sp. had similar results as for the trial 1.

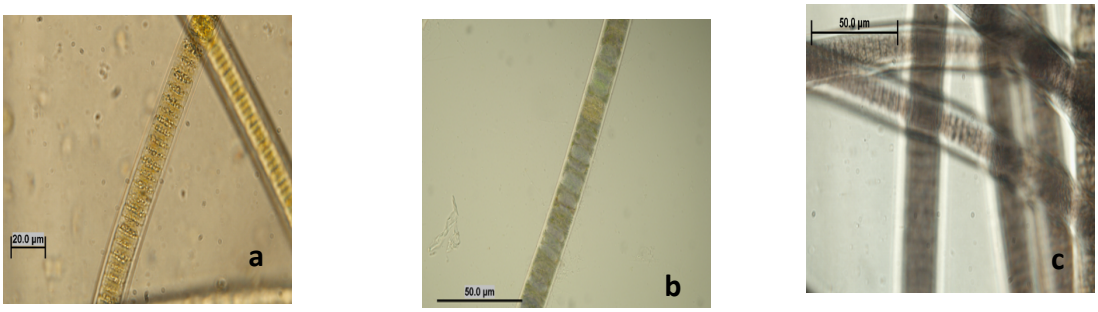


Figure 20: *Lyngbya* 1 sp., in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 1 ppm c) Nano ZnO 1 ppm.

The *Lyngbya* 2 sp. had a morphology change in color. The control had a brownish green color, the bulk at 1 ppm Zn showed a brown purple hue, and the nano at 1 ppm underwent a change color to purple. In trial 2, *Lyngbya* 2 sp. had similar results as for the trial 1.

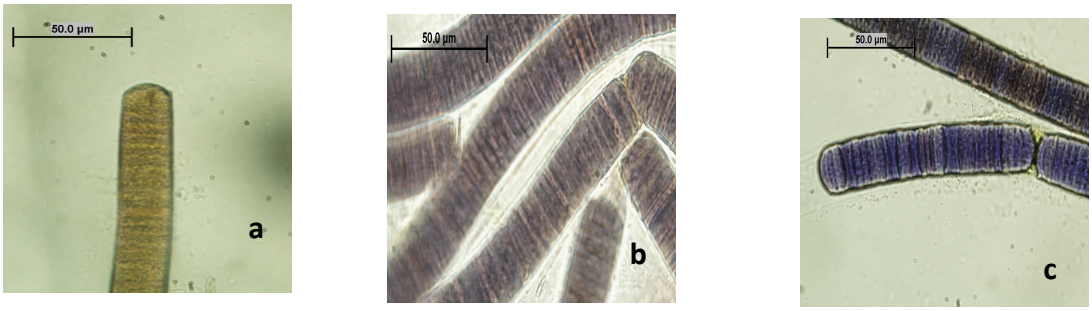


Figure 21: *Lyngbya* 1 sp.., in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 1ppm c) Nano ZnO 1 ppm.

For the *Porphoridium* sp., there was also a morphology in terms of its color transition. The control had a pink color. The bulk at 1 ppm Zn had some microalgae convert to a pink hue, but some turned green. The microalgae nano at 1pm experienced a turn to green (Figure 22). In trial 2, the *Porphoridium* sp. had similar results.

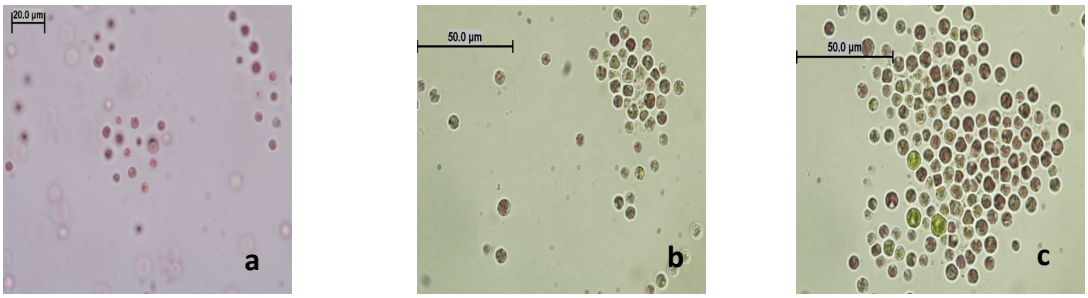


Figure 22: *Porphoridium* sp., in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 1ppm c) Nano ZnO 1 ppm.

For the *Roseofilum* sp., there was also a morphology change in the coloration of the microalgae. The control yielded a pink color and a high concentration of filaments. The bulk at 1 ppm Zn showed some microalgae turn a light green, while the nano at 1 ppm turned vert light green which was almost transparent and with less concentration on filaments (Figure 23). The trial 2 of the *Roseofilum* sp. yielded similar results.

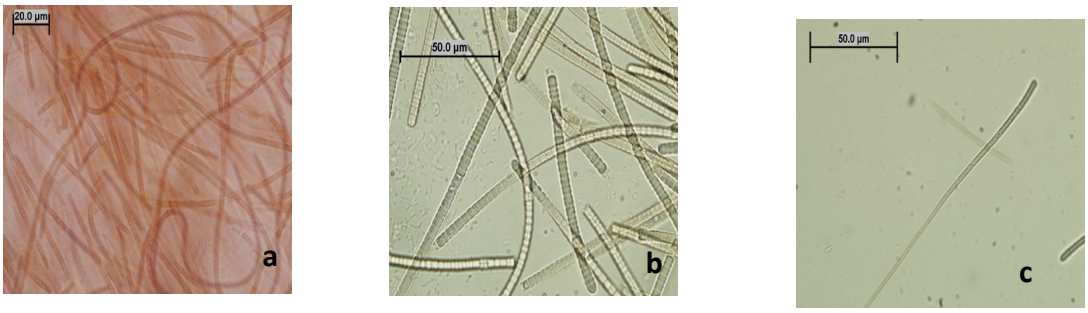


Figure 23: *Roseofilum* sp., in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 1ppm c) Nano ZnO 1 ppm.

For the *Apistonema* sp., a morphology change from the control was noted. The control displayed round shapes. The bulk at 1 ppm Zn showed a round shape however larger than the control and also an agglomeration occurred. The nano at 1 ppm yielded a very similar result to the bulk in terms of its agglomeration (Figure 24). In trial 2, *Apistonema sp*. had similar results.

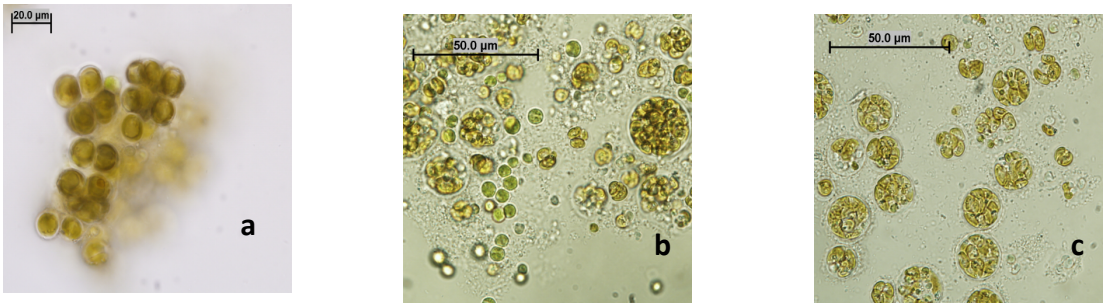


Figure 24: *Apistonema* sp., in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 1ppm c) Nano ZnO 1 ppm.

4.1.2 Growth response

The results for the two-way mixed (ANOVA) Lower dosage experiments with: Cyno 1: *Limnothrix sp.,* Cyno 2: Leptolyngbya sp.*,* Cyno 3: *Lyngbya* 1 sp.*,* Cyno 4: *Lyngbya* 2 sp. Cyno5: *Porphoridium* sp., Cyno 6: *Roseofilum* sp. Cyno 7: *Apistonemas.* The codification for dosages was as follow: B1: 0.125ppm Bulk ZnO, B2: 0.25 ppm Bulk ZnO, B3 0.5 ppm: Bulk ZnO, B4: 1 ppm Bulk ZnO, N1: 0.125ppm NPs ZnO, N2: 0.25 ppm NPs ZnO, N3: 0.5 ppm NPs ZnO and N4: 1 ppm NPs ZnO.

After the assumptions were met the ANOVA was conducted. The Test of between-subjects' effects was done to a statistically significant difference in absorbance concentration between intervention of treatments. There was a statistically significant difference in absorbance concentration between intervention of treatments $F(8, 15.762) = 6.521$, $p = .000$, partial $\eta^2 = .592$ for *Limnothrix* sp. ; $F(8, 32) = 6.521$, $p = .000$, partial $\eta^2 = .704$ for Leptolyngbya sp. ; $F(8, 3.449) = 48.911$, $p = .000$, partial $\eta 2 = .431$ for *Lyngbya* sp. ;$F(8, 31)=13.116$, $p=0.000$ $\eta 2 = 1.736$ for *Lyngbya* sp;$F(8, 35)=1.208$, $p=.000$ $\eta 2 = .014$ for *Porphoridium* sp. ; $F(8, 37)=10.814$, $p=.000$ $\eta 2 = .138$ for *Roseofilum* sp. and $F (8, 34) = 7.588$, $p=.000$ $\eta 2 = .499$ for *Apistonema* sp.

Multiple Comparisons was used to know the sadistically significantly between treatments. For *Limnothrix* sp.: Absorbance concentration was statistically significantly greater in the control compare all of the other treatments (0.125ppm, 0.25 ppm, 0.5 ppm , 1 ppm , bulk and nano ZnO) For example: Control and b1(0.125ppm ZnO bulk) (M =.935510, *SE* =.1738216 nm, $p = .000$). The absorbance concentration in all the treatments excluding control was not statistically significantly lower than. For example: the control group b1 and n4 ($M = .194290$, $SE = .1738216$ nm, $p = .968$).

For *Leptolyngbya* sp.: For the results in these microalgae had many variety results absorbance concentrations were statistically significantly greater: Control with all the treatments (b1-n4); All bulk (b1-b4) with all nano (n1-n4). For example: Control with b1 (M= .204250, SE= .0371248, p=0). The absorbance concentration in all bulk with all bulk treatments (b1-b4) and all nano with nano treatments (n1- n4) were not statistically significantly greater. For example, b1 with b3 (M= .001100, SE= .0350016, p=1.00).

For *Lyngbya* 1 sp.: Absorbance concentration was statistically significantly greater in the control compare all of the other treatments (b1-n4). For example: Control and b1 (M = .528250, SE = .0331947 nm, p = .000). The absorbance concentration in all the treatments excluding control was not statistically significantly lower than. For example: the control group b1 and n4 (M = .053313, SE = .0314912, p = .746).

For *Lyngbya* 2 sp: Absorbance concentration was statistically significantly greater in the control compare all of the other treatments (b1-n4) and for all bulk with N4. For example: B1 and N4 (M= .078875, SE=.0087693, p=.004). The absorbance concentration in most treatments excluding control was not statistically significantly lower than. For example: n1 and n4 (M = .020400, SE = .0355980, p = .999).

For *Porphoridium* sp.: The absorbance concentration for all the treatments was not statistically significantly lower than. There is no significance difference between the treatments.

For *Roseofilum* sp.: Absorbance concentration was statistically significantly greater in the control with b4 and N4; B1 with N4; B2 with N2 and N4; B3 with N2 and

N4; N1 and N4. For example, control group and b4 (M= .113563, SE= .0339681, p= 0.004); The absorbance concentration in some treatments was not statistically significantly lower. For example: control and b1 (M= .035375, SE=.0339681, p=.979).

For *Apistonema* sp.: Absorbance concentration was statistically significantly greater in the control with all bulks (b1-b4); B4 with B1. For example, control group and b4 (M= .118787, SE= .0268076, p= 0.002). The absorbance concentration most treatments was not statistically significantly lower than for control with all nano (n1-n4), Nano with bulk. For example: B1 and b2 (M= .014750, SE= .0330920, p=1.00).

The two-way interaction is statistically significant for all the cyanobacteria but *Lyndya 1* sp. did not pass the Levene's test of equality of error variance meaning there was no variance of growth was equally distributed across groups for each of the comparison days**.** Also, the *Apistonema* sp. there was no homogeneity of variances because it fails the Box's test of equality of covariance matrices there was no other variants.

For *Limnothrix* sp., there were no significant outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances with the exception of days 5 and 7 for cyanobacteria 1 and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects' effects: There was a statistically significant interaction between the intervention and days on absorbance, F(3, 108) = 67.223, p =0.00, partial η2 = .651. Absorbance concentration was statistically

significantly greater in the control compare all of the other treatments (b1-n4). For example: Control and b1 (M = .935510, SE =.1738216 nm, p = .000). The absorbance concentration in all the treatments excluding control was not statistically significantly lower than. For example: the control group b1 and n 4 (M = .194290, SE = .1738216 nm, p = .968).

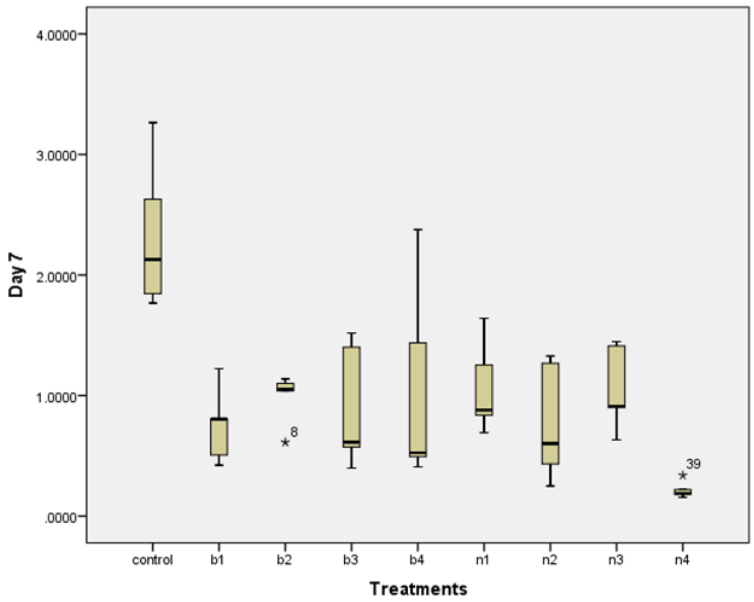


Figure 25: Box plot's shows the effects of ZnO Nano and bulk particles on *Limnothrix* sp., growth at day 7.

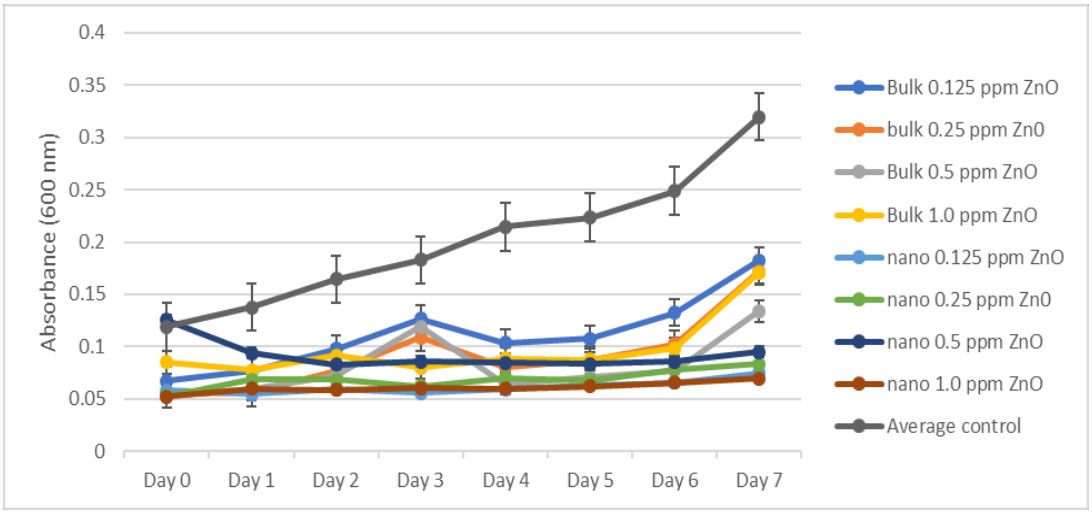


Figure 26: The effects of ZnO Nano and bulk particles on *Limnothrix* sp., growth.

For *Leptolyngbya* sp, there were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances with the exception of days 5 and 7 for cyanobacteria 1 and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects' effects: There was a statistically significant interaction between the intervention and days on absorbance $F(1,96)= 26.758$; $p=.000$, partial $\eta 2 = 6.362$. For the results in this microalgae had many variety results. Absorbance concentration was statistically significantly greater: Control with all the treatments (b1-n4); B1 with all nano (n1-n4); B2 with all nano (N1-n4); B3 all nano (N1-n4); B4 with all nano (N1-n4). For example: Control with b1 ($M= .204250$, $SE= .0371248$, $p=0$). The absorbance concentration in all bulk with bulk treatments (B1 with b2, b3, b4) and all nano with nano treatments (N1 with n2, n3, n4). For example, b1 with b3 ($M= .001100$, $SE= .0350016$, $p=1$).

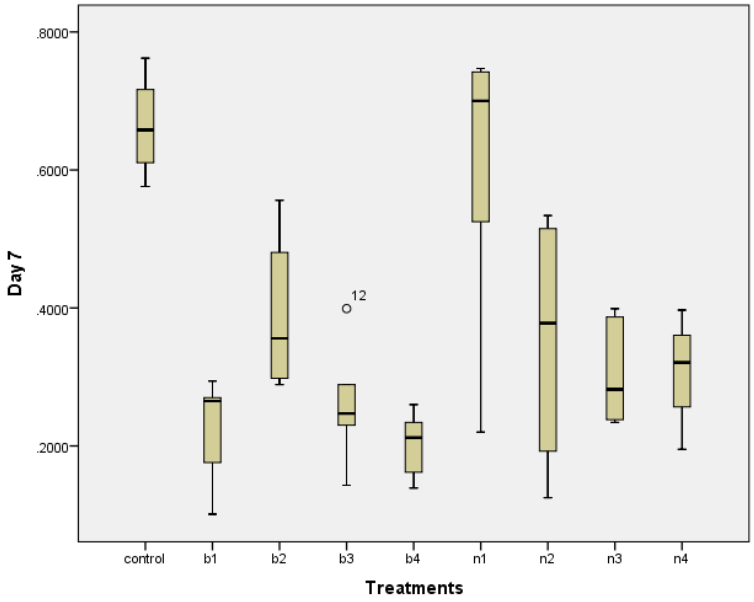


Figure 27: Box plot's shows the effects of ZnO Nano and bulk particles on *Leptolyngbya* sp., growth at day 7.

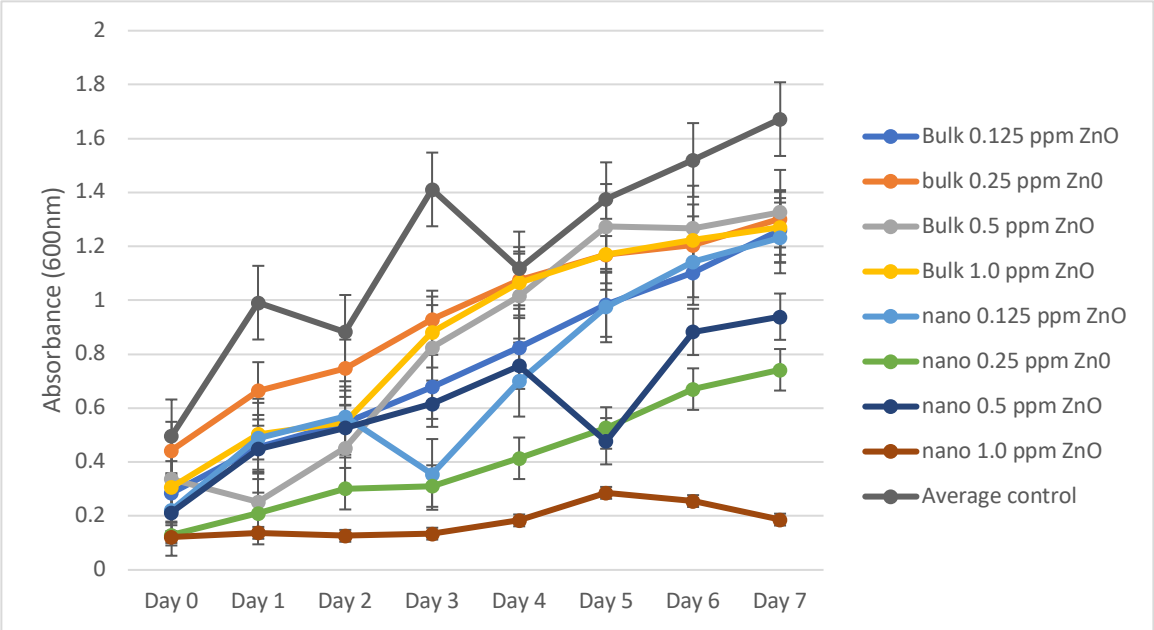


Figure 28: The effects of ZnO Nano and bulk particles on *Leptolyngbya* sp., growth.

For *Lyngbya* 1 sp., there were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was no homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances but the Box's M test did pass. Unfortunately, transformations were not successful and there are no robust mixed ANOVA methods available in SPSS Statistics because the Leven's was violated but the analysis and testing continued regardless. Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects' effects: There was a statistically significant interaction between the intervention and days on absorbance (3,90) = 11.959 p=.000, partial $\eta 2 = .056$. Absorbance concentration was statistically significantly greater in the control compare all of the other treatments (b1-n4). For example: Control and b1 (M = .528250, SE = .0331947 nm, p = .000). The absorbance concentration in all the treatments excluding control was not statistically significantly lower than. For example: the control group b1 and n4 (M =. 053313, SE = .0314912, p =. 746).

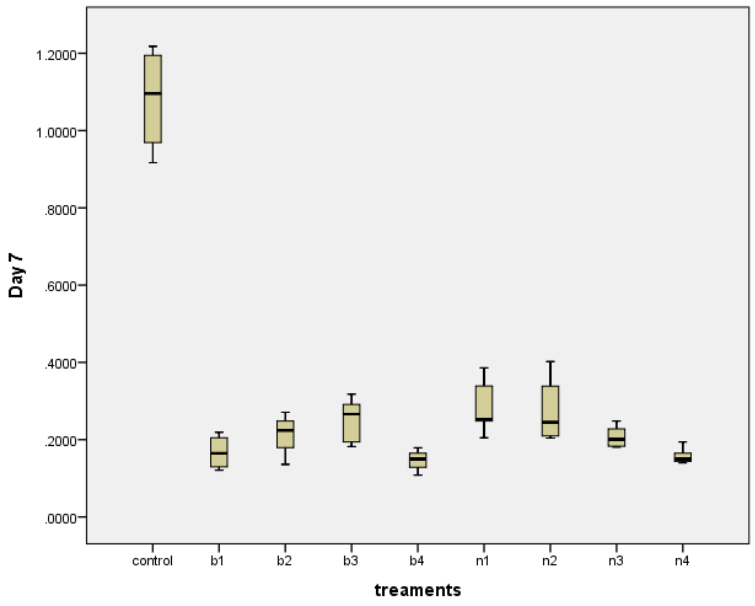


Figure 29: Box plot's shows the effects of ZnO Nano and bulk particles on *Lyngbya* 1 sp., growth at day 7.

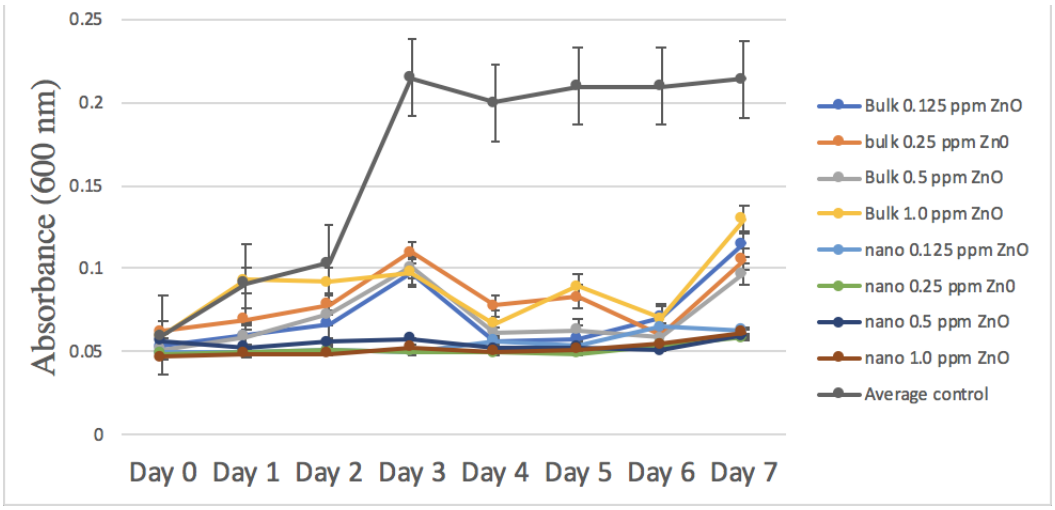


Figure 30: The effects of ZnO Nano and bulk particles on *Lyngbya* 1 sp., growth at day 7.

For *Lyngbya* 2 sp., there were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances with the exception of days 5 and 7 for cyanobacteria 1 and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects' effects: There was a statistically significant interaction between the intervention and days on absorbance $F(3,93) = 8.248$ $p=.000$, partial $\eta 2 = .027$. Absorbance concentration was statistically significantly greater in the control compare all of the other treatments (b1-n4); b3 with n3 ($M= .078875$, $SE=.0087693$, $p=.004$). The absorbance concentration in most treatments excluding control was not statistically significantly lower. For example: the control group b1 and n4 ($M = .020400$, $SE = .0355980$, $p = .999$).

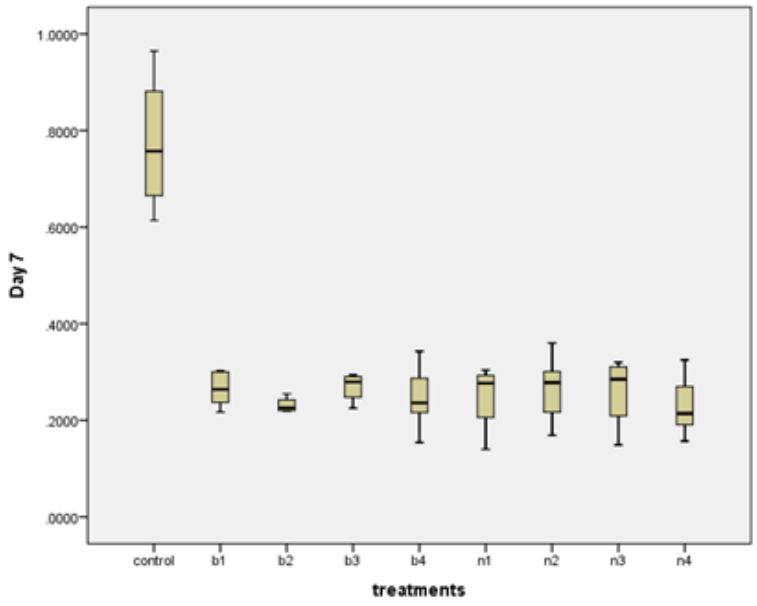


Figure 31: Box plot's shows the effects of ZnO Nano and bulk particles on *Lyngbya* 2 sp., growth at day 7.

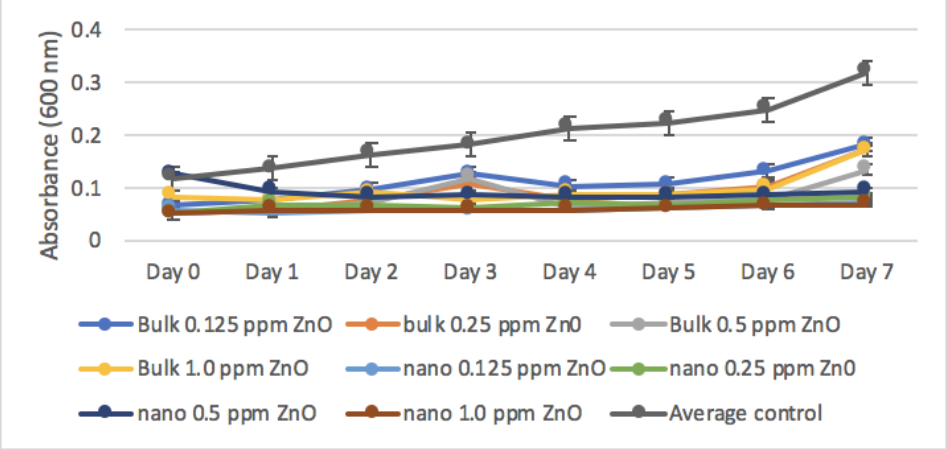


Figure 32: The effects of ZnO Nano and bulk particles on *Lyngbya* 2 sp., growth.

For *Porphoridium* sp., there were no significant outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances with the exception of days 5 and 7 for cyanobacteria 1 and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of within-subjects' effects: There was a statistically significant interaction between the intervention and days on absorbance $F (3, 105) = 55.043$, $p = .00$ $\eta 2 = .261$. The absorbance concentration for all the treatments was not statistically significantly lower than. There is no significance difference between the treatments.

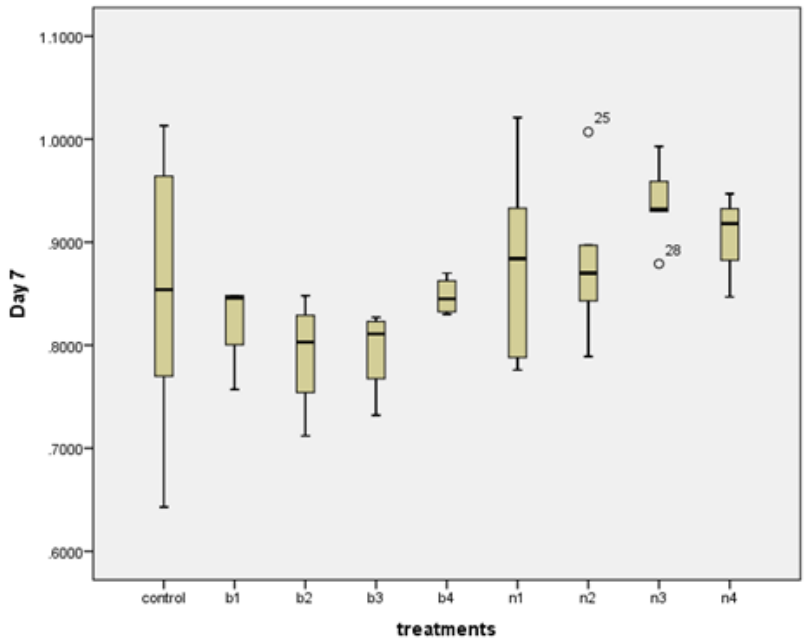


Figure 33: Box's plot of effects of ZnO Nano and bulk particles on *Porphoridium* sp., growth at day 7.

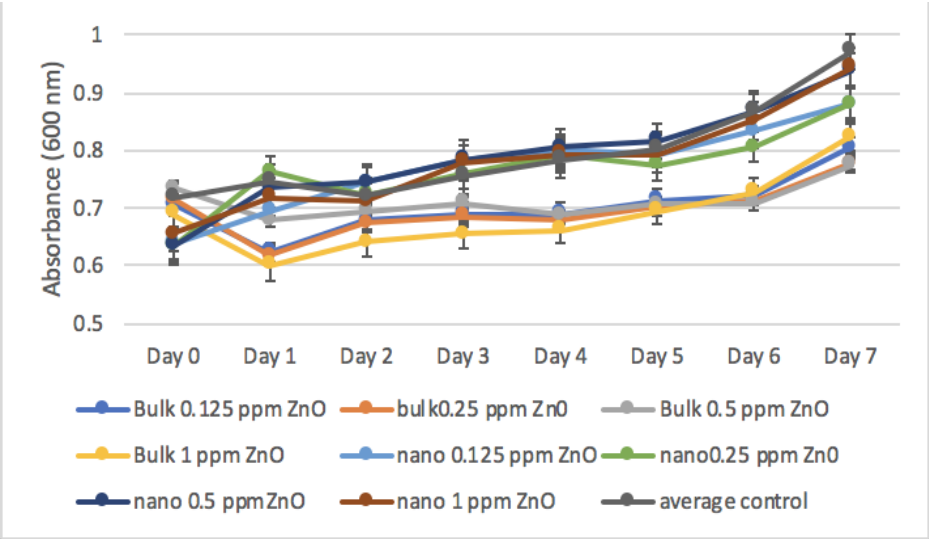


Figure 34: The effects of ZnO Nano and bulk particles on *Porphoridium* sp., growth.

For *Roseofilum* sp., there were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances with the exception of days 5 and 7 for cyanobacteria 1 and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of within-subjects' effects: There was a statistically significant interaction between the intervention and days on absorbance $F(3,111) = 51.799$, $p = .00$ $\eta 2 = 1.209$. Absorbance concentration was statistically significantly greater in the control with b4 and N4; B1 with N4; B2 with N2 and N4; B3 with N2 and N4; N1 and N4. For example, control group and b4 ($M = .113563$, $SE = .0339681$, $p = 0.004$). The absorbance concentration in some treatments was not statistically significantly lower. For example: control and b1 ($M = .035375$, $SE = .0339681$, $p = .979$).

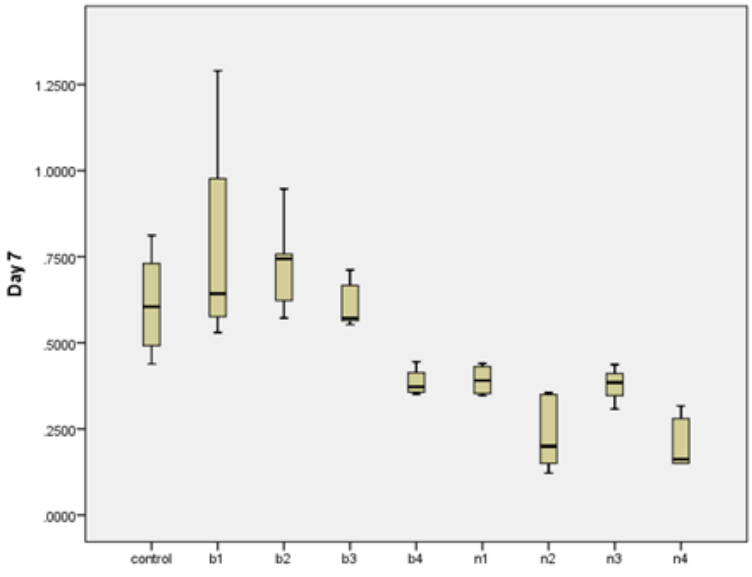


Figure 35: Box's plot of effects of ZnO Nano and bulk particles on *Roseofilum* sp., growth at day 7.

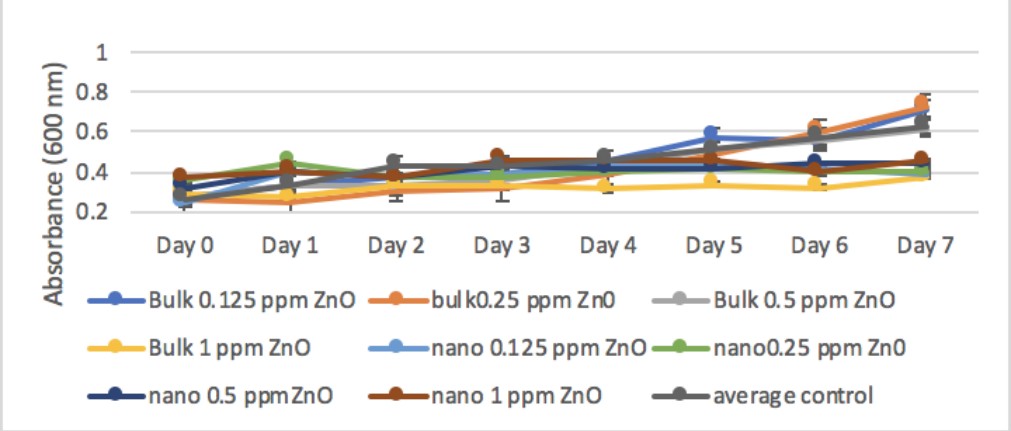


Figure 36: The effects of ZnO Nano and bulk particles on *Roseofilum* sp.., growth.

4.2 Cyanobacteria -Exposure to Low Zn Concentrations with Additional Zn Source

The results of the multi-well plate-based experiment with four cyanobacteria: *Limnothrix* sp. *(173-10-1), Leptolyngbya (146-5-2), Neolyngbya* sp. *(EK17-3A)*, and *Oscillatoria* sp. *(Bpm 173F*) Observation following the incubation of multi-well plates, growth inhibition due to nano Zn was clearly indicated by visible total loss of pigmentation (in row two, Figure 37).

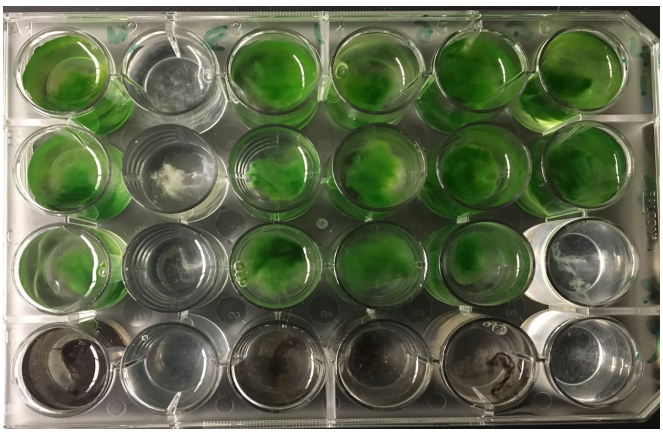


Figure 37: 24 well plates with different concentration of nano zinc and cyanobacteria.

4.2.1 Optical microscope

After seven days, the four cyanobacteria were observed in 100x magnification. For the *Limnothrix* sp., there was a significant morphology change between control and high concentration of zinc. The control had a bright green color while those exposed to the high concentrations of zinc were transparent and showed almost no filaments (Figure 38).

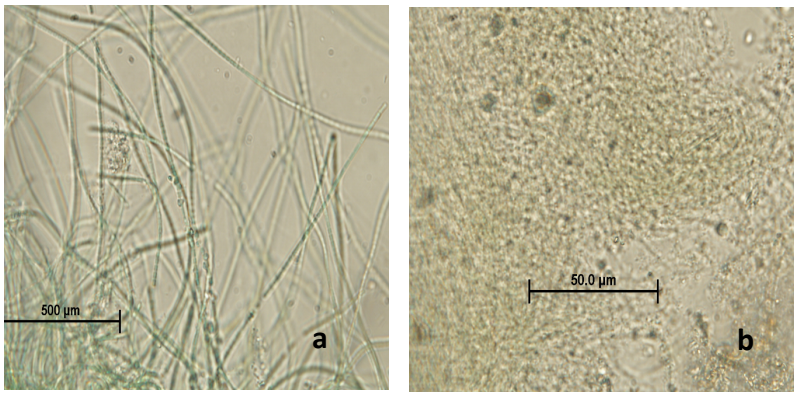


Figure 38: *Limnothrix* sp., in BG 11 media with a) standard $ZnSO_4$ b) High concentration of $ZnSO_4$.

For the Leptolyngbya sp., there was a prominent morphology change between control and high concentration of zinc. The control had a bright green color while those exposed to the high concentrations of zinc were transparent and showed small size filaments (Figure 39).

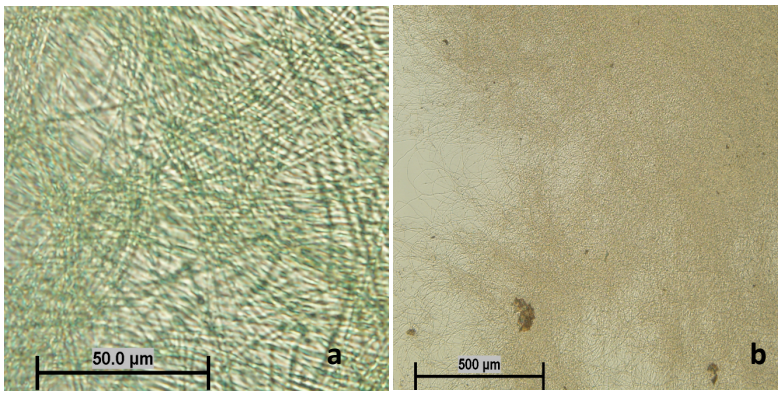


Figure 39: *Leptolyngbya* sp. in BG 11 media with a) standard $ZnSO_4$ b) High concentration of $ZnSO_4$.

For the *Lyngbya* 1 sp., there was a distinguished morphology change between control and those with high zinc concentration exposure. The control had a bright green color while the high concentration of zinc was transparent and there was a size reduction (Figure 32).

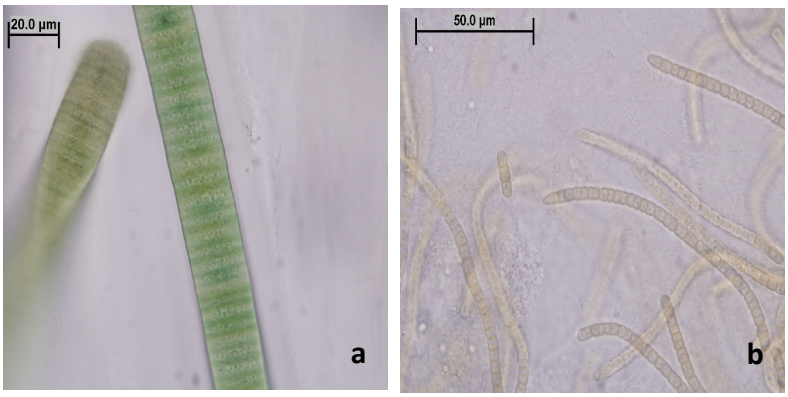


Figure 40: *Lyngbya* 1 sp. in BG 11 media with a) standard $ZnSO_4$ b) High concentration of $ZnSO_4$.

For the *Lyngbya* 2 sp., there was a noted morphology change between control and high concentration of zinc. The control had a bright green color while those with high concentration of zinc were transparent and experienced a size reduction (Figure 33).

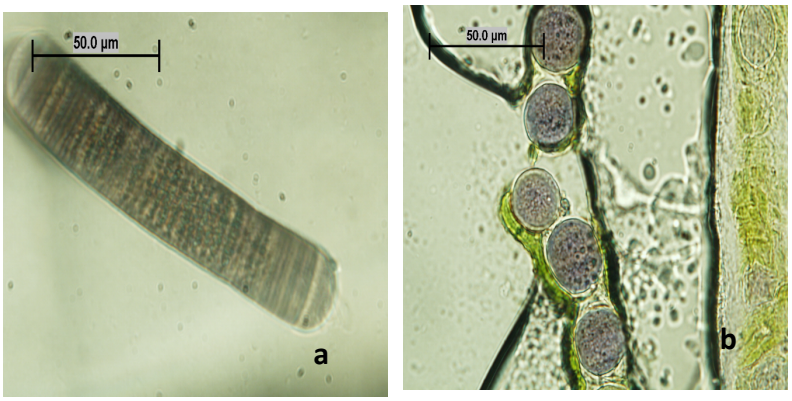


Figure 41: *Lyngbya* 2 sp. in BG 11 media with a) standard $ZnSO_4$ b) High concentration of $ZnSO_4$.

4.2.2 Statistical Analysis

The results for the two-way mixed (ANOVA) with higher dosage of Zinc experiments with: *Limnothrix* sp., *Leptolyngbya* sp., *Lyngbya* 1sp. and *Lyngbya* 2 sp. The codification for dosages was as follow: Extra: High concentration of ZnSO4, B1: 0.125ppm Bulk ZnO, B2: 0.25 ppm Bulk ZnO, B3 0.5 ppm: Bulk ZnO, B4: 1 ppm Bulk ZnO, N1: 0.125ppm NPs ZnO, N2: 0.25 ppm NPs ZnO, N3: 0.5 ppm NPs ZnO and N4: 1 ppm NPs ZnO.

After most the assumption was met the results were reported. There was a statistically significant difference in absorbance concentration between intervention of treatments $F(9, 23) = 4.139$, $p = .003$, partia $\eta 2 = .387$ for *Limnothrix* sp.; $F(9, 22) = 31.527$, $p = .000$, partial $\eta 2 = 2.419$ for *Leptolyngbya* sp; $F(9, 28) = 5.423$, $p = .000$, partial $\eta 2 = .104$ for *Lyngbya* 1 sp. and $F(9, 28) = 14.984$, $p=0.000$ $\eta 2 = .042$ for *Lyngbya* 2 sp.

The multiple Comparisons table was analyzed to determine which treatments were significantly different. For *Limnothrix* sp., the absorbance concentration was statistically significantly greater in the control treatments with all the treatments. For example, Extra and control (M = .531222SE = .1140085 nm, p =.004). The absorbance concentration in all the treatments excluding control was not statistically significantly lower than. For example: extra and b1 (M = .024611, SE = .1248900 nm, p = 1.00).

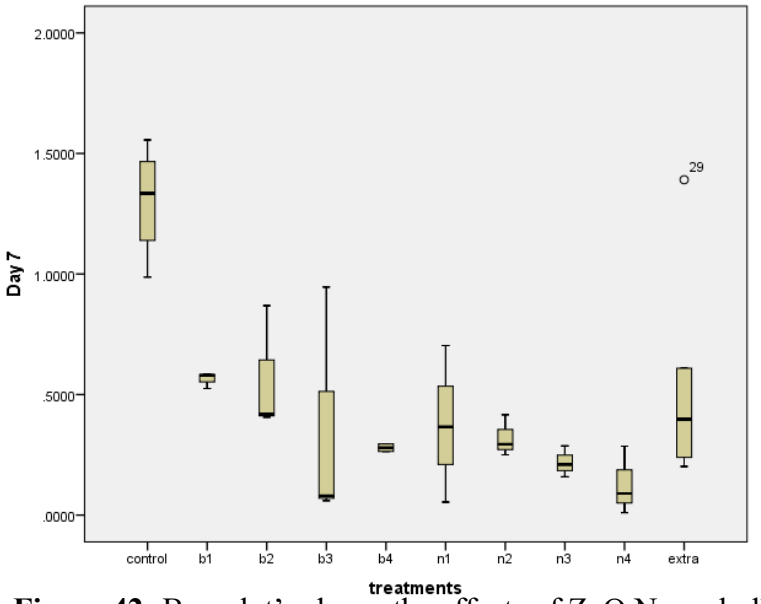


Figure 42: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Limnothrix* sp., growth at day 7.

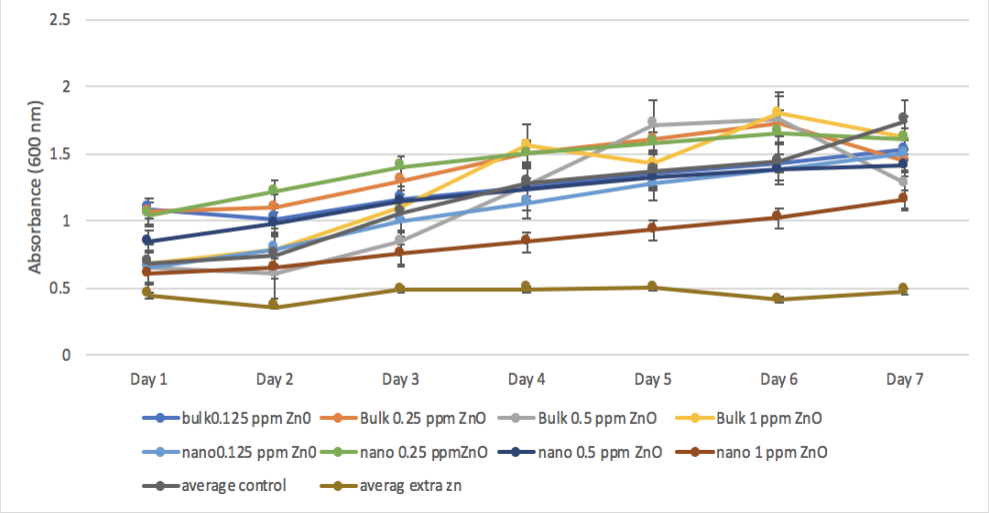


Figure 43: The effects of ZnO Nano, bulk particles and extra zinc on *Limnothrix* sp.

For *Leptolyngbya* sp. the absorbance concentration was statistically significantly greater in the control treatments with all the treatments. For example, extra and control (M = .908417 SE = .1130879 nm, p =.000). The absorbance concentration in all the treatments excluding control was not statistically significantly lower than. For example: extra and b1 (M = .006694, SE = .1221489 nm, p = 1.00).

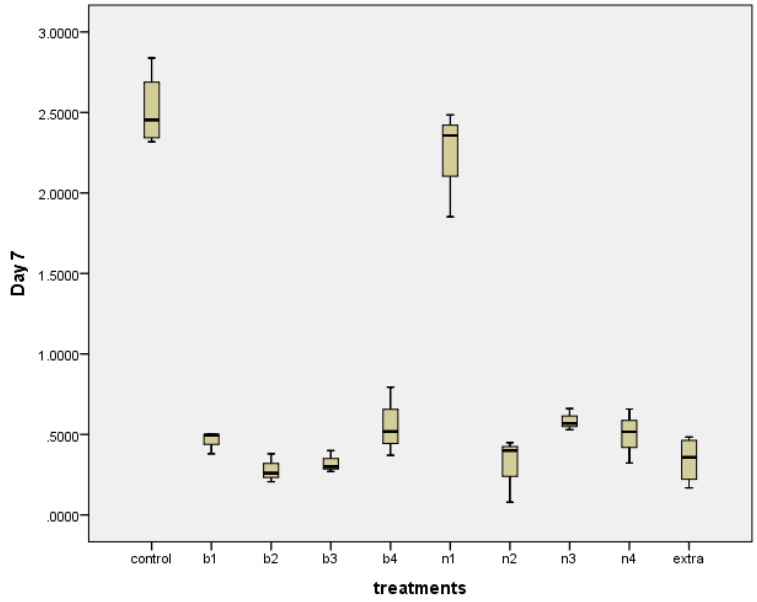


Figure 44: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Leptolyngbya* sp. growth at day 7.

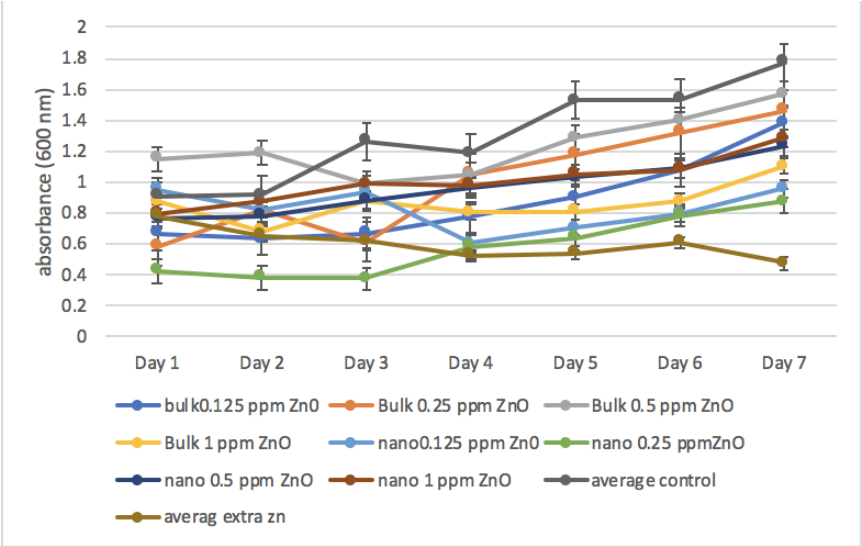


Figure 45: The effects of ZnO Nano, bulk particles and extra zinc on *Leptolyngbya* sp.

For *Lyngbya* 1 sp. the absorbance concentration was statistically significantly greater in the control compare to some treatments but not all. For example: Control and b1 (M = .287300, SE = .0541944 nm, p = .000). The absorbance concentration in extra zinc with all bulk and nano was not statistically significantly lower. For example, the control group extra and n4 (M = .053313, SE = .0314912, p = .746).

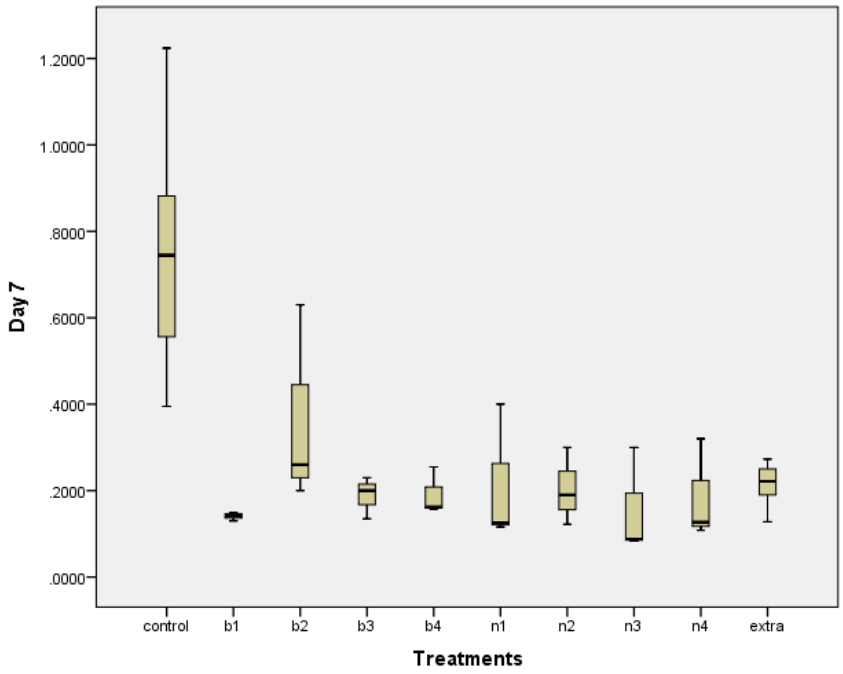


Figure 46: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Lyngbya* 1 sp. growth at day 7.

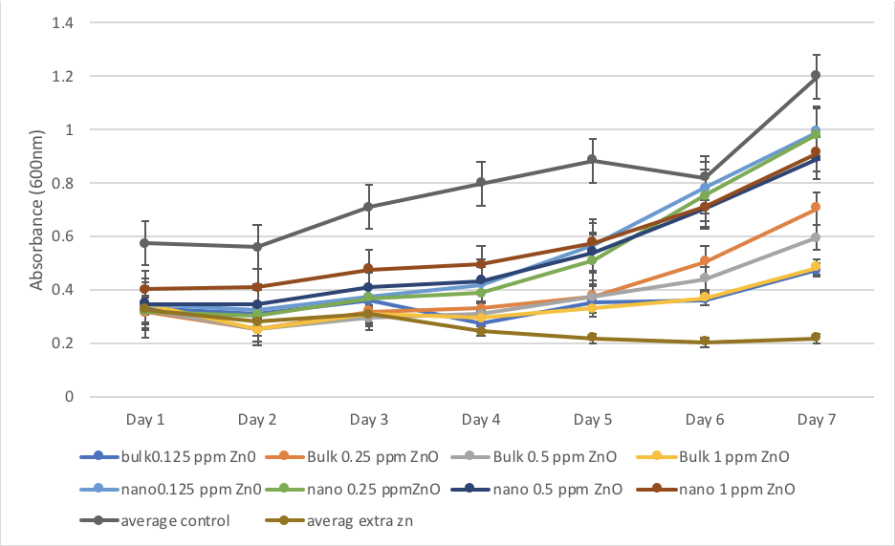


Figure 47: The effects of ZnO Nano, bulk particles and extra zinc on *Lyngbya* 1 sp.

For *Lyngbya* 2 sp., the absorbance concentration was statistically significantly greater in the control treatments with all the treatments. For example, extra and control (M = .155931 SE = .0164524 nm, p =.000). The absorbance concentration in all the treatments excluding control was not statistically significantly lower than. For example, extra and b1 (M = .002833, SE = .0215413 nm, p = 1.00).

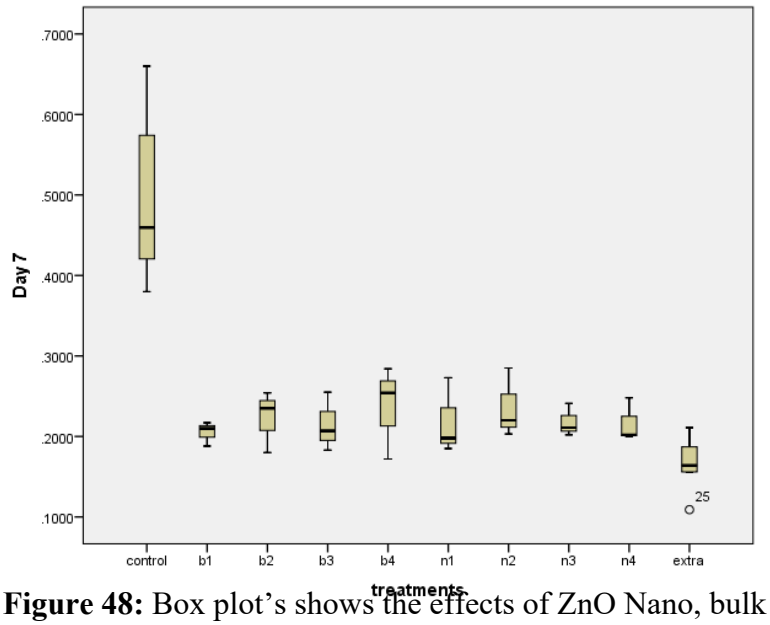


Figure 48: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Lyngbya* 2 sp. growth at day 7.

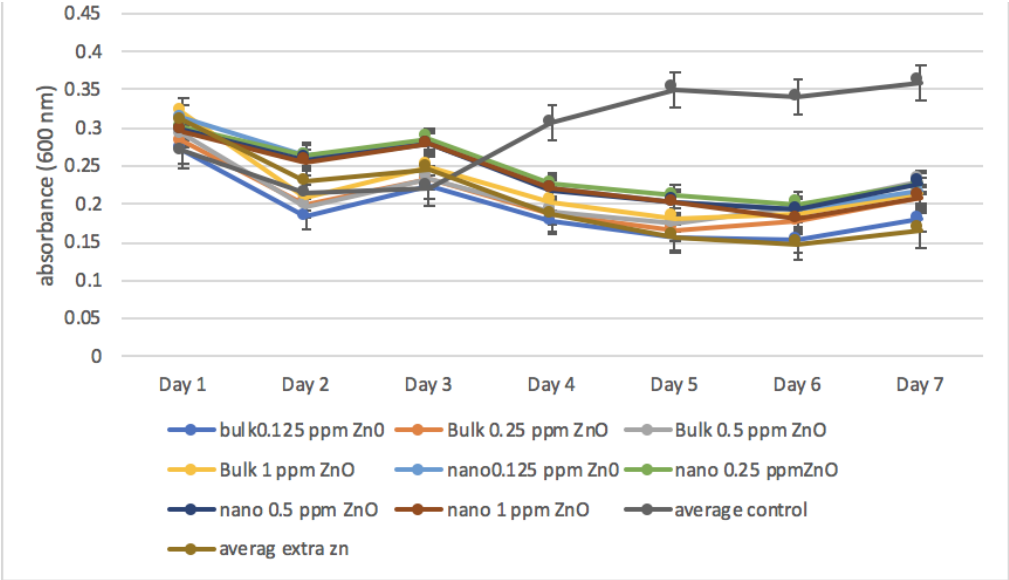


Figure 49: The effects of ZnO Nano, bulk particles and extra zinc on *Lyngbya* 2 sp.

There was a statistically significant interaction between the intervention and days on absorbance, for *Limnothrix* sp.: F (2, 46) = 6.630, p =0.03, partial η2 = .285 and for *Leptolyngbya* sp.: F (2, 44) = 8.241, p =0.03, partial η2 = .458. There was no significant statistically significant interaction between the intervention and days on absorbance, for Lyngbya 1 sp.: F (2, 56) = 3.919, p =0.026, partial η2 = .123, and for Lyngbya 2 sp.: F (2, 56) =34.023, p =0.03, partial η2 = .055.

Pairwise comparison was analysis to know the statistically significant between days. For all cyanobacteria, the absorbance concentration was not statistically significantly difference for all of the days. For *Leptolyngbya* sp.: The absorbance concentration was not statistically significantly different between day 0 -4 (M= .100, SE=.075, p=.529); day 4-7 (M=.140, SE=.060, p=.088) but the absorbance concentration was statistically significantly difference at day 0-7 (M=.119, SE=.017, p=.000). For the *Lyngbya* 1 sp.: The absorbance concentration was not statistically significantly different between all days. For *Lyngbya* 2 sp.: The absorbance concentration was not statistically significantly different between day 4 - 7(M= -.028, SE =.011, p=0.58); but the absorbance concentration was statistically significantly difference at day 0-4 (M = .079, SE=.007, p=.000), day 0-7 (M= .052, SE=.011, p=.000).

4.3 Green Microalgae – Exposure to Low Zn Concentrations

For the first experiment, green microalgae were exposed at low concentrations (0.125 ppm, 0.25 ppm, 0.5 ppm, and 1 ppm). The results show that most of the green microalgae have some tolerance to bulk and nano ZnO. The results will be divided by microscope and the absorbance. The trial conducted with three green microalgae used

were: 146-2-6 *Chlorococcum* sp., 146-2-10 *Chlamydomonas* sp., and 146-2-16 *Chlorella*. Then, a second trial was performed which yielded similar results.

4.3.1 Optical microscope

After the seven days, the three microalgae were observed in 100x magnification. For the *Chlorococcum* sp. (Figure 50), *Chlamydomonas* sp. (Figure 51) and *Chlorella* sp. (Figure 52), no morphology changes in all the concentration were observed. These microalgae kept their shape and color.

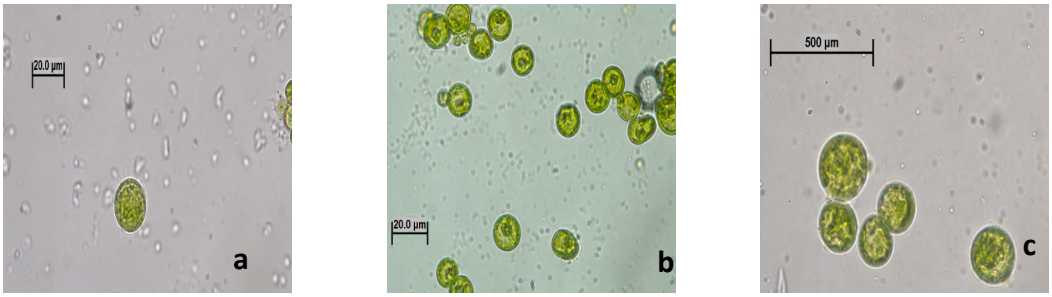


Figure 50: *Chlorococcum* sp., in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 1 ppm c) Nano ZnO 1 ppm.

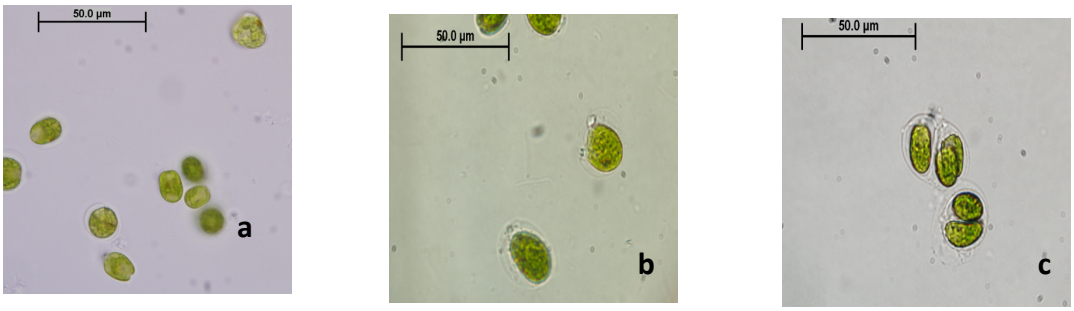


Figure 51: *Chlamydomonas* sp., in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 1 ppm c) Nano ZnO 1 ppm.

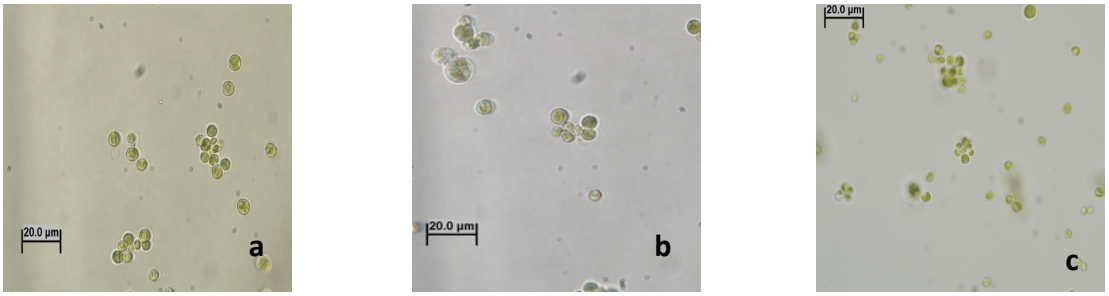


Figure 52: *Chlorella* sp., in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 1 ppm c) Nano ZnO 1 ppm.

4.3.2 Statistical Analysis

The results for the two-way mixed (ANOVA) Lower dosage experiments with: *Chlorococcum* sp., *Chlamydomonas* sp., and *Chlorella* sp., dosages was as follow: B1: 0.125 ppm Bulk ZnO, B2: 0.25 ppm Bulk ZnO, B3 0.5 ppm: Bulk ZnO, B4: 1 ppm Bulk ZnO, N1: 0.125 ppm NPs ZnO, N2: 0.25 ppm NPs ZnO, N3: 0.5 ppm NPs ZnO and N4: 1 ppm NPs ZnO.

After all the assumption was met the results were reported. Test of between-subjects effects was analysis to determine if there was a statistically significant difference in absorbance concentration between intervention of treatments $F(8, 38) = 6.521$, $p = .000$, $\eta^2 = .043$ for *Chlorococcum* sp. ; $F(8, 34) = 4.217$, $p = .000$, partial $\eta^2 = .193$ for *Chlamydomonas* sp. and $F(8,34) = 6.303$, $p = .000$, $\eta 2 = .088$ for Chlorella sp.

Multiple Comparisons was analyzed know if the absorbance concentration was or not statistically significantly for the treatments. For *Chlorococcum* sp., the absorbance concentration in all the treatments was not statistically significantly lower. For example, the control group and n4 ($M = .035775$, $SE = .039786$ nm, $p = .992$). For *Chlamydomonas* sp. the absorbance concentration was statistically significantly greater in the control compare with n3 and n4. For example, control and n4 (M= .155350, SE=

.0322812, p=.001). The absorbance concentration in most treatments was not statistically significantly different. For example: the control group b1 and n4 (M = .041512 SE = .0395362, p = .979). For *Chlorella* sp., the absorbance concentration in all the treatments was not statistically significantly lower. For example: the control group and n4 (M = -.183887, SE = .0629066 nm, p = .119).

There was statistically significant interaction between the intervention and days on absorbance, F (3, 114) = 103.874, p =0.00, η^2 = 1.212 for *Chlorococcum* sp.; F(3, 34) = 684.391, p =0.00, η2 = 10.450 for *Chlamydomonas* sp. and F(3, 102) = 270.412, p =0.00, η2 = 1.876 for *Chlorella* sp. .

The Pairwise comparison table was used to know if there was statistically significant between days of testing. The absorbance concentration was not statistically significantly different between day 0 and day 2 (M = -.032 SE = .037, p = .021), but the absorbance concentration was statistically significantly difference at for the rest of the days for *Chlorococcum* sp. For *Chlamydomonas* sp. and *Chlorococcum* sp., the absorbance concentration was statistically significantly difference for all days.

The results were reported, for *Chlorococcum* sp.: There were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality (p > .05). There was homogeneity of variances (p > .05) and covariance (p > .05), as assessed by Levene's test of homogeneity of variances with the exception of day 0 for *Chlorococcum* sp. and Box's M test, respectively. “Mauchly’s test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects effects: There was a statistically significant interaction between the intervention and days on absorbance, g F(3, 114) = 103.874. The absorbance concentration in all the treatments

was not statistically significantly lower. For example, the control group and n4 (M = .035775, SE = .039786 nm, p = .992).

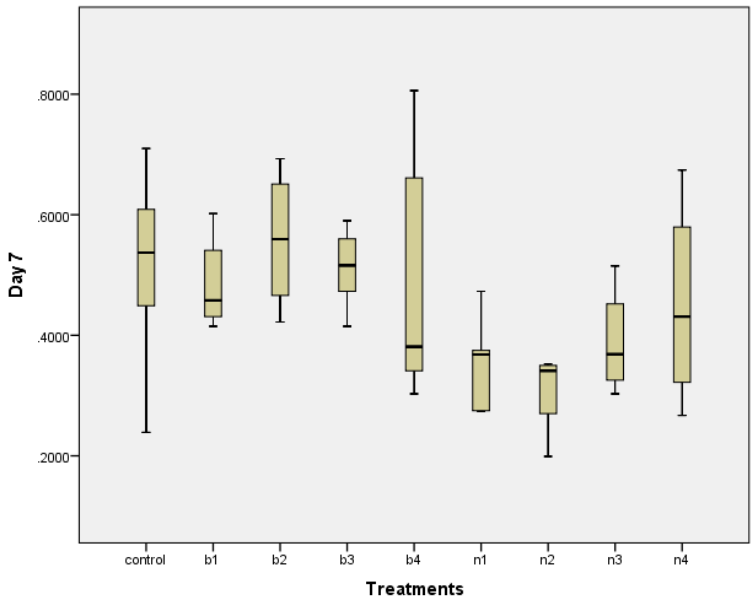


Figure 53: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Chlorococcum* sp. growth at day 7.

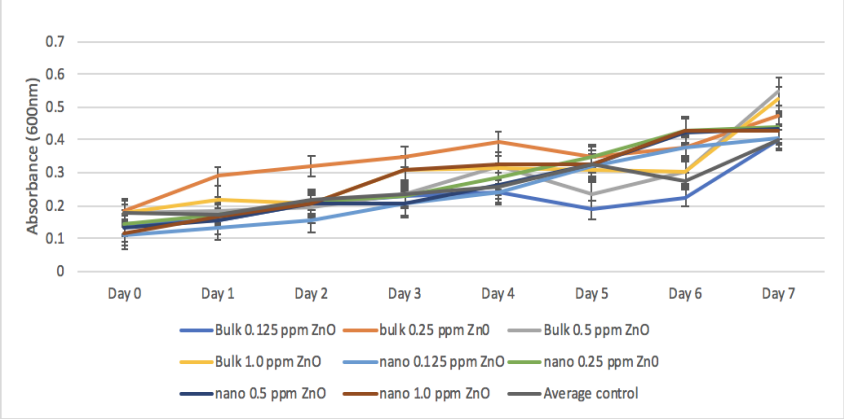


Figure 54: The effects of ZnO Nano, bulk particles and extra zinc on *Chlorococcum* sp. growth.

For *Chlamydomona*s sp.: There were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects effects: There was a statistically significant interaction between the intervention and days on absorbance $F (3, 102) = 270.412$, $p = 0.00$, $\eta 2 = 1.876$: Absorbance concentration was statistically significantly greater in the control compare with n3 and n4. For example, the control and n4 yielded M= .155350, SE= .0322812, and p=.001. The absorbance concentration in most treatments excluding control was not statistically significantly lower than it. For example, the control group b1 and n4 (M = .041512 SE = .0395362, p = .979).

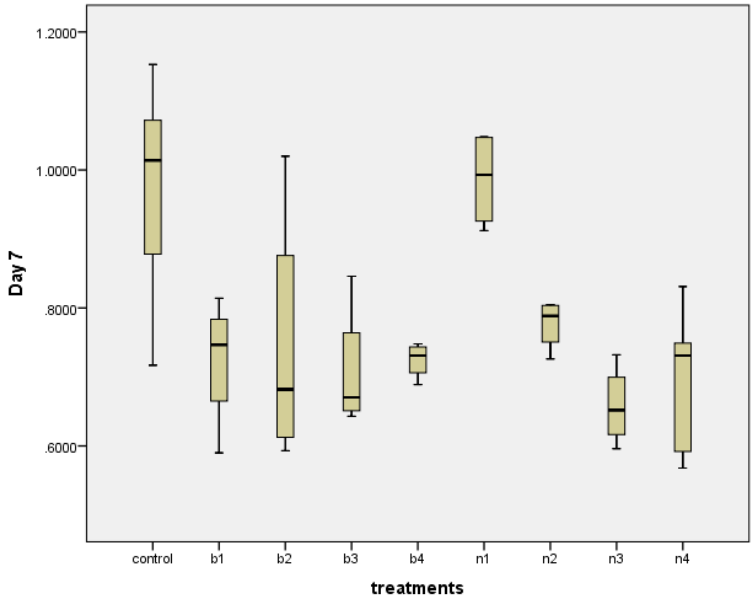


Figure 55: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Chlamydomonas* sp. growth at day 7.

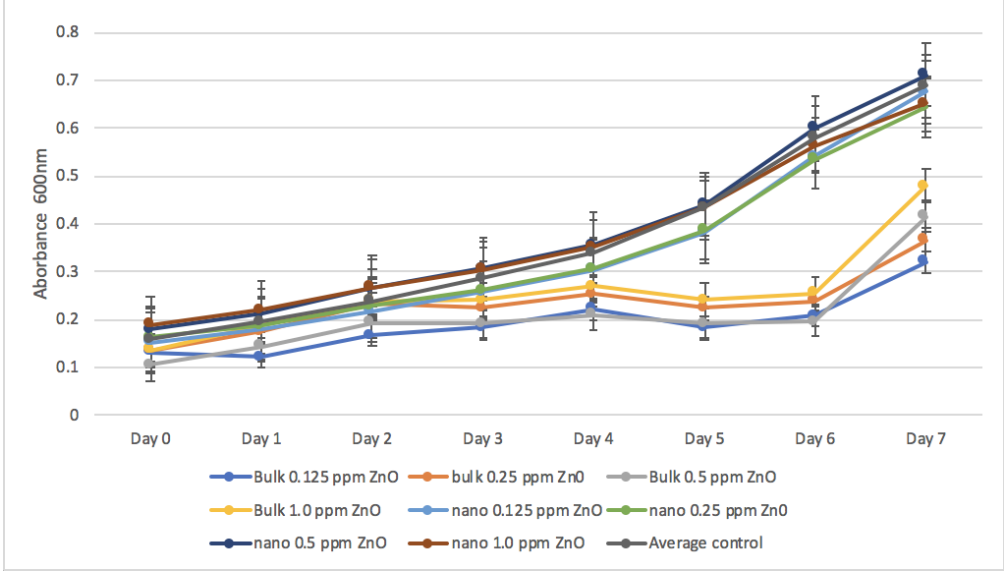


Figure 56: The effects of ZnO Nano, bulk particles and extra zinc on *Chlamydomonas* sp. growth.

For *Chlorella* sp.: There were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There

was homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances with the exception of days 7 and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects effects: There was a statistically significant interaction between the intervention and days on absorbance $F(3, 34) = 684.391$, $p = 0.00$, $\eta 2 = 10.450$. The absorbance concentration in all the treatments was not statistically significantly lower than. For example, the control group and n4 ($M = -.183887$, $SE = .0629066$nm, $p = .119$).

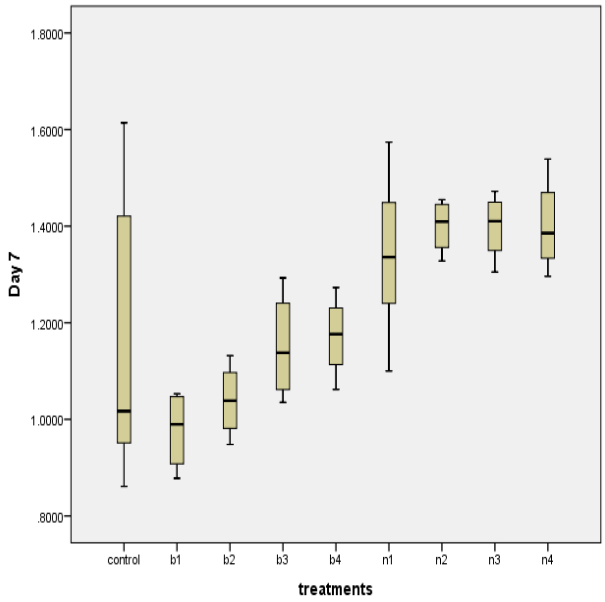


Figure 57: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Chlorella* sp. growth at day 7.

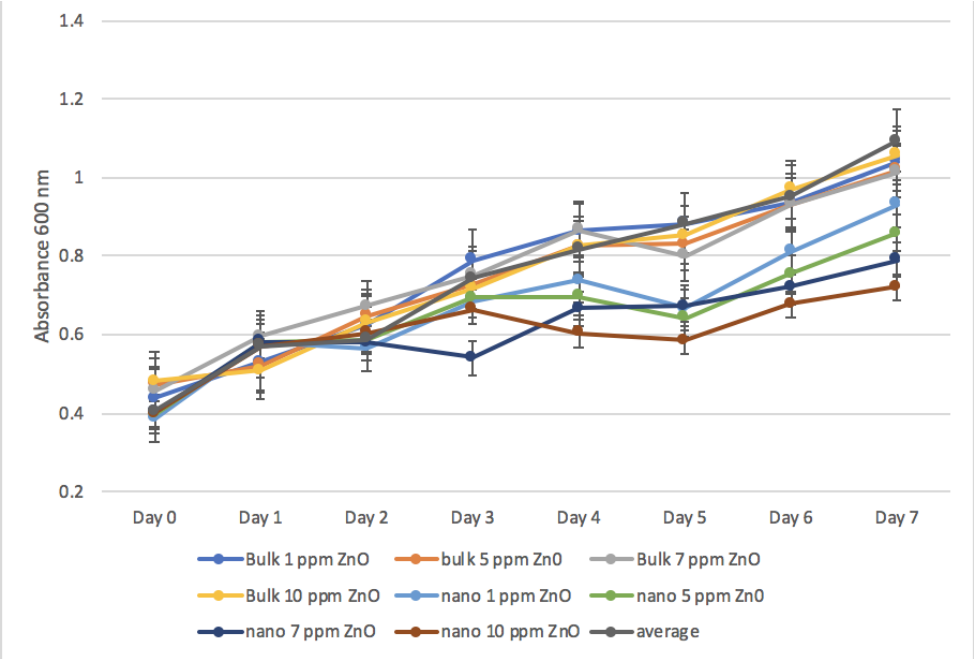


Figure 58: The effects of ZnO Nano, bulk particles and extra zinc on *Chlorella* sp. growth.

4.4 Green Microalgae – Exposure to High Zn Concentrations

For the second experiment, which was exposed to high concentration (2.5 ppm, 5 ppm, 7.5 ppm, 10 ppm, and 20 ppm), showed some tolerance again. The results will be divided by microscope and the absorbance. This experiment was performed with four cyanobacteria again with the 24 wells: 146-2-6 *Chlorococcum* sp., 146-2-10 *Chlamydomonas* sp., and 146-2-16 *Chlorella*.

4.5.1 Optical Microscope

After seven days of testing, the three microalgae were observed in 100x magnification. For the *Chlorococcum* sp. (Figure 59), the *Chlamydomonas* sp. (Figure 60), and the *Chlorella* sp. (Figure 61) some morphology changes in high nano concentrations was observed. These microalgae retained their original shape and color.

For the *Chlorococcum* sp. (Figure 59), from control to nano it retained the same color, however there was small noted morphology change for the 20 ppm nano. *Chlamydomonas* sp. (Figure 60) for the nano 20 ppm appears to have some sensitivity as is indicated with the clear parches observed. The *Chlorella* sp. (Figure 61) also did not show any morphology change (i.e. tolerance).

Table 4: Potential Tolerance and Sensitivity of the Microalgae

Microalgae	Tolerance or Sensitive
Chlorococcum sp.	Tolerance
Chlamydomonas sp.	Sensitivity
Chlorella sp.	Tolerance

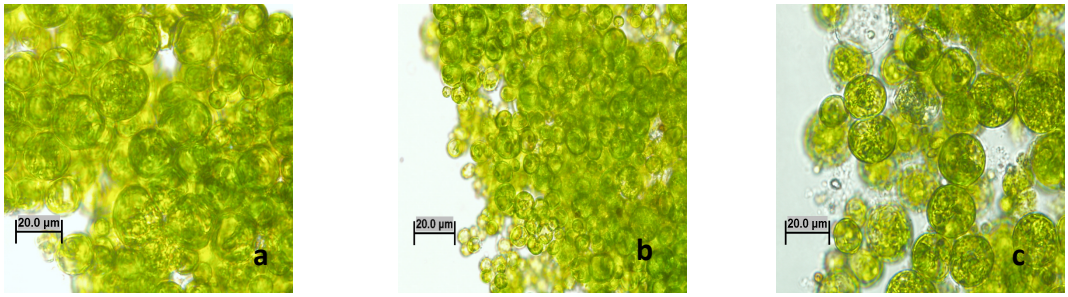


Figure 59: *Chlorococcum* sp., in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

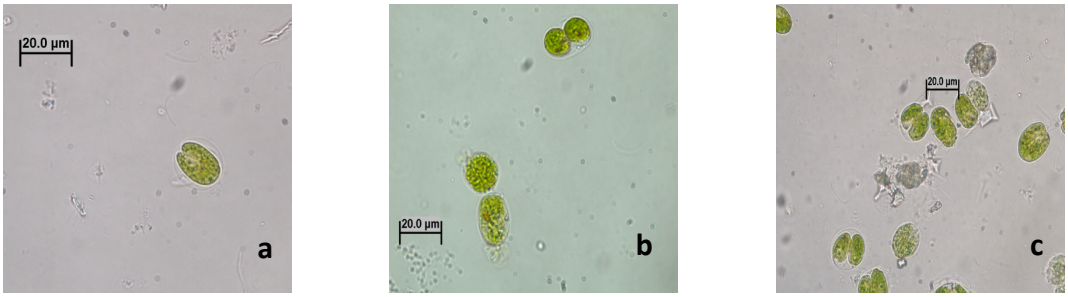


Figure 60: *Chlamydomonas* sp. in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

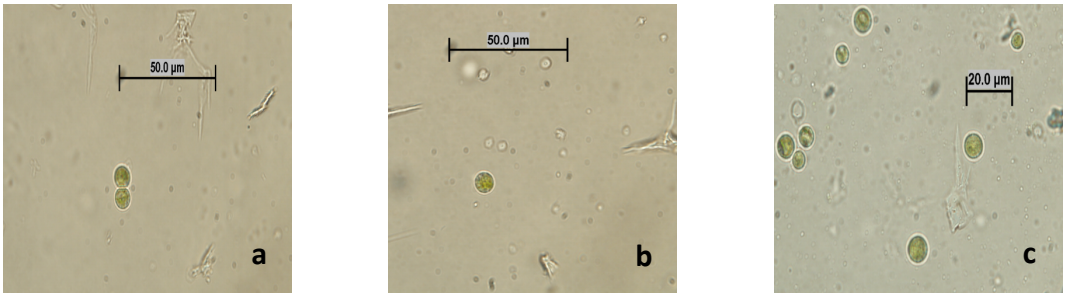


Figure 61: *Chlorella* sp. in BG 11 media with a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

4.5.2 Statistical Analysis

The results for the two-way mixed (ANOVA) with higher dosage with *Chlorococcum* sp., *Chlamydomonas* sp. and *Chlorella* sp.; the dosage was coded as follows: B1: 2.5 ppm Bulk ZnO, B2: 5 ppm Bulk ZnO, B3: 7.5 ppm: Bulk ZnO, B4: 10 ppm Bulk ZnO, N1: 2.5 ppm NPs ZnO, N2: 5 ppm NPs ZnO, N3: 7.5 ppm: NPs ZnO and N4: 10 ppm NPs ZnO.

After all the assumption was met the results were reported. Test of between-subjects' effects was analysis to determine if there was a statistically significant difference in absorbance concentration between intervention of treatments $F(8, 38) = 2.451$, $p = .000$, $\eta^2 = .045$ for *Chlorococcum* sp.; $F(8, 33) = 4.646$, $p = .001$, $\eta^2 = .199$ for *Chlamydomonas* sp. and $F(8, 34) = 6.346$, $p = .000$, $\eta 2 = .090$ for *Chlorella* sp.

Multiple Comparisons was analyzed to see if the absorbance concentration was or was not statistically significant for the treatments. For *Chlorococcum* sp., the absorbance concentration in all the treatments was not statistically significantly different. For

example: the control and n4 (*M* = .039750, *SE* =. 0399180nm, *p* = .984). For *Chlamydomonas* sp., the absorbance concentration was statistically significantly greater in the control to n2, n3 and n4. For example: Control and n4 (M= .157857, SE = .0323052 nm, p = .001). The absorbance concentration in was not statistically significantly for most of the treatments except control and n4/n3/n2. For example: the control group and b1 (M= .116345, SE =. .0348937 nm, p = .047). For *Chlorella* sp., the absorbance concentration in all the treatments was not statistically significantly. For example: the control and n4 (M =. 198951, SE =. .0621882 nm, p = .065).

There was a statistically significant interaction between the intervention and days on absorbance, for *Chlorococcum* sp.: F (3, 114) = 105.894, p =0.00, partial η^2 = .399.; for *Chlamydomonas* sp.: F (3,99) = 491.399, p=.000, η2 = 4.91 and for *Chlorella* sp.: F (3,102) = 267.584 p=.000, partial η2 = 1.875.

The Pairwise comparison table was used to know if there was statistically sig. between days. The absorbance concentration was not statistically significantly different between day 0 and day 2 (M = .030, SE = .010, p = .019), but the absorbance concentration was statistically significantly difference at for the rest of the days for *Chlorococcum* sp. For *Chlamydomonas* sp. and *Chlorococcum* sp., the absorbance concentration was statistically significantly difference for all days.

The results were reported, for *Chlorococcum* sp.: There were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality (p > .05). There was homogeneity of variances (p > .05) and covariance (p > .05), as assessed by Levene's test of homogeneity of variances and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test

of Within-subjects' effects: There was a statistically significant interaction between the intervention and days on absorbance: F (3, 114) = 105.894, p =0.00, η2 = .399. The absorbance concentration in all the treatments was not statistically significantly. For example: the control and n4 (M = .039750, SE = .0399180nm, p = .984).

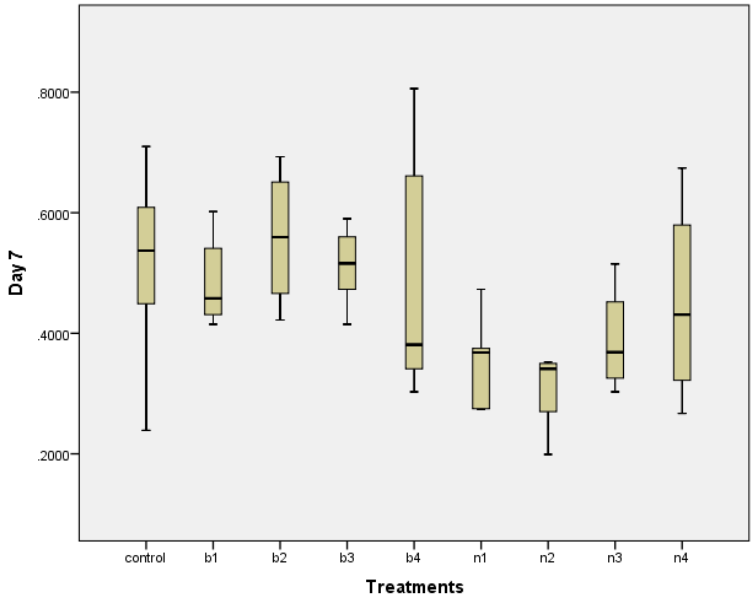


Figure 62: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Chlorococcum* sp. growth at day 7.

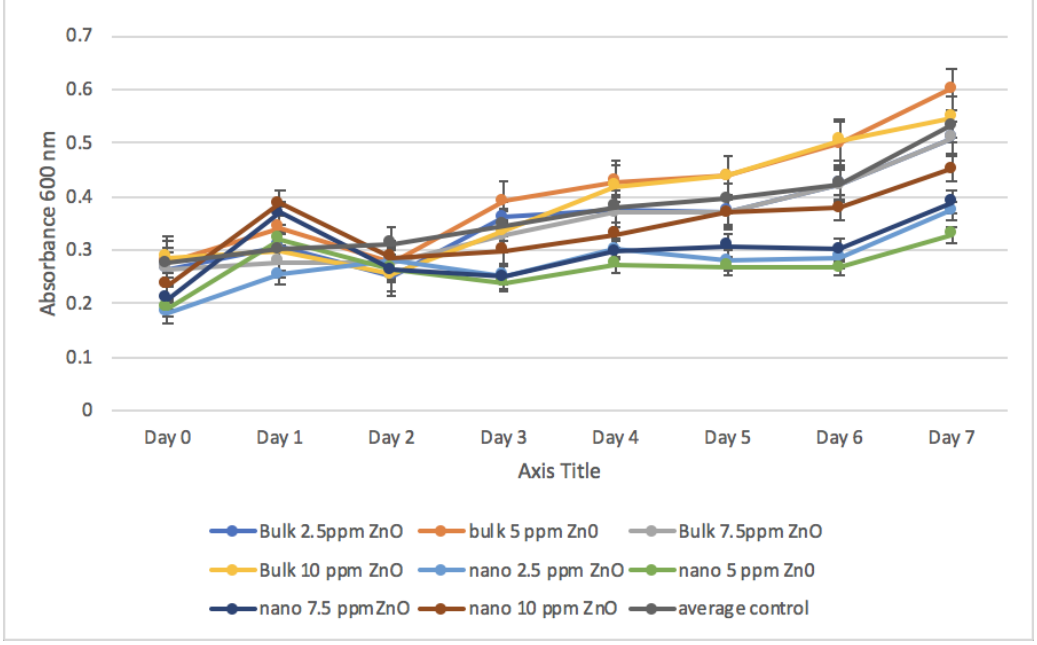


Figure 63: The effects of ZnO Nano, bulk particles and extra zinc on *Chlorococcum* sp. growth.

For *Chlamydomonas* sp.: There were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects effects: There was a statistically significant interaction between the intervention and days on absorbance $F(3,99) = 491.399$; $p=.000$, $\eta 2 = 4.911$. Absorbance concentration was statistically significantly greater in the control to n1, n3, and n4. For example: Control and n4 (M= .157857, SE = .0323052 nm, p = .001). The absorbance concentration in was not statistically significantly for most of the treatments except control and n1/ n4/n3. For example: the control group and b1 (M= .116345, SE =. .0348937 nm, p = .047).

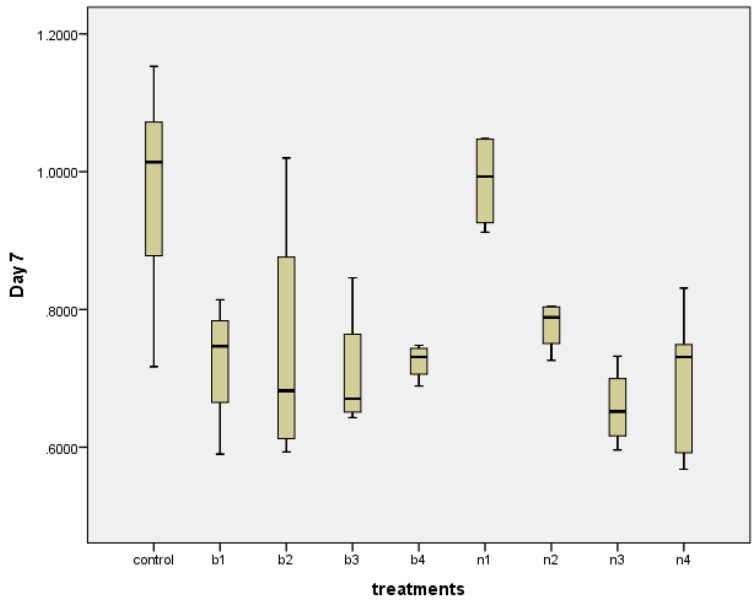


Figure 64: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Chlamydomonas* sp. growth at day 7.

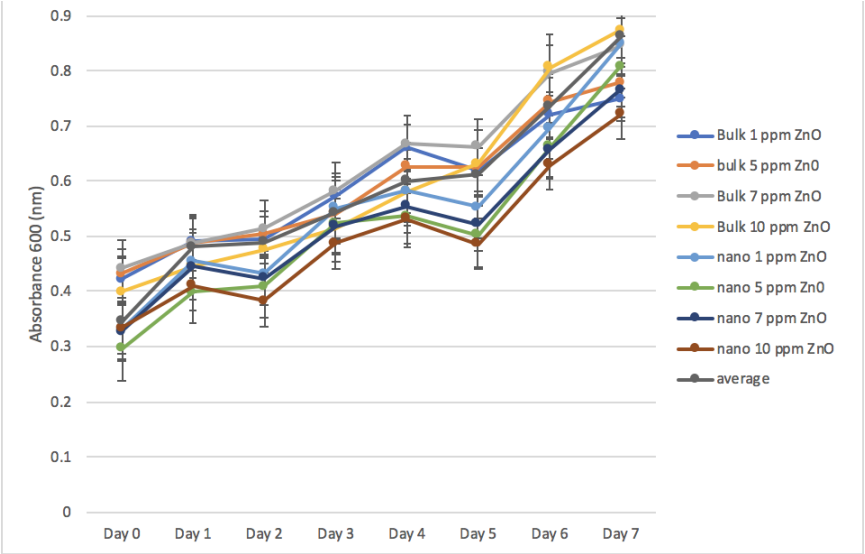


Figure 65: The effects of ZnO Nano, bulk particles and extra zinc on *Chlamydomonas* sp.

For *Chlorella* sp.: There were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances with the exception of days 0 and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects' effects: There was a statistically significant interaction between the intervention and days on absorbance: $F(3,102) = 267.584$. $p=.000$, partial $\eta 2 = 1.875$. The absorbance concentration in all the treatments was not statistically significantly. For example: the control and n4 (M =. 198951, SE = .0621882 .0399180nm, $p = .065$).

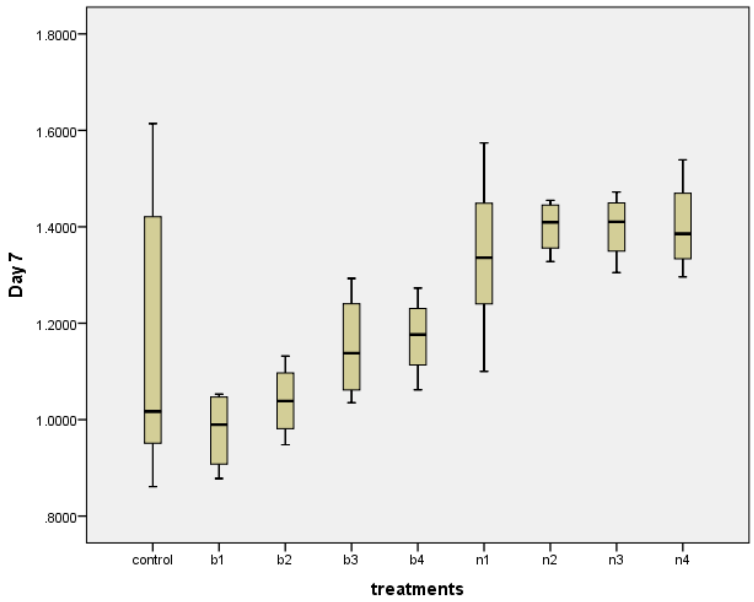


Figure 66: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Chlorella* sp. growth at day 7.

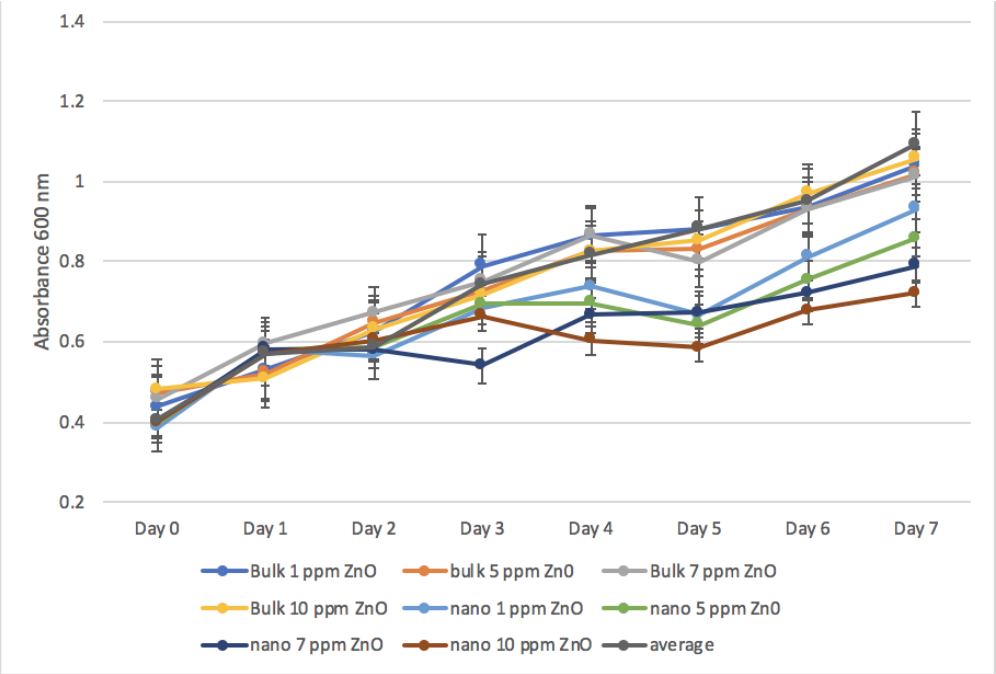


Figure 67: The effects of ZnO Nano, bulk particles and extra zinc on *Chlorella* sp.

4.5 Flask Experiment

Two trials were conducted for the flask experiment. The first trial was performed with *Chlorococcum* sp., *Chlamydomonas* sp., and *Chlorococcum* sp. however this specimen was found to have been contaminated. For the *Chlamydomonas* sp., the bulk grew more than control and nano (Figure 68) as observed visually. Trial two was performed with *Chlorococcum* sp. and *Chlorella* sp. This time the *Chlorococcum* sp. was not contaminated, and there was no color change visually between control, bulk and nano. There was measurement done with a microscope. The *Chlorella* sp. showed some

sensitivity in the control and bulk as they can be seen to have the same green color, while the nano has a lighter color.

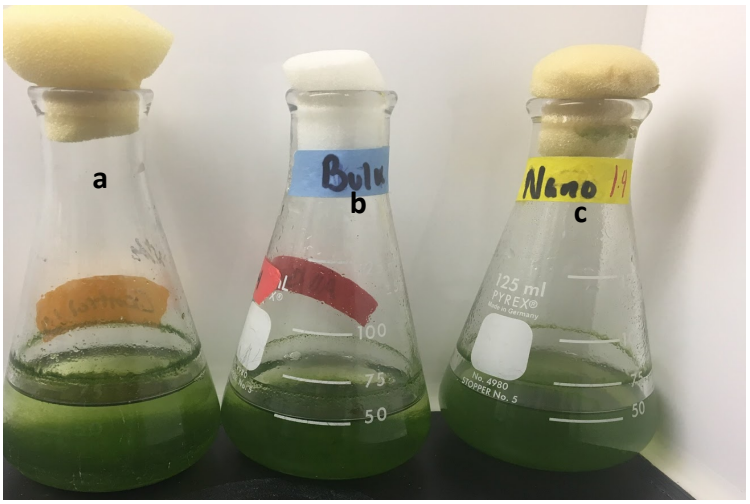


Figure 68: *Chlorococcum* sp. in flask experiment BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

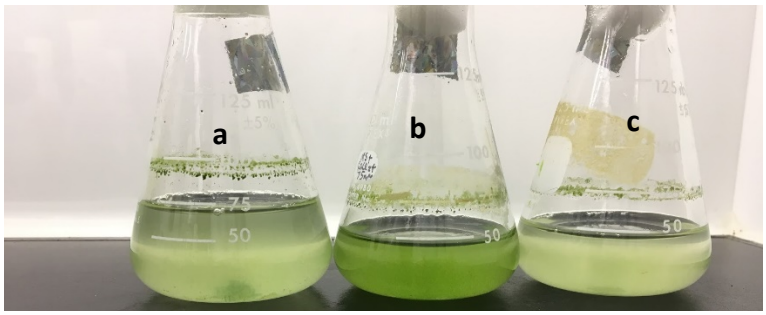


Figure 69: *Chlamydomonas* sp. in flask experiment BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

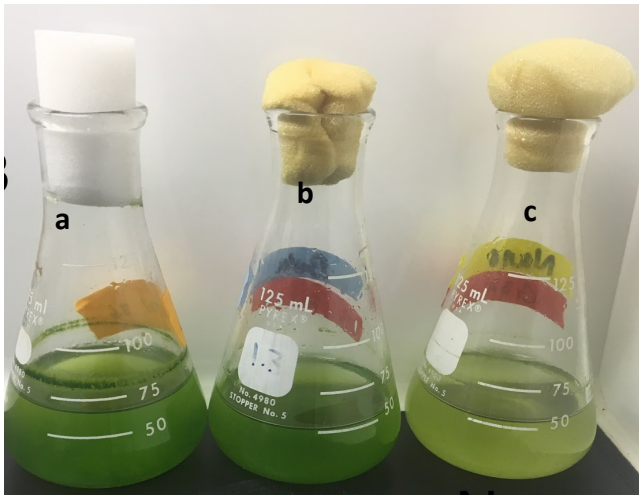


Figure 70: *Chlorella* sp. in flask experiment BG 11 media with a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

4.6.1 Optical Microscope

The results for *Chlorococcum* sp. were unexpected. The *Chlorococcum* sp. was unanticipatedly contaminated with a type of filamentous microalgae, however the nano flask NPs were not contaminated (Figure 71). For the *Chlamydomonas* sp. (Figure 72), the control and bulk had similar morphology and color. However, the 20 ppm nano experienced some discoloration and disruption of the cells. The *Chlorella* sp. (Figure 73) exhibited some discoloration from control to nano. The *Chlamydomonas* sp. (Figure 74) was not contained such as in trial 1. There was no visual morphology change. However, when seen through the microscopes, there is a notable difference from control and nano. The 20 ppm ZnO NPs can be observed starting to lose their green discoloration.

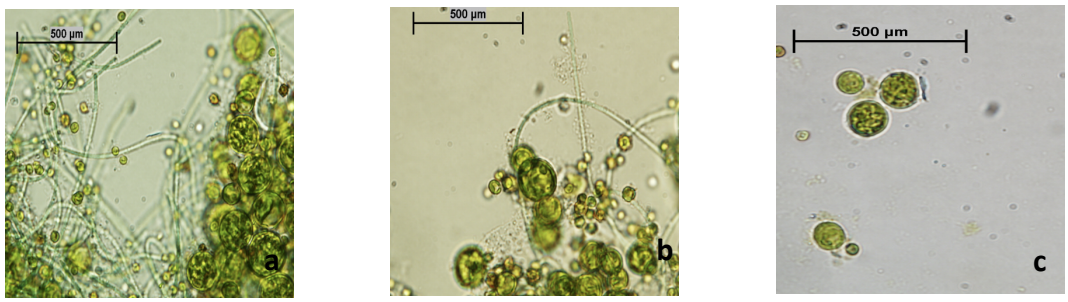


Figure 71: *Chlorococcum* sp. in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

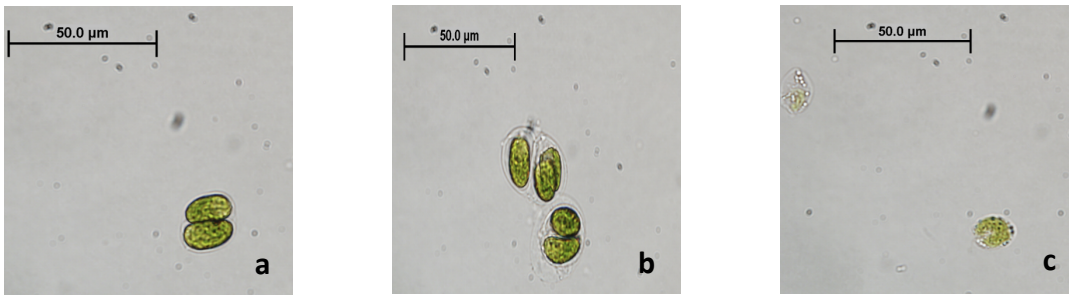


Figure 72: *Chlamydomonas* sp. in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

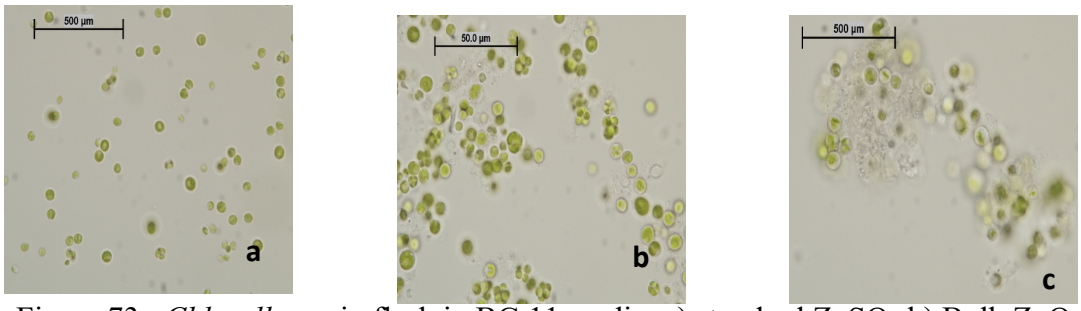


Figure 73: *Chlorella* sp. in flask in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

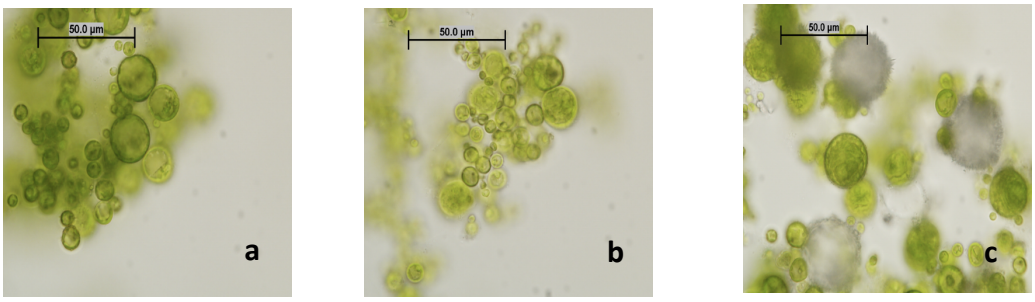


Figure 74: *Chlorococcum* sp. in flask in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

4.6.2 Cell Counts

The cell was counted for day 0 and day 7 on trial 1 and 2. In trial 1, *Chlamydomonas* sp. (Figure 76) exhibited an increase of cell amount for bulk and control, but for the nano there was a noted decrease. *Chlorella* sp. showed an increase for all three treatments, but the nano had less cells comparably (Figure 77). *Chlorococcum* sp. had an increased number of cells (Figure 75).

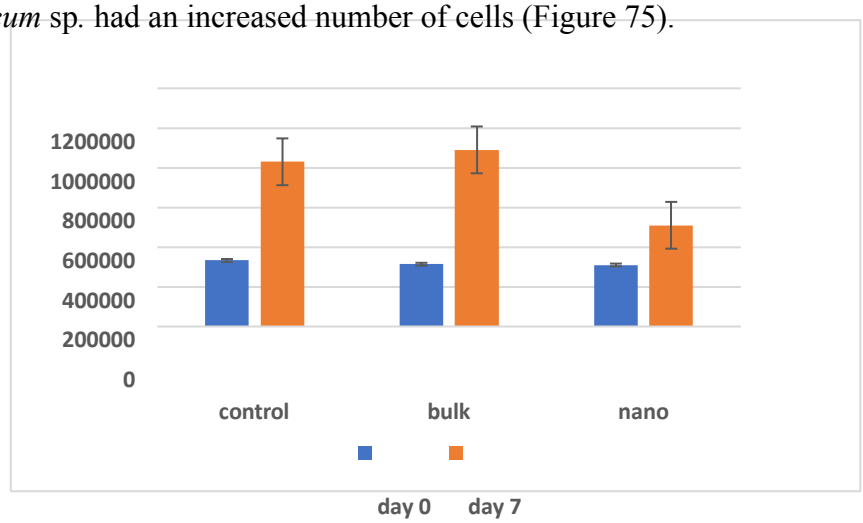


Figure 75: *Chlorococcum* sp. cells counted for day 0 and day 7.

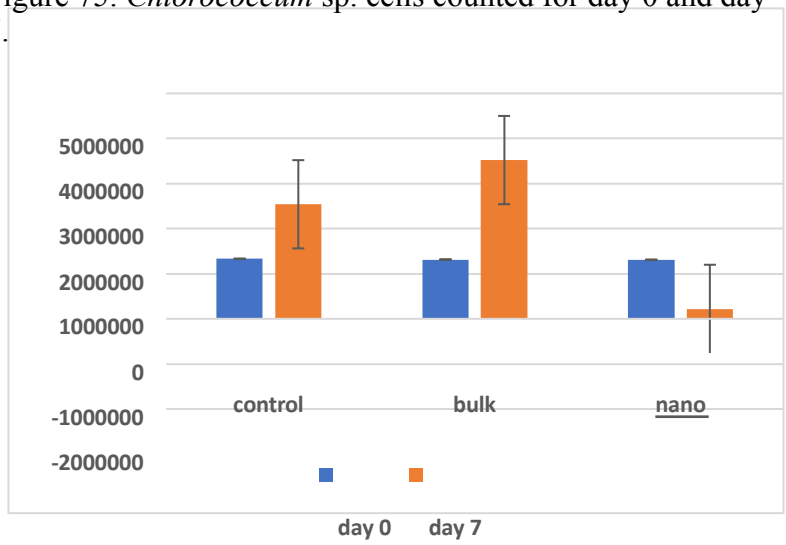


Figure 76: *Chlamydomonas* sp. cells counted for day 0 and day 7.

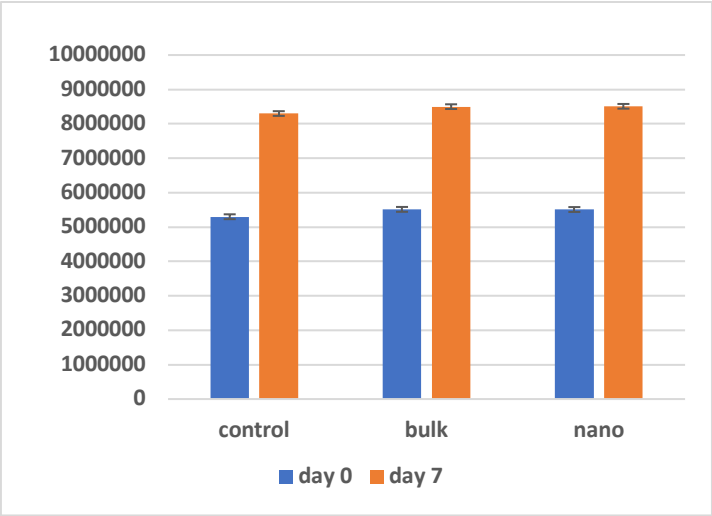


Figure 77: *Chlorella* sp. cells counted for day 0 and day 7.

4.6.3 SEM and EDS

The SEM and EDS were used to analyze *Chlorococcum* sp. (Figure 78). In three treatment trials, the microalgae retained their round morphology, and the nano had small particles attached were possible NPs can be noted. For the EDS (Figure 79), a spot test was conducted in order to determine the Zn concentration. The control Zn concentration was at 2.652 wt.%, following with bulk having the lowest concentration of Zn at 1.189 wt.%, and finally the nano which had the highest Zn concentrations 5.032 wt.%.

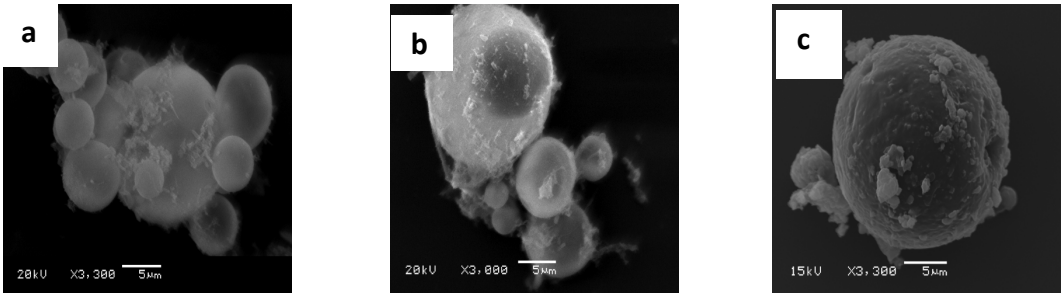


Figure 78: *Chlorococcum* sp. in BG 11 media: a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

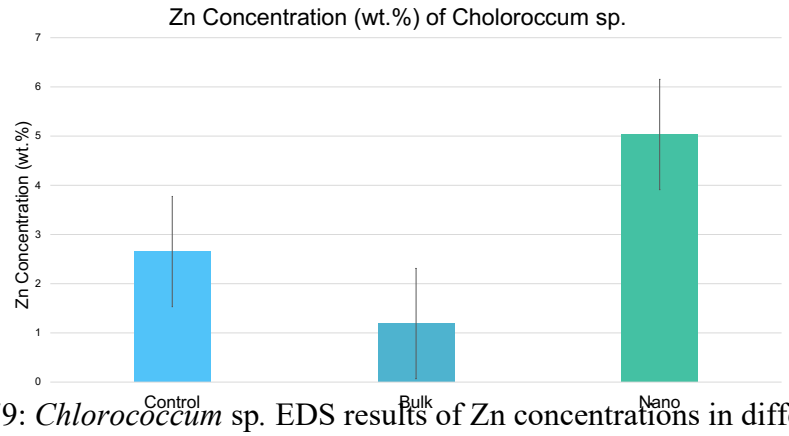


Figure 79: *Chlorococcum* sp. EDS results of Zn concentrations in different treatments

The SEM and EDS were used to analyze *Chlamydomonas* sp. It is important to note that the drying portion of the experiment was not done properly because the tank CO_2 inadvertently ran out. This maybe the reason why the *Chlamydomonas* sp. morphology got disturbed (Figure 80). For the results, the flagella were found in most of the microalgae in the control, however in the bulk and the nano the flagella no longer appeared. For the EDS (Figure 79), spot testing was performed to determine the Zn concentration. The control Zn concentration was the lowest at 4.377 wt. %, following with bulk at 9.695 wt.%, and finally nano had the highest Zn concentrations at 11.274 wt.%.

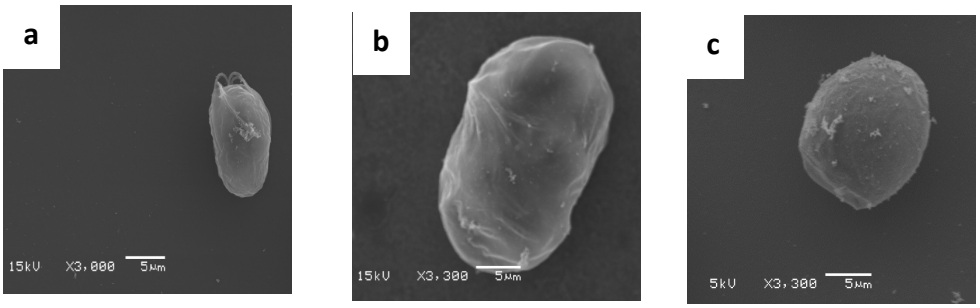


Figure 80: *Chlamydomonas* sp. in BG 11 media with a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.

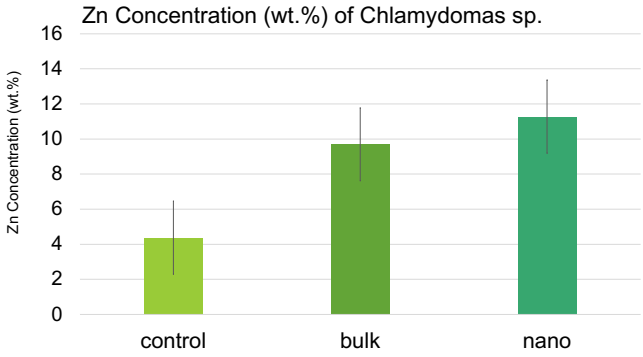


Figure 79: *Chlamydomonas sp.* EDS results of Zn concentrations in different treatments.

The SEM and EDS were used to analyze: *Chlorella* sp. (Figure 82). In the three treatment trails, the microalgae retained their small round morphology, even though the nano and bulk were observed to have small particles and filamentous structures. The EDS (Figure 83) spot test was conducted to determine the Zn concentration. As can be seen, the control Zn concentration had the lowest concentration at 4.204 wt.%, following with bulk at 6.706wt.%, and finally the nano having the highest Zn concentrations at 8.239 wt.%.

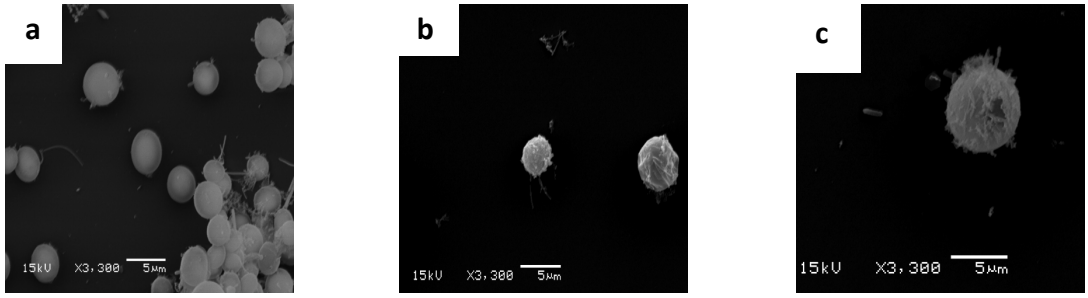
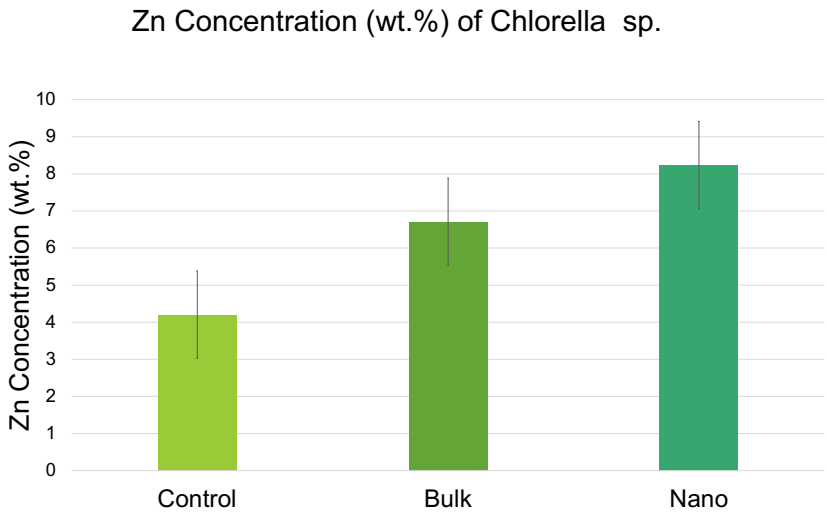


Figure 82: *Chlorella* sp. in BG 11 media with a) standard $ZnSO_4$ b) Bulk ZnO 20 ppm c) Nano ZnO 20 ppm.



4.6.4 Statistical Analysis

The results for the two-way mixed (ANOVA) with 20 ppm ZnO with *Chlorococcum* sp., *Chlorococcum* sp. And *Chlorella* sp.; the dosage was coded has follow B 20: 20 ppm Bulk ZnO; N 20: 20 ppm NPs ZnO.

After all the assumption was met the results were reported. Test of between-subjects' effects was analysis to determine if there was a statistically significant difference in absorbance concentration between intervention of treatments: F (2, 15) = .532 p = .000, η2 = .110 for *Chlorococcum* sp. ; F (2, 13) = 23.601p = .000, η2 = .020 for *Chlamydomonas* sp.; F (2, 15) = 11.985p = .000, η2 = 1.074 for Chlorella sp. .

Multiple Comparisons was analyzed know if the absorbance concentration was or was not statistically significantly for the treatments. For *Chlorococcum* sp., the absorbance concentration in all the treatments was not statistically significantly different. For example: the control and n20 (M = .004750 SE = .0124869, p = .924). For *Chlamydomona*s sp., the absorbance concentration was statistically significantly greater in the control and nano; bulk and nano. For example: Control and nano (M= .078600, SE

= .0124255 nm, p = .000). The absorbance concentration in was not statistically significantly for control and bulk. For example: the control group and bulk (M= .013200, SE =. 0129780 nm, p = .580). For *Chlorella* sp.; Absorbance concentration was statistically significantly greater in the control and nano. For example: Control and nano (M= .597333, SE = .1221930nm, p = .001). The absorbance concentration in was not statistically significantly for control and bulk; bulk and nano. For example: the control group and bulk (M= .270167, SE = .1221930 nm, p = .102).

There was a statistically significant interaction between the intervention and days on absorbance, for *Chlorococcum* sp.: F (1, 15) = 149.527, p =0.00, partial η^2 = 23.704; for green2: F(1, 13) = 34.375, p =0.00, partial η2 = .035. For *Chlorella* sp.: F (1, 15) = 72.307, p =0.00, partial η^2 = 9.011.

The Pairwise comparison table was used to know if there was statistically significant between days. For all the green microalgae the absorbance concentration was statistically significantly different for all days.

The results were reported, for *Chlorococcum* sp.: There were no significant outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality (p > .05). There was homogeneity of variances (p > .05) and covariance (p > .05), as assessed by Levene's test of homogeneity of variances and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects effects: There was a statistically significant interaction between the intervention and days on absorbance F (1, 15) = 149.527, p =0.00, partial η2 = 23.704. The absorbance concentration in all the treatments was not

statistically significantly. For example: the control and n20 (M = .004750 SE = .0124869, p = .924).

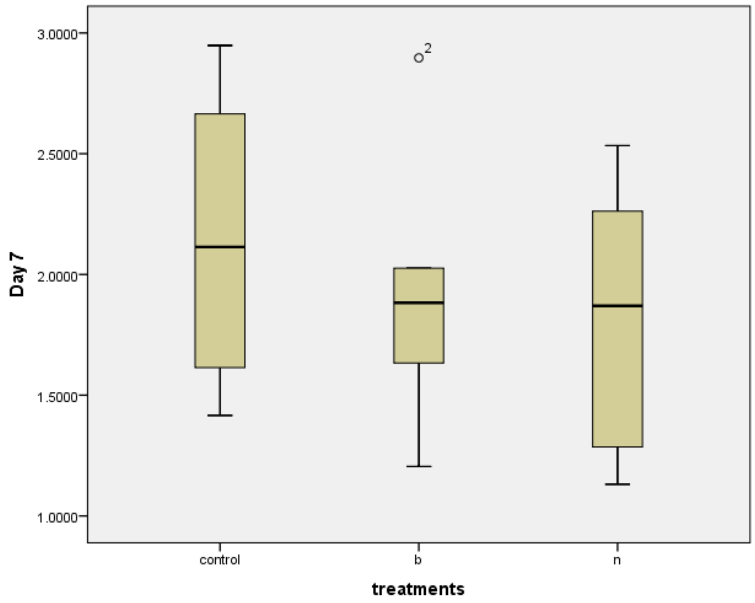


Figure 84: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Chlorococcum* sp. growth at day 7.

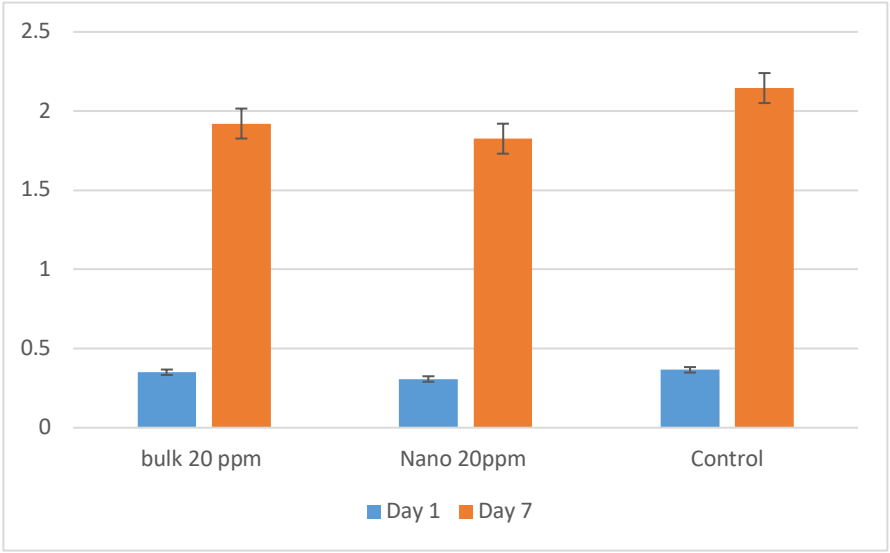


Figure 85: The effects of ZnO Nano, bulk particles and extra zinc on *Chlorococcum* sp. growth.

For *Chlamydomonas* sp.: There were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects effects: There was a statistically significant interaction between the intervention and days on absorbance $F(1, 13) = 34.375$, $p = 0.00$, partial $\eta 2 = .035$. Absorbance concentration was statistically significantly greater in the control and nano. For example: Control and nano ($M = .078600$, $SE = .0124255$ nm, $p = .000$). The absorbance concentration in was not statistically significantly for control and bulk. For example: the control group and bulk ($M = .013200$, $SE = .0129780$ nm, $p = .580$).

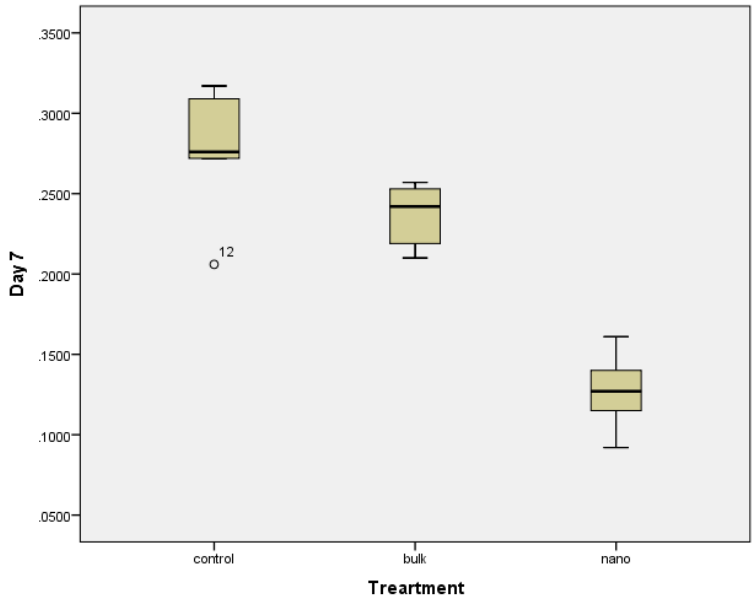


Figure 86: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Chlamydomonas* sp. growth at day 7.

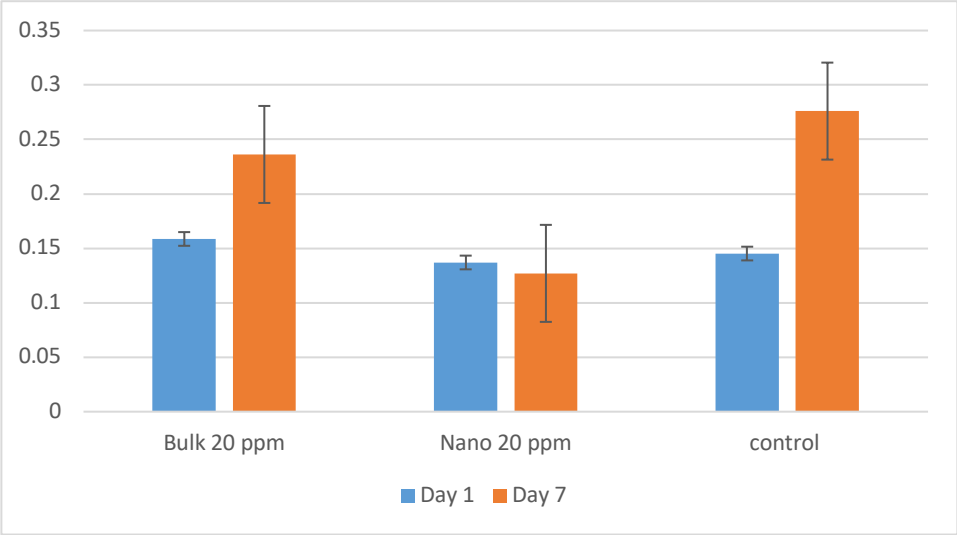


Figure 87: The effects of ZnO Nano, bulk particles and extra zinc on *Chlamydomonas* sp. growth.

For *Chlorella* sp.: There were no outliers, as assessed by boxplot. The data was normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was homogeneity of variances ($p > .05$) and covariance ($p > .05$), as assessed by Levene's test of homogeneity of variances with the exception of days 0 and Box's M test, respectively. "Mauchly's test of sphericity was used to examine the assumption of sphericity. The Huynh-Feldt epsilon was used to interpret the two-way interactions. Test of Within-subjects effects: There was a statistically significant interaction between the intervention and days on absorbance $F(1, 15) = 72.307$, $p = 0.00$, partial $\eta 2 = 9.011$. The Absorbance concentration was statistically significantly greater in the control and nano. For example, Control and nano (M= .597333, SE = .1221930 nm, p = .001). The absorbance concentration in was not statistically significantly for control and bulk. For example, the control group and bulk (M= .270167, SE = .1221930 nm, p = .102).

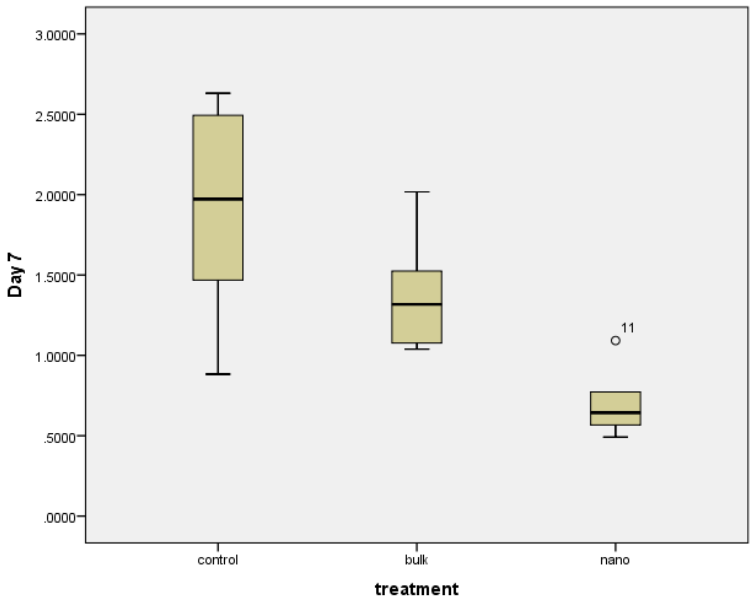


Figure 88: Box plot's shows the effects of ZnO Nano, bulk particles and extra zinc on *Chlorella* sp. growth at day 7.

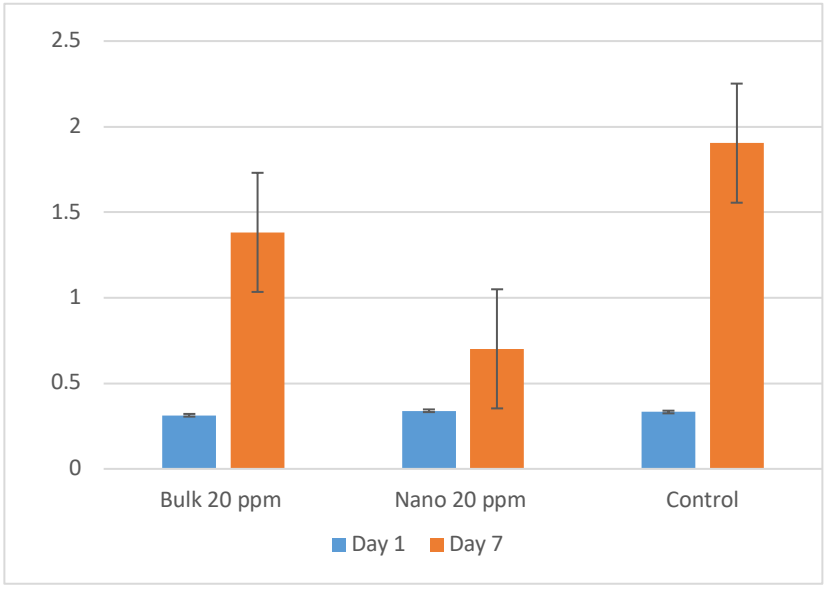


Figure 89: The effects of ZnO Nano, bulk particles and extra zinc on *Chlorella* sp. growth.

4.7 Enrichment and Isolation of Microalgae Tolerant to High Nano-ZnO Concentration

4.7.1 Optical microscope

After three months, samples from the four enrichment flasks showing turbidity were observed under microscope. Initial enrichment flasks contained a variety of organisms from purple filaments, to round brown microalgae, and green microalgae (Figure 90). Repeated enrichment transfers showed that only one flask consistently showed algal growth, based on initial observation the cells appeared to be Chlorella sp. (Figure 90 f).

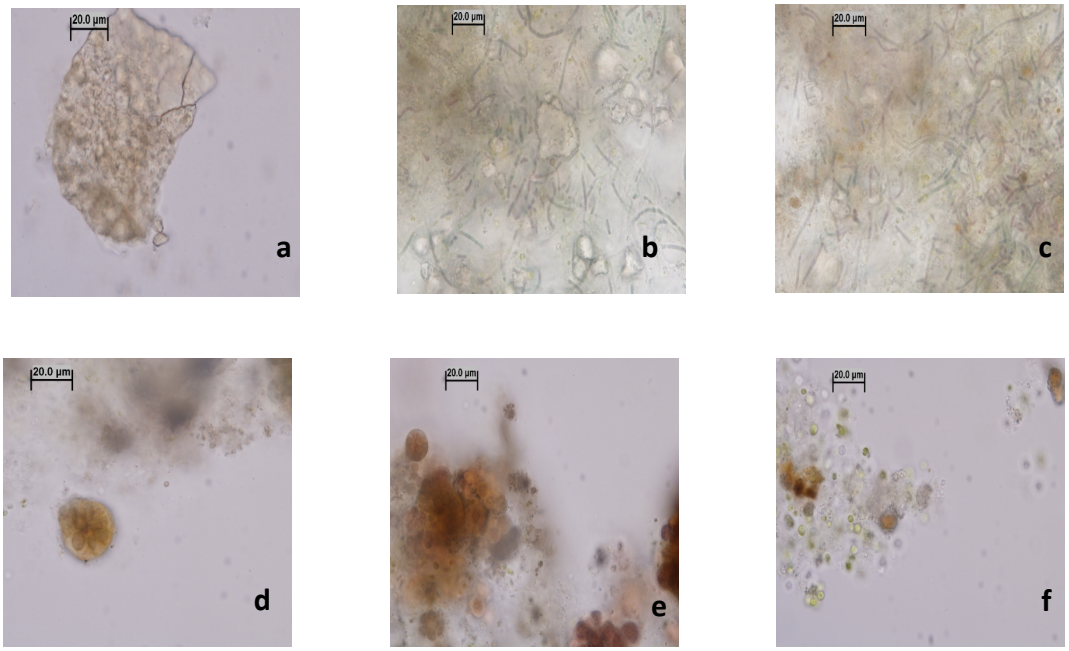


Figure 90: All of the images were taken from South Point Park Beach and exposed to 100 ppm.

Section 5: Discussion

5.1 Cyanobacteria

Overall, the results showed that cyanobacterial species were more sensitive to nano ZnO than the green microalgae. Cyanobacteria differ from the green micro algae because they are prokaryotic gram-negative bacteria, while microalgae are eukaryotic. There have been multiple studies done with the *E. coli* bacteria, which is gram negative like the cyanobacteria, about their toxicity to bulk and ZnO NPs. Most of the studies showed that there is damage within the *E. coli* cells due to disorganization in the gram-negative triple membrane, which is highly unusual for these species (Brayner et al., 2006). Experiments have also been done with cyanobacteria showing similar results. For example, the cyanobacteria A. *flosaquae* and *E. gracilis* showed a decrease of photosynthetic activity for both when exposed to ZnO NPs (Brayner et al., 2010). Another study using *Spirulina (Arthrospira) platensis* discovered that ZnO NPs triggered substantial cytotoxicity within the *S. platensis* and caused cell death (Djearamane et al., 2018). Similar to all of these results, this research showed that most of the select group of cyanobacteria have some sensitivity to bulk and/or nano ZnO.

Even though most of the cyanobacteria showed sensitivity to both bulk and nano ZnO, *Leptolyngbya* sp. showed more sensitivity to nano ZnO than to bulk ZnO. This corresponds with the microscope picture, as there is a noticeable morphological difference between nano and bulk ZnO. These results are similar to research using A. *flosaquae* , that showed (Figure 4) how nano ZnO has a lower growth than the bulk ZnO (Nandi et al., 2012). Surprisingly, the *Porphoridium* sp. showed no significant difference between all treatments. Although this microalga showed sensitivity with its color change

from pink to green, the microplate reader may be faulty and not detect this change in color.

Another technical problem occurred when using a higher concentration of bulk and nano ZnO with the species: *Limnothrix* sp., *Oscilatoria* sp., *Leptolyngbya* sp., and *Lyngbya* sp., which could be attributed with the fact they were not new. Nevertheless, the majority of cyanobacteria in this experiment showed a clear sensitivity to both nano and bulk. Other than by absorbance measurements, this was proven visually with the pictures, which showed clear morphology change for both nano and bulk. These results raised a new hypothesis: If cyanobacteria are sensitive to both bulk and nano ZnO, are they also sensitive to the zinc?

This hypothesis was researched by doing a new experiment with higher concentration of "normal" Zinc. The experiment of higher Zn concentration was done for *Limnothrix* sp., Leptolyngbya sp., *Lyngbya* 1 sp. and Lyngbya 2 sp. The results indicate that these cyanobacteria are not necessarily sensitive to the Nano "effect" but to the zinc concentration itself. Also, the optical microscope pictures confirmed that all of these cyanobacteria were sensitive to Zn and not only to nano and bulk. There was a study done by Djearamane et al. (2018), which showed that *Cyanobacterium Synechococcus* sp. was toxic when exposed to a high concentration of Zinc.

5.2 Green Microalgae

Green microalgae showed a tolerance to bulk and nano ZnO compared with these cyanobacteria. This could be due to the complexity of eukaryotic organisms (green microalgae) versus the simpler prokaryotic cell (cyanobacteria). To our knowledge, there has not been a comparative study of a mechanism of how the ZnO NPs' toxicity compares in the eukaryotic vs prokaryotic organisms. There has been research that shows

NPs of metal oxides can induce cell death in eukaryotic cells (Nel 2006; Long et al., 2006) and growth inhibition in prokaryotic cells (Huang et al., 2008) due to cytotoxicity.

At all levels of NPs ZnO tested (0.125 ppm, 0.25 ppm, 0.5 ppm, and 1 ppm) the three green microalgae showed marked tolerance (OD more than 0.8618). Due to the tolerance results of the low concentration experiment, all three green algal species were selected for assessing response to higher ZnO concentration levels. All the three green microalgae tested showed tolerance to higher concentration of nano ZnO based on growth response. These tolerances to ZnO NPs may be because the green microalgal cells are negatively charged, providing a set of binding sites for metal cations like Zn^{+2}, which have the potential to lower toxicity in the microalgae (Monteiro, Fonseca, Castro, & Malcata, 2010). However, for *Chlamydomonas* sp., there showed minor sensitivity when exposed to ZnO NPs. Also, abnormal cell morphology and clumping of cells were observed when exposed to nano ZnO NPs at concentrations of 7.5 ppm and 10 ppm Zn.

After the 24 wells experiments, a flask experiment was conducted. The flask experiment was with the three green microalgae and 20 ppm NPs and bulk ZnO. The results on the flask versus the wells were different. Both *Chlorococcum* sp. and *Chlorella* sp. showed tolerance to ZnO Nano and bulk. The results for the morphology do not agree with these results. For *Chloroccoum* sp., there were transparent circle patches and for *Chlorella* sp. there was a transparent patch. Studies showed that the green microalgae S. *obliquus* can tolerate higher Zn concentrations than *D. pleiomorphus* (Monteiro, Fonseca, Castro, & Malcata, 2010). Another study showed *Raphidocelis subcapitata* and *Chlorella vulgaris* are proven to have a tolerance to Zinc (Muyssen & Janssen, 2001). For the species *Tetraselmis sucica*, there was no effect in a high

concentration of 100 mg/L ZnO NPs (Castro-Bugallo, González-Fernández, Guisande, & Barreiro, 2014)

For *Chalydomonas* sp., there were significant differences between the control with nano and bulk with nano, indicating that there is a clear sensitivity to NPs ZnO. This can also be seen by the microscope morphology results, where the cell has less pigmentation and a very small size. A study done with the *Chalydomonas* (*D.tertiolecta*) showed different results, with toxicity at a much higher concentration of 133 mg/L ZnO NPs (Miglietta et al., 2011); but another study done with the same algae had toxicity at a lower concentration of 2.42 mg/L ZnO NPs (Manzo et al., 2013).

There have been other studies that showed some sensitivity to green microalgae. In one study done with *S. obliquus* and *D. pleiomorphus*, researchers discovered ZnO NPs are toxic to them (Monteiro, Fonseca, Castro, & Malcata, 2010). *Alexandrium minutum*, *Pseudokirchneriella subcapitata* and *T.pseudonana* had a high inhibition of 80% with a very low concentration of 0.01 mg/L ZnO NPs (Lee & An, 2013).

In the flask experiment, interesting results occurred for the first trial with *Chloroccoum* sp. The *Chloroccoum* sp. was contaminated with a filamentous structure, which could potentially be a cyanobacteria. For the Nano ZnO 20 ppm flask results, the contamination was no longer there. Potentially the NPs ZnO eliminated the contamination of the cyanobacteria because the cyanobacteria from the previous experiment are sensitive to zinc.

For SEM and EDS results, we wanted to see the morphology change as well as the Zn concentration on the cell. There was not much change in the morphology for *Chlorococcum* sp. and *Chlorella* sp., but as expected in the nano, some patches on the cells can be observed, which can potentially be the NPs. For the EDS, these both

microalgae had different zinc concentrations for each treatment, but it was not significant. For *Chlamydomonas* sp., there was a considerable morphology change where the flagella disappeared for bulk and for nano at 20 ppm. Also, in the EDS results, nano had more than a 50% difference than the control, indicating that there is a lot of Zn concentration in the cell.

5.3 Zn NPs tolerant algae

Long term incubation of enrichment cultures of sea water samples on high concentration of Zn NP showed no algal growth in all but one flask. Visible green algal growth in enrichment flask took close to one month. Light microscopic observation of microalgal cells growing in enrichment flask containing 100 ppm Zn NP most likely belong to *Chlorella* sp. In a study on *Chlorell*a sp. (Chen, Powell, Mortimer, & Ke, 2012), it was observed that *Chlorella* sp. is able naturally adapt to discharged nanomaterials because these algal cells have the capability of self-protection by minimizing their surface area through aggregation mediated. The mechanism is able to be achieved by the oppositely charged metal ions and suppressing zinc ion release from the NPs. Due to *Chlorella* sp. proven to have a natural resistance to ZnO NPs, the possibility of *Chlorella* sp. being the species able to survive the 100ppm ZnO NPs, is high. But additional purification of culture and subsequent DNA sequence analysis is needed to confirm the identification of the species.

Section 6: Conclusions

These findings provide a strong impetus for screening a larger collection of diverse indigenous microalgae for tolerance to NPs, potentially yielding viable candidates for bioremediation of NPs and also sensitive indicator species for Eco toxicological testing. Due to the results, cyanobacteria are more sensitive to ZnO NPs and bulk than the

green microalgae, especially the one with a filamentous shape. The filamentous shape makes them more vulnerable to high concentration of zinc; meaning that filamentous cyanobacteria can be seen as an indicator species for ecotoxicology testing. While most of green microalgae show tolerance to ZnO NPs and bulk especially the ones with circular shape, *Chlorella* sp. was confirming to be tolerant not only in the lab setting but also nature even when tested at 100 ppm experiment. *Chlorella* sp. can potentially be used for bioremediation since it is tolerant to such high concentrations of ZnO NPs.

Section 7: Recommendations

The Zn concentration of the Miami Beach, FL area has never been historically measured because it is not one of the elements required to be tested by the city's and state's regulations. Due to it being a high tourist region, it would be ideal to analyze the potential concentrations in popular beach areas in South Florida. For future studies, it would be desirable to try to create a regular and historical testing routine in order to be able to look for patterns over time. This will enable researchers and health and safety monitoring entities to check for any potential impacts for both the aquatic environment and for humans who come into contact with it. It would also be appropriate to learn more about the mechanisms of how *Chlorella* sp. is able to tolerate such high concentrations and why cyanobacteria are not.

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