2020 年臺灣國際科學展覽會
優勝作品專輯

作品編號 **180014**

參展科別 **地球與環境科學**

作品名稱 **Chlorella vulgaris chlorophyll a fluorescence as a potential indicator for zinc and nickel detection**

得獎獎項 **大會獎：二等獎**

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關鍵詞 **Chlorella vulgaris, heavy metal, chlorophyll fluorescence**

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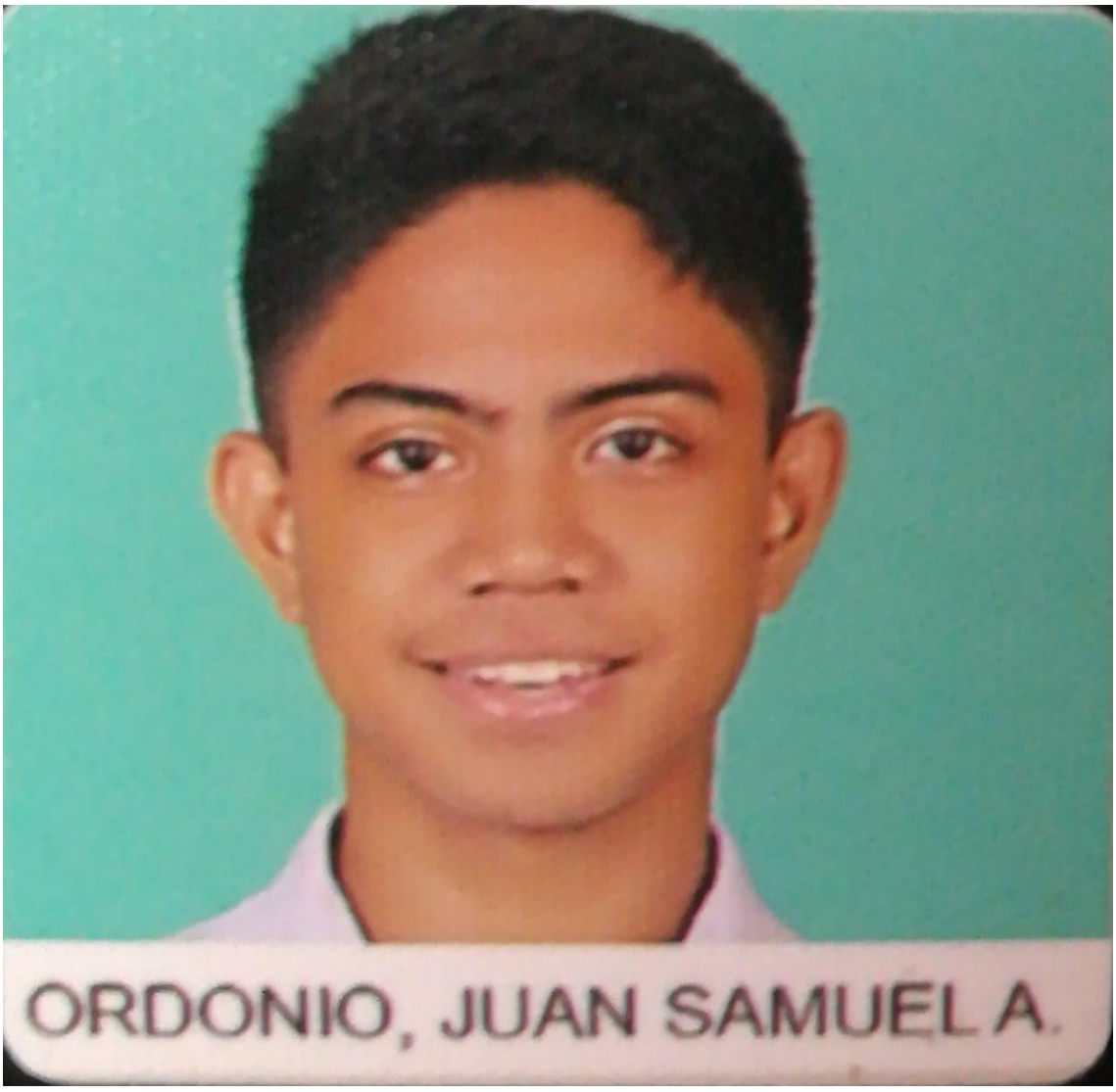

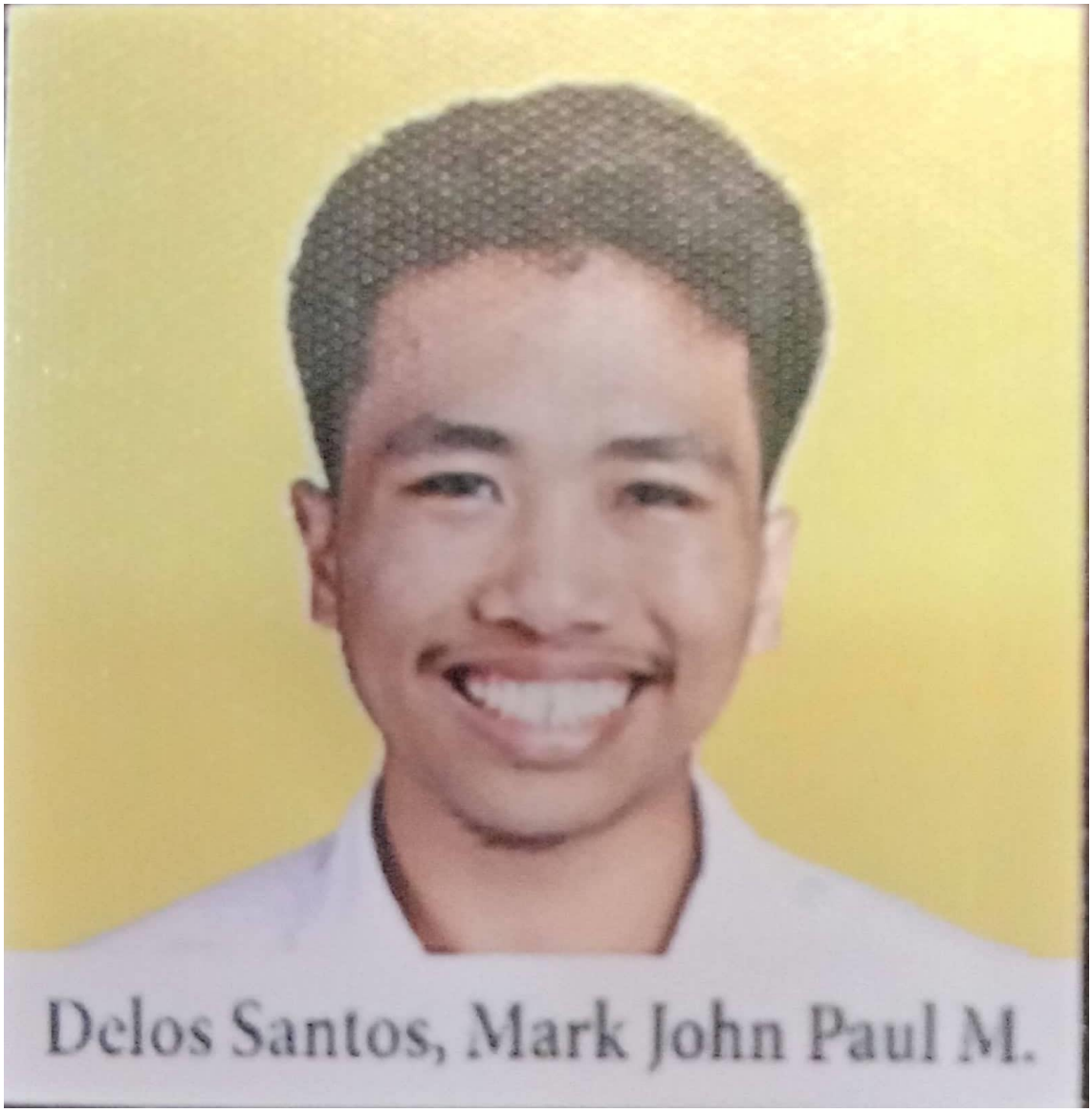
Delos Santos, Mark John Paul M.

Abstract

Heavy metals contaminate many bodies of water, posing a health risk to not only organisms that live and use the water in these areas, but also to the humans that live nearby. *Chlorella vulgaris*, a microalga, is one organism whose chlorophyll a fluorescence can indicate the presence of these substances, detecting any changes in concentrations using fluorescence microscopy and other fluorescence devices. The study explores the sensitivity of *C. vulgaris* to the heavy metal zinc where the algae was exposed to five concentrations of zinc: 0 ppm, 5 ppm, 10 ppm, 50 ppm, and 100 ppm. The fluorescence of the samples was observed with a fluorescence microscope on days 0, 4, 7, and 12, where the algal samples were adapted to the dark for 5 minutes, then exposed to light for 90 seconds. The values of the minimal and maximal fluorescence of the samples in the dark were noted. There is a significant difference in the values of the minimal fluorescence, maximal fluorescence, and maximum quantum yield, a value derived from the minimal and maximal fluorescence, at the highest concentration, 100 ppm, from the other treatments for the entirety of the experiment. The significantly low values at 100 ppm and the calculated EC_{50} of 75.70 ppm indicate that *C. vulgaris* is indeed a viable indicator for zinc detection at this and higher concentrations of zinc.

Introduction

Heavy metals are metallic elements of relatively high density. Most are essential in small amounts, but can be carcinogenic and can have adverse effects on vital organs (Fu & Wang, 2011). As larger amounts accumulate in marine animals, such as fish and shellfish, and in crops, heavy metals can negatively affect ecological diversity and even reach a concentration that can kill off those that consume these organisms (Bosch, O'Neill, Sigge, Kerwath, & Hoffman, 2015).

Zinc is a trace element that plays a major role in different processes in the body, such as in the synthesis of insulin (Lopez-Lopez, Rojas-Sobarzo, Arredondo-Olguin, 2018). A curious point about the presence of zinc in the body is that humans do not naturally have a storage for the metal, which means it is necessary to have a daily intake of zinc (Emsley, 2011). Despite this, larger amounts of zinc can cause harmful damage to the environment and the organisms that live in it. A study by Giardina et al. in 2009 showed that twenty years after a zinc spill, the water and fish living in the stream were still adversely affected.

Due to *Chlorella vulgaris* having similar photosynthetic and metabolic processes to terrestrial plants, it is an ideal microorganism for research involving photosynthesis and plant metabolism. Its high growth rate and tolerance for temperatures from 15 to 40 °C ensures growth in extreme situations, and can battle contamination by other microalgae (Masojidek & Torzillo, 2014). The chlorophyll a fluorescence of this algae is currently being studied as a possible indicator of stress in these organisms (Kumar et. al., 2014; Wu et. al., 2012). By analyzing the fluorescence emitted by algae, different stressors and their amount in their environment could be determined.

The onset of industrialization and continuous urbanization have contributed to the increase of zinc levels in aquatic ecosystems (Al Mukaimi et al., 2018). This not only affects the organisms living in these environments, but also the ecosystem and humans who depend on the resources from the affected areas. Because of the danger these metals pose, it is important that significant levels of and zinc are detected as soon as possible.

Through this study, efficient and non-invasive methods of detecting heavy metals, specifically zinc, could be introduced. It is essential that such methods are studied for the possibility of the use of *C. vulgaris* as a biological indicator for high levels of certain toxic substances in an aquatic environment.

Methodology

Culture of *Chlorella vulgaris*

The *Chlorella vulgaris* algae stored in the school research laboratory was revived by first removing the culture medium the algae initially lived in. Then, the *C. vulgaris* was placed in a long-necked glass bottle with 750 mL of fresh BG-11 culture medium (Bartosh & Banks, 2007). The bottle was continuously illuminated only by a white fluorescent bulb. A polyvinyl chloride (PVC) tube was connected to an air pump and was placed inside the culture to aerate it. To ensure that the air flowed from the middle of the culture for uniform distribution, a balloon stick was connected to the PVC tube and put into the culture. Two holes were made in the cap of the bottle: one to allow the tube into the culture, and the other to allow the flow of air while minimizing contamination (Oukarroum, Bras, Perreault, & Popovic, 2012). The mother culture was transferred into a six liter plastic bottle once it was an intense green color. Three subcultures were then made from this mother cultured, as adapted from “Culturing Algae” by James (2012). All four cultures followed the same aeration method. In addition, the mother culture and the subcultures were refilled with BG-11 culture medium when needed, as the culture medium had the tendency to evaporate.

Preparation of the heavy metal solutions

Five erlenmeyer flasks were sterilized with an autoclave. A 100 mgL solution of $ZnNO_3$ was first created which would later be divided in making the heavy metal solutions of 5 ppm, 10 ppm, 50 ppm, and 100 ppm (Kumar,

Han, Choo, Kong, & Han, 2009). This was done by placing 0.035 g of $ZnNO_3$ in a 500-mL erlenmeyer flask, and 350 mL of distilled water was added. For the 5 mgL solution, 7.5 mL of the 100 mgL solution was measured using a graduated cylinder and distilled water was added until there was 150 mL of solution. This was then added to an Erlenmeyer flask and swirled to evenly mix the solution. The same procedure was done for the 10 mgL and 50 mgL solutions, with 15 mL and 75 mL of the 100 mgL solution, respectively.

Exposure of *C. vulgaris* to $ZnNO_3$

Fifteen 250-mL Florence flasks were filled with 150-mL of algae. At random, 50 mL of each concentration was transferred to each of the set-ups, repeated by having 3 set-ups for each concentration, 5ppm, 10 ppm, 50 ppm, and 100 ppm, of $ZnNO_3$. The positive control set-ups contained just the algae and the culture medium. The flasks were continuously illuminated by a fluorescent bulb for 14 days.

Chlorophyll a fluorescence imaging of algae

Fifteen 1.5 mL samples of *C. vulgaris* were collected and were centrifuged at 5000 rpm for 15 minutes with a temperature of 25°C to separate the algae from the culture medium, which was then decanted (Bagjuz, 2002; Converti, et al, 2009). For every sample, 15 µL of the separated algae was wet mounted onto glass slides, covered with a cover slip, and then sealed with clear nail polish to prevent the samples from leaking out. Samples were chosen at random using an unbiased random number generator. The algae was dark adapted for five minutes, before being observed under a fluorescence microscope with the excitation wavelength set at 630 nm - 640 nm to record the maximal fluorescence (F_m) (Šetlíková, Šetlík, Küpper, Kasalický, & Prášil, 2005; Kumar et al., 2009). The samples were then exposed to light for 90 seconds and observed under the same excitation wavelength to record the minimal fluorescence (F_o). For the F_m and F_o values, the maximum, minimum, and mean values of the fluorescence of the algae were recorded. A microscopy camera was used to to take pictures of the samples for analysis with the software ImageJ. This whole procedure was done on days 0, 4, 7, and 12 of the exposure of the algae set-ups to the heavy metal solutions.

Analysis of data

The software ImageJ was used to analyze the chlorophyll a fluorescence yield; namely, the maximum fluorescence of the algal samples during maximal fluorescence (F_m) and minimal fluorescence (F_0). The Shapiro-Wilk test was used to check for normality. Since the data was found to be normally distributed, the two-way repeated measures ANOVA was used to compare the results of the algae samples with a p-value of $p < 0.05$. In addition, since the results were statistically significant, the Tukey test was used to determine which of the groups were statistically different.

Results

Results of Two-way Repeated Measures ANOVA

Over the course of twelve days, the chlorophyll a fluorescence of the *C. vulgaris* cells exposed to the different concentrations of zinc decreased over time. The tests, however, show that only the highest concentration of zinc showed a significant difference on the last day of the experiment for minimal (F_o), maximal (F_m) fluorescence, and maximum quantum yield.

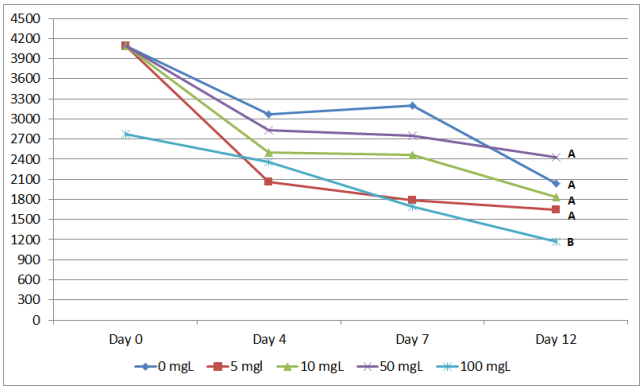

Figure 1. Minimal fluorescence of *C. vulgaris* after different periods of exposure to zinc.

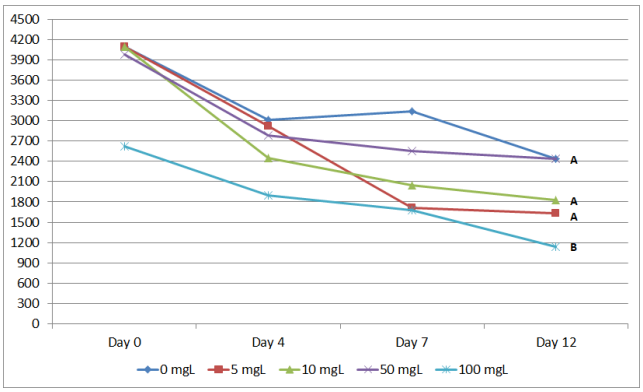

Figure 2. Maximal fluorescence of *C. vulgaris* after different periods of exposure to zinc.

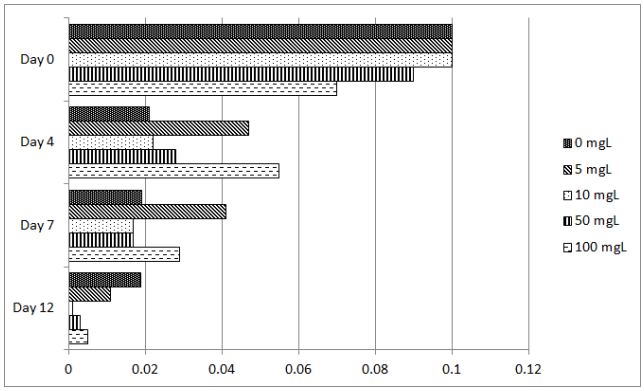

Figure 3. Maximum quantum yield of *C. vulgaris* after different periods of exposure to zinc.

Table 1

P values of the maximal fluorescence, minimal fluorescence, and maximum quantum yield.

Parameter	P value
F_m	0.003
F_0	< 0.001
Maximum quantum yield	< 0.001

Results of Tukey Post-Hoc Test

In the Tukey test, a significant difference in the algae exposed to the zinc concentration of 100 ppm was observed for F_m and F_0.

The same trend can be observed from the p-values from the Tukey test for the F_0 and maximum quantum yield of *C. vulgaris*.

Discussion

These findings could be attributed to the low values of 100 ppm recorded in the parameters measured in this concentration, which could suggest that 100 ppm of zinc is toxic for the algae. This is further supported by the calculated EC_{50}, 75.70 ppm, which suggests that at this concentration, 50% of the *Chlorella* cells were inhibited by the zinc metal, and that concentrations lower than this cannot inhibit growth. Moreover, the concentrations below EC_{50} might even serve as a micronutrient for *C. vulgaris*, as zinc is present in trace amounts in the BG-11 culture medium used for culturing the algae (Bartosh & Banks, 2007). The decrease in F_v/F_m over the twelve days suggest, however, that prolonged exposure to such levels may be detrimental to the algae.

Summary and Conclusion

Chlorella vulgaris was shown to have a weaker fluorescence yield as zinc concentration increased and as the algae was exposed for longer; moreover, the highest concentration of 100 ppm was shown to significantly affect the algae's fluorescence. The significantly low values at 100ppm and the calculated EC_{50} of 75.70 ppm indicate that *C. vulgaris* is indeed a viable indicator for zinc detection at this and higher concentrations of zinc.

The exposure of the algae to the heavy metals was in a ratio of 3 : 1, specifically 150 mL of the algae to 50 mL of the heavy metal solution. Should this study be replicated, the ratio of 1 : 1 could be used, or the concentrations of the heavy metal solutions could be altered. In addition, *Chlorella vulgaris* may be tested on other heavy metals, such as methylmercury, arsenic, and nickel to compare and contrast the sensitivity of the algae to the other metals. Other species of algae may also be tested as a biomarker for zinc to also compare how these algae react to the metal.

Acknowledgement

We thank our research adviser, Mr. Mc Jervis S. Villaruel, and our research teacher, Mrs. Donna Salve C. Hipolito, for advising and guiding us through this project. We also thank all the teachers and adults who helped us during the experimentation and culturing processes. We also thank our school for providing the funding needed to use the necessary equipment for examining the *C. vulgaris* samples. Lastly, we thank the Multidimensional Imaging Center of the University of the Philippines - National Institute of Molecular Biology and Biotechnology for allowing us to use their equipment and facilities for examining the *C. vulgaris* samples.

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【評語】180014

Heavy metals contamination in water bodies is a major concern for public health and environmental safety. This study provides a feasible and quick solution for detecting the zinc and nickel concentrations in natural waters. It is a good research topic. The authors took the chlorophyll of a microalgae (Chlorella vulgaris) as the indicator for zinc and nickel concentrations and observed the sensitivity and performance of C.Vulgaris to the different concentration solutions of heavy metal solutions and proved their study is viable in detecting the high concentrations heavy metals. Their study method is sound but can be improved for a better efficiency in terms of detection days and lab solution preparation. The limitation of concentration detection for zinc (76 ppm) and nickel (723 ppm) can be refined in a future pursue. There's a lot of literature on this type of research, this study should be compared with them and highlight their strengths.