The quantification of the correlation between water nitrogen level and the phosphorus uptake of *Chlorella sorokiniana* (freshwater green alga) in simulated nutrient-contaminated freshwater

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Abstract

Eutrophication is a phenomenon wherein there is an oversaturation of nutrients, principally phosphorus and its partner nutrient nitrogen, in an ecosystem. It induces algal blooms which causes water anoxia and overall water quality deterioration. This has become an issue of increasing concern, especially in agricultural countries. Biological removal methods have proven to be cost-effective and efficient against this phenomenon, specifically microalgal bioremediation. Though microalgae have proven to remediate nitrogen and phosphorus, studies that investigate the effect of the presence of nitrogen on phosphorus removal are uncommon. In this study, the correlation between water nitrogen level and the phosphorus uptake of *Chlorella sorokiniana*, a locally available algae species, was investigated in simulated nutrient-contaminated freshwater. Batch cultures were exposed to treatments of 0, 5, 10, and 15 mg/L of nitrogen and a uniform amount of phosphorus for a period of 11 days, after which the samples were analyzed using UV-vis spectrophotometry. The results showed that a statistically significant positive correlation exists between water nitrogen level and percentage phosphorus removed.

Keywords: *Chlorella sorokiniana, eutrophication, limiting nutrients, nitrogen, phosphorus*

Introduction. Excess nutrient inputs from agricultural wastelands cause eutrophication which refers to the over-enrichment of lakes with limiting nutrients such as phosphorus (P) and nitrogen (N) [1,2,3]. Accumulating nutrients cause the overgrowth of algae and plants limiting sunlight and oxygen in water. It causes the aquatic environment to become oxygen-deficient [2], and negatively affects other oxygen-dependent aquatic species [4]. Widespread eutrophication due to nutrient input has become an increasing concern worldwide, and this is linked to increasing levels of industrial, urban, and agricultural activities such as land conversion and fertilizer use. It can be attributed as well to the increase in human population, demand for food, and nitrogen deposition in the recent years [1,4].

A wide array of treatments and technology for controlling eutrophication exists. However, biological remediation (bioremediation) methods, which use the uptake mechanisms of living organisms to consume and break down environmental contaminants such as nutrients, are favored over chemical methods of remediation because they are more cost-effective, and they produce less toxic waste and harmful by-products [4]. In this context, uptake refers to the movement or transfer of environmental contaminants into the tissues of biological organisms.

The usage of microalgae for bioremediation has become popular in recent research due to established findings proving their effectivity in remediating various types of contaminants, particularly phosphorus and nitrogen [5]. Biomass from microalgal bioremediation may also be used in post-experimental applications [6].

Chlorella sorokiniana, a locally available species of microalgae, has been proven to be able to remove organic pollutants [6]. It also has applications in the field of aquaculture such as its usage as fishmeal [7]. The significance of this species supports the need to further study it. Learning about its nutrient uptake mechanisms will give substantial information on the removal efficiency of certain nutrients. It can serve as beneficial information for the application of microalgal treatments for eutrophicated waters as well.

In the mitigation of eutrophication, nutrient control and reduction are essential. However, the control of multiple nutrients can be complicated by the effects of the presence of other nutrients impacting the uptake of another [1,2,3]. Nitrogen and phosphorus, as limiting nutrients that cause eutrophication, have been hypothesized to have a correlation that explains how they affect each other's uptake [1]. Along with this, the possibility of having various factors as to why nitrogen and phosphorus

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could either hamper or assist the removal of each other exists [1,3,8].

Phosphorus removal characteristically has a high organic loading rate (OLR), which is a measure of the quantity of substrate or contaminant entering the system per unit time, and high sludge retention time (SRT), which is the average time a contaminant is contained in a system. Meanwhile, the removal of nitrogen possesses a low OLR and a high SRT. From those characteristics, a negative correlation can be expected between water nitrogen content and phosphorus uptake. Several studies, however, show that a positive relationship may also exist, more specifically an increase in nitrogen input may increase phosphorus uptake via stimulation of microalgal growth and metabolism rates and vice versa [9]. This shows that the relationship between nitrogen and phosphorus may be variable depending on the dominant effect of nutrient presence on uptake.

Though both nutrients contribute to eutrophication, it is suggested to focus on the removal of phosphorus rather than nitrogen. This is because nitrogen remediation is a more delicate process, susceptible to triggering nitrogen-fixing cyanobacterial blooms that result in adverse effects in the ecosystem and decreasing the activity of phosphorus removal in eutrophicated waters when unregulated. It also involves a prolonged timeframe for denitrification and nitrification. Thus, the cost is relatively high. Furthermore, phosphorus is regarded as a critical limiting factor in lakes, making its removal more costly but more beneficial to the environment on a long-term basis [3,4].

Studies investigating the individual remediation efficiency of phosphorus along with nitrogen are prevalent in recent research. However, studies on the remediation of both in relation to one another, specifically in the freshwater setting, are rare. There is a significant lack of research involving the relationship of nitrogen levels and the phytoremediation efficiency of phosphorus, especially in microalgal and water treatment studies. With this lack of information, this study sought to address the knowledge gap by conducting a study that aimed to describe and evaluate the correlation between water nitrogen content and phosphorus uptake of *Chlorella sorokiniana* in nutrient-contaminated freshwater in different levels of nitrogen. It specifically aimed to:

(i) measure the percentage of phosphorus removed by *Chlorella sorokiniana* in different levels of nitrogen using ultraviolet-visible (UV-Vis) spectrophotometry through the ascorbic acid method; and

(ii) quantify the correlation between the concentration of nitrogen input in the water and the percentage of phosphorus removed through Pearson R Correlation and Linear Regression.

Methods. The utilized algae culture was obtained, then subjected to microalgal cell counting. The four utilized treatment solutions were then prepared. After this, the replicate samples per treatment were prepared, then inoculated with algal culture. After an exposure period of 11 days based on the study of Patel [6], the samples were analyzed through UV-vis spectrophotometry through the ascorbic acid method, and absorbances were read at 880 nm. Obtained data was then ran through statistical analysis using Pearson R Correlation and Linear Regression.

Procurement of Microalgal Culture. A culture of *Chlorella sorokiniana* was purchased from SEAFDEC, Tigbauan, Iloilo. The culture was sealed in a plastic bottle for transport and was then stored in a refrigerator at 3°C prior to usage.

Counting of Microalgal Cells via Hemocytometer. Ten milliliters (10 mL) of the algae culture was pipetted into a test tube. It was then agitated in a vortex mixer for five seconds. Ten microliters (10 µL) of the culture was pipetted into slot A of the hemocytometer. Algae colonies in the five smaller squares of the hemocytometer's central square were counted. Colonies that overlapped between squares were not counted. The concentration of algae per milliliter was computed based on the number of microalgal cells per microliter (µL).

Preparation of Treatment Solutions. Four liters (4 L) of distilled water was measured and poured into a clean plastic container. After this, 57.3 mg of potassium dihydrogen phosphate (KH_2PO_4) was measured and mixed into the water via agitation to create the control (base) solution. One liter (1 L) of the KH_2PO_4 solution was then measured for each of the four treatments using a graduated cylinder and transferred into labelled beakers. From the separated solution, 180 mL was measured using a graduated cylinder and was transferred into each of the five Erlenmeyer flasks designated to hold the replicates of the control setup with the treatment containing 0 mg of nitrogen. The remaining 350 mL of the solution was stored inside a beaker. This process was then repeated for the three other treatments wherein 5 mg, 10 mg, and 15 mg of nitrogen as potassium nitrate (KNO_3) was added respectively to the one liter of base solution for each treatment.

Preparation of Batch Cultures. Twenty milliliters (20 mL) of *Chlorella sorokiniana* culture cultured in Conway medium was inoculated into each Erlenmeyer flask. The flasks were linearly arranged in a ventilated, isolated room. The cultures were subjected to light exposure with a 20-watt fluorescent lights distance of 40.64 cm (16 in) away from the flasks on a light/dark regimen with a 14/10 h simulation of the day and night light exposure cycle [6]. After submerging the connected tubing into the filled flasks, the water aerator, set on low, was switched on to induce agitation of the medium by continuously bubbling the air in the flasks. These conditions were maintained for the entire duration of the observation period.

Observation Period. The setup was monitored for a span of nine days. The lights were switched on and off at 6:26 AM and 8:26 PM, respectively. This simulated a 14/10-hour day and night cycle.

Ascorbic Acid Method. Fifteen milliliters (15 mL) of 36 N concentrated sulfuric acid (H_2SO_4) was diluted to 108 mL to form 5 N sulfuric acid.

Potassium antimonyl tartrate ($K_2Sb_2(C_4H_2O_6)_2$) solution was made through dissolving 1.37 g of the compound in 400 mL of distilled water in a beaker. Twenty grams (20 g) of ammonium molybdate ($(NH_4)_2MoO_4$) was also dissolved in 500 mL of distilled water. An ascorbic acid solution was prepared through mixing 1.76 g of ascorbic acid with 100 mL of distilled water in another beaker. A combined reagent was then made through mixing 50 mL of 5 N sulfuric acid, 5 mL of potassium antimonyl tartrate solution, 15 mL of the ammonium molybdate solution and 30 mL of ascorbic acid solution, for a total of 100 mL of combined reagent that was used for the chemical analysis of the samples [10].

From each sample, 50 mL of the solution was pipetted into an Erlenmeyer flask. One drop of phenolphthalein indicator was added. If a red color developed, 5 N H_2SO_4 was added drop by drop to discharge the color. Eight milliliters (8 mL) of the combined reagent was added to the samples and mixed thoroughly. This process was repeated for each individual replicate for all treatments.

UV-vis Spectrophotometry. After the 20-minute exposure to the combined reagent, the absorbance of each sample was measured at 880 nm, using distilled water as a blank solution for reference. After the individual analysis of the samples per replicate, the absorbance of the Conway medium content in the samples was analyzed and then subtracted from the sample absorbances, providing the values for the phosphorus remaining in the samples, disregarding the phosphorus content of that of the Conway medium.

$$A_{actual} = A_{sample} - A_{Conway}$$

Where: A_{sample} *= absorbance of sample*
A_{Conway} *= absorbance of Conway medium*
A_{actual} *= absorbance of sample disregarding Conway*

Computation of Phosphorus Concentration. The Beer-Lambert Law was used to compute for the concentration of phosphorus remaining in the samples based on their absorbances as shown below.

$$A_{actual} = \mathcal{E}\, c\, L$$

Where: A_{actual} *= absorbance of sample disregarding Conway*
$\mathcal{E}$ *= molar extinction coefficient*
L *= path length of light*
c *= concentration of chemical*

Data Analysis. The data was analyzed through Pearson R Correlation and Linear Regression analysis using the IBM Statistical Package for the Social Sciences (SPSS®) application.

Safety Procedure. After the conduct of the experiment, the remaining algae culture and glassware were filled with bleach solution and were left for 24 hours as prescribed by standard protocol. After the 24-hour exposure period, the glassware were then washed and rinsed thoroughly with detergent and running water followed by a final rinsing using distilled water. The excess solutions from chemical analysis were poured into a container labeled accordingly to be turned over to the Science Research Assistant (SRA) of the school for proper storage and disposal. The glassware that were used for culturing and UV-Vis analysis were subjected to autoclaving to eliminate any microorganisms that may still be present even after the conduct of the experiment.

Results and Discussion. Shown in Table 1 is the mean percentage ± standard deviation of phosphorus removed by the replicates exposed to each nitrogen level treatment. Percentage values were determined through calculation of the change in concentration over the initial concentration of phosphorus in the solution.

Table 1. Mean ± standard deviation of the percentages of phosphorus removed by the algae in different levels of nitrogen.

Nitrogen Level (mg/L)	Percent Removed (%)
0	90.40 ± 2.497
5	91.96 ± 0.946
10	93.26 ± 0.606
15	93.64 ± 0.861

The average removal percentages were seen to increase in treatments of higher concentrations of nitrogen input. This is illustrated in Figure 1 below.

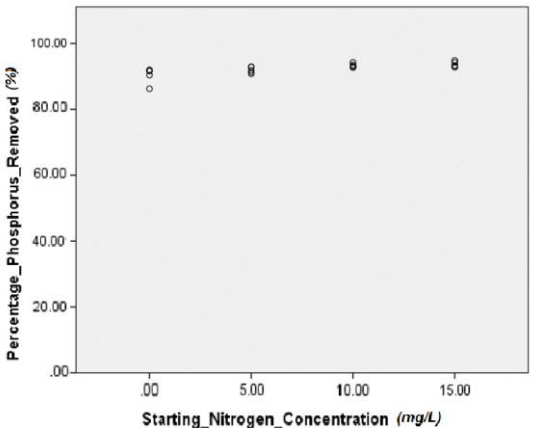

Figure 1. Scatterplot of phosphorus removed from samples for each nitrogen level.

The trend exhibited indicated a positive relationship between the involved variables.

The Pearson R correlation analysis was conducted to determine: (1) the strength of the linear correlation between water nitrogen content and phosphorus uptake, and (2) the type of correlation that exists between the variables, which may be either positive or negative.

The Pearson R correlation was run at a 95% confidence interval. The p-value derived from the data was 0.001, a value less than the significance level (0.01), indicating a significance in the results. The resultant coefficient from the test is a positive value of 0.694.

A value of 0.694 in the Pearson R correlation test indicates that a positive correlation exists between nitrogen input and phosphorus removal. This confirms that in the case of this study, the increase of one variable leads to an increase of another. Nitrogen input and phosphorus removal are found to be directly proportional.

The Linear Regression was used to represent the correlation between water nitrogen content and phosphorus uptake as a graph or linear function which shows their relationship as variables. A significant difference between the mean value of sample groups was first evaluated in order to proceed with the linear regression analysis.

A linear equation where x is the nitrogen level and y is the amount of phosphorus removed was derived from the linear regression analysis of the data conducted at a 95% confidence interval (p=0.05). This equation is shown below.

$$y = 0.2213x + 90.66$$

The equation mathematically models the relationship between water nitrogen level and phosphorus uptake of *Chlorella sorokiniana.* It can be observed that a positive correlation exists between nitrogen level and the amount of phosphorus removed as illustrated by Figure 2.

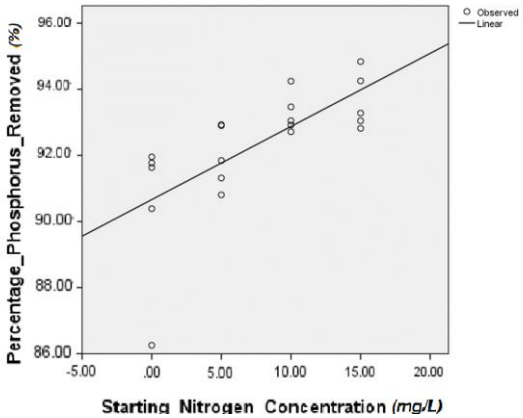

Figure 2. Linear representation of the relationship between water nitrogen level and phosphorus removed.

The linear equation, having a positive slope, is an indicator of a positive relationship between the variables and shows that as the nitrogen level increases, so does the amount of phosphorus removed from the water.

The relationship and effects of water nitrogen levels on the uptake of phosphorus are dependent on the dominant mechanism of their uptakes relative to each other's presence, which may vary between species. The mechanisms behind the removal of phosphorus and nitrogen may be attributed to their uptake through microalgal biological processes such as nitrification and denitrification, and stripping phenomena such as ammonia volatilization and phosphorus precipitation [5]. Ammonia volatilization is the conversion of ammonium into ammonia gas at high pH levels [11]. Biological phosphorus removal is done by phosphorus-accumulating organisms (PAOs) like algae through the absorption dissolved phosphorus in wastewater and storing them as granules (precipitate) within the cells [12]. These phenomena are induced by photosynthetic microalgal growth alongside the adsorption on the cell surface of the algae. Aside from uptake, adsorption to the cell surface is among the most significant processes in the removal of phosphate by algae. Phosphate removal and biomass production are known to positively correlate [5]. Despite this, various studies show differing hypothetical understanding as to what the effect of remediating both phosphorus and nitrogen is.

One point of basis for prediction of the possible dominant relationship between the uptake of the two nutrients of an algal species is the differences in the SRT and the OLR required for the efficient bioremediation of nitrogen and phosphorus. Nitrogen, requiring a low OLR, interferes with the efficiency of the loading of phosphorus, whose uptake requires a higher, more rapid rate of entry into the algal cells. On the other hand, phosphorus has a characteristically low SRT, which means that it is not retained in the algal cells for a prolonged period in its remediation. Nitrogen, having a high SRT, has a tendency to interfere with the efficiency of the outflow of P from the algal cell as it is retained longer, and has a less rapid rate of outflow itself [3]. Due to the interplay of these factors, a negative trend and correlation between phosphorus uptake and nitrogen concentration may exist.

However, studies such as those by Graciano [8], Bennett [9], and Xin et al. [13] have shown that positive trend and correlation between these two variables may be observed. Bennett [9] discusses that increasing the presence of phosphorus and nitrogen, nutrients essential for plant and algae growth, increases the rate of growth of an organism, leading to an increase of nutrient demand to sustain its growth, and ultimately resulting to an overall increased rate of the uptake of the two nutrients.

In the case of the results of this study, the statistical analysis of the data shows that a positive correlation exists between water nitrogen content and phosphorus uptake of *Chlorella sorokiniana* which is aligned with the results of the three aforementioned studies.

Limitations. The scope of the procedures in the study is limited to the usage of laboratory simulated freshwater. The simulation of freshwater medium utilized in the experiment constituted of only four major components: phosphorus (in the form of KH_2PO_4), nitrogen (in the form of KNO_3), Conway medium which is a nutrient replete culturing medium, algae, and distilled water. Other constituents linked to natural freshwater such as additional nutrient input, pH, and temperature are not included in the scope of the study and procedures.

Conclusion. It was determined that the replicates exposed to the treatment with the highest N content (15 mg/L) exhibited the highest percentage of phosphorus removal at 93.64%. In contrast, the replicates exposed to the treatment with the lowest N content (0 mg/L) exhibited the lowest percentage of phosphorus removal at 90.40%. A positive

relationship between nitrogen content and phosphorus removal was observed to exist represented by the linear equation, $y = 0.2213x + 90.66$. This may have been caused by accelerated microalgal growth stimulated by the presence of increasing concentrations of growth-stimulating nutrients.

Recommendations. It is recommended to implement measures to minimize inaccuracies due to human error such as the usage of a timed electrical switch for turning the lights on and off for the 14/10 day and night cycle simulation. It is also recommended for the procedures of the study to be conducted using naturally sourced freshwater from local sources. Lastly, the conduct of a study of similar nature involving the testing of more nitrogen levels is recommended to produce a more accurate representation of the linear relationship between the nitrogen input and phosphorus uptake.

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