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## Examination of Nitrogen to Phosphorus Ratio in Nutrient Removal from Wastewater through *Chlorella vulgaris*

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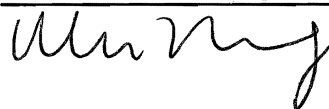
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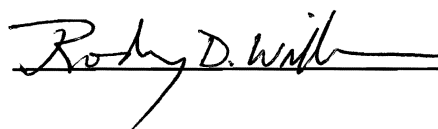
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Examination of Nitrogen to Phosphorus Ratio in Nutrient Removal from  
Wastewater through *Chlorella vulgaris*

An Undergraduate Honors College Thesis

in the

Department of Civil Engineering  
College of Engineering  
University of Arkansas  
Fayetteville, AR

by

Courtney Leah Hill

Examination of Nitrogen to Phosphorus Ratio in Nutrient Removal from Wastewater  
through *Chlorella vulgaris*

**Courtney Hill**

**Advisor: Wen Zhang**

**Abstract**

A need for phosphorus removal is becoming increasingly evident as some wastewater treatment plants struggle to meet wastewater effluent nutrient requirements. High nutrient levels in wastewater effluents have also caused ongoing tension between the state of Oklahoma and Arkansas regarding the pollution of the Illinois River. This research seeks to establish the relationship between nitrogen to phosphorus ratio in wastewater and the level of nutrient reduction using *Chlorella vulgaris*. Seed cultures of *Chlorella vulgaris* were added into solutions with various ratios of nitrate to phosphate representative of treated domestic wastewater and the removal of nitrogen and phosphorus were measured in this study. Results showed phosphate is the limiting factor for *Chlorella vulgaris*. The removal of nitrate from phosphate-limited wastewater effluent increased significantly by the growth of *Chlorella vulgaris* when supplemented with phosphate. This suggests that algal nutrient removal might not be suitable as a tertiary treatment for all wastewater effluent. This study will enhance wastewater treatment plants' knowledgebase about further nutrient reduction techniques, and in turn, make more informed decisions.

## **Introduction**

### ***Background***

Wastewater treatment technology has advanced over the past few decades. The three major categories of nutrients in domestic wastewater that can harm the environment when left untreated are organic carbon, nitrogen and phosphorus. While the traditional secondary treatment of wastewater (activated sludge system) removes most organic carbon and transforms nitrogen effectively, the phosphorus level in the effluent raises concerns over time due to the lack of sufficient removal.

Phosphorous is a limiting nutrient for algal growth, which can lead to eutrophication when discharged into a water body from wastewater effluent. The decay of excessive algal biomass in eutrophication depletes the dissolved oxygen in the natural water body to where aquatic life cannot be sustained (Davis, 2010). Phosphorous removal from wastewater can be achieved using chemical and biological approaches. Through chemical processes, phosphorous is removed through the use of calcium, aluminum, or iron salts to form a precipitate in the wastewater and then removed (Davis, 2010). Though effective, the non-reusable chemical sludge can only be disposed in landfill, which increases the cost significantly. In biological processes, phosphorus can be removed by phosphate accumulating organisms (PAOs) in activated sludge. However, due to the nature of PAOs, an anaerobic phase is required and most treatment plants need to go through major system updates to modify their existing facilities. In addition, the phosphorus level in the effluent can only be brought down to a certain level (approximately 0.1-1 mg/L in Westside Wastewater Treatment Facility, Fayetteville AR) when operating at optimal conditions.

Currently, the State of Arkansas follows the NPDES permit, which allows 1 mg/L of P discharged into the nearby water bodies. However, for years there has been pressure on Arkansas to regulate phosphorous due to the high amount discharged into the Illinois River from poultry production. In 2002, Oklahoma passed a regulation that total phosphorus concentration in scenic rivers cannot exceed 0.037 mg/L due to the pollution of the Illinois River (Soerens, 2003). In the foreseeable future, there will be stringent regulations on phosphorus discharge in Arkansas as well, which creates an urgent need for wastewater facilities to prepare for effective phosphorus removal.

One possibility of tertiary wastewater treatment for sufficient phosphorus removal is the use of microalgae (Wang et al., 2012). Nutrient removal in wastewater through microalgae has multiple benefits. They not only utilize these nutrients for biomass growth, but also sequester carbon dioxide and produce oxygen gas to release into the environment. The reduction of carbon dioxide will also decrease the carbon footprint of the wastewater treatment plant if successfully applied.

Another advantage for using microalgae for nutrient removal is that the algal biomass produced can be used as a potential as a feedstock for biofuel production through either biomass or lipid extraction (Wang et al., 2012). As fossil fuels are recognized as a diminishing energy resource, attention towards investigating renewable energy sources has increased. Current available biofuels are bioethanol that come from crops such as soybeans and oilseed rape (Demirbas, 2009). Because these biofuels come from crops, they not only use resources that are used as food but also land that could be used to grow food. As the world's population continues to grow exponentially, these crops and the land used to grow them become increasingly vital (Demirbas, 2009). *Chlorella vulgaris* and

other algal biomass used to remove nitrogen and phosphorus have the potential to replace these much needed agriculture related resources as a source for biofuel. It has been investigated that microalgae can produce more biomass than common biofuel crops when comparing land area required for growth (Dismukes et al., 2008). *Chlorella vulgaris* was chosen in this study to further reduce nutrients from wastewater.

When comparing microalgae and crops as source for biofuel, microalgae also holds an advantage in that it can be grown with minimal freshwater while current crops grown for biofuels have a higher demand for water (Rodolfi et al., 2009). As a result, microalgae could be grown near the sea and utilize seawater for growth, in turn, providing for more freshwater to be used for drinking and agriculture.

Both the potential for biofuel production and the elimination of costly chemical treatment that produces non-reusable chemical sludge appeal to the economic and environmental components of the Brundtland Commission's "triple bottom line" (economically, environmentally, and socially sustainable) (Brundtland Commission, 1987).

### ***Previous Work***

Previous work regarding microalgae in wastewater treatment has resulted in various, sometimes contradicting, conclusions. In 2012, the Aquatic Ecohealth group at the Institute of Urban Environment in China grew *Chlorella* in both influent and effluent that was diluted to four different levels. It was found that the slowest growth in *Chlorella* was observed in wastewater influent and wastewater effluent that were not diluted, providing evidence that the algal growth was dependent on the initial nutrient content of the wastewater. It was also observed that the high concentration of nutrients in the

wastewater flasks that were not diluted actually inhibited algal growth (Wang et al., 2012). In 1993, a similar experiment was conducted that cultivated *Chlorella vulgaris* in a medium containing varied concentrations of  $\text{NO}_3^-$  that found no growth was inhibited for any of the nitrate concentration but that nitrate concentration did have an effect on biomass production (Jeanfils et al., 1993). It was concluded that *Chlorella vulgaris* could survive in high nitrate concentrations although a certain level of nitrate concentration could inhibit growth, similar to the 2012 study (Jeanfils et al., 1993).

In 2009, a group in Minnesota inoculated *Chlorella vulgaris* in wastewater and measured algal growth and nutrient content, also finding a strong relationship between nutrient levels (Wang, 2010). Although this study supported the 1993 and 2012 studies' conclusions that wastewater nutrient content affects algal growth, it found that higher levels of nitrogen and phosphorus were found to assist rather than deter algal growth, contradictory to both studies previously discussed. The 2009 study also found high phosphorus limitation in effluent correlated with limited algal growth, in turn, efficient nutrient removal, also contradicting the 1993 and 2012 studies.

Other studies have even shown results that contradict both the 2009 and 2012 study. The University of Minnesota investigated the growth and nutrient removal of *Chlorella vulgaris* on dilutions of pretreated swine manure (Bing, 2009). *Chlorella vulgaris* survived in all culture media and ammonium and inorganic nitrogen were almost completely removed in five days. The removal of phosphorus showed no relation to algal growth, unlike the results from both the 2009 and 2012 study (Bing, 2009).

Additional studies have investigated the effects of protozoa and cyanobacteria during nutrient removal and other important factors to consider if *Chlorella vulgaris* is to



be put into widespread use for treating wastewater. In 1994, an experiment investigated *Chlorella vulgaris* inoculated in primary settled sewage and Bristol medium and found the algae growth was less in the collected sewage due to the presence of indigenous bacteria and protozoa (Lau et al., 1994). The results suggest that employing a superconcentrated culture into wastewater treatment would be best, as it would minimize retention time for nutrient removal. A similar study conducted by Tam and Wong reported significantly higher removal in an open system, also suggesting that bacteria and protozoa may influence the efficiency of nutrient removal by algal systems (Tam and Wong, 1989).

These contradicting conclusions about the effects of *Chlorella vulgaris* on nutrient removal, supplemented by the potential impacts of bacteria and protozoa on nutrient removal through algal systems, highlight the need to further investigate this topic. The objective of this study is to investigate *Chlorella vulgaris*' ability to reduce nitrogen and phosphorus in wastewater with various nitrogen to phosphorus ratios. This project seeks to look deeper into the feasibility of algal nutrient removal, giving input that will be helpful in developing a more definitive conclusion in regards to the practicality of utilizing algal systems as a widespread mechanism for nutrient removal.

## **Materials and Methods**

*Chlorella vulgaris* was purchased from UTEX algae center at the University of Texas at Austin (Austin, Texas) and cultivated in an environmental growth chamber at  $25 \pm 1$  °C. The culture was exposed to 4 foot cool-white fluorescent light tubes mounted approximately 10 inches above the culture. During the life of the culture, it was not

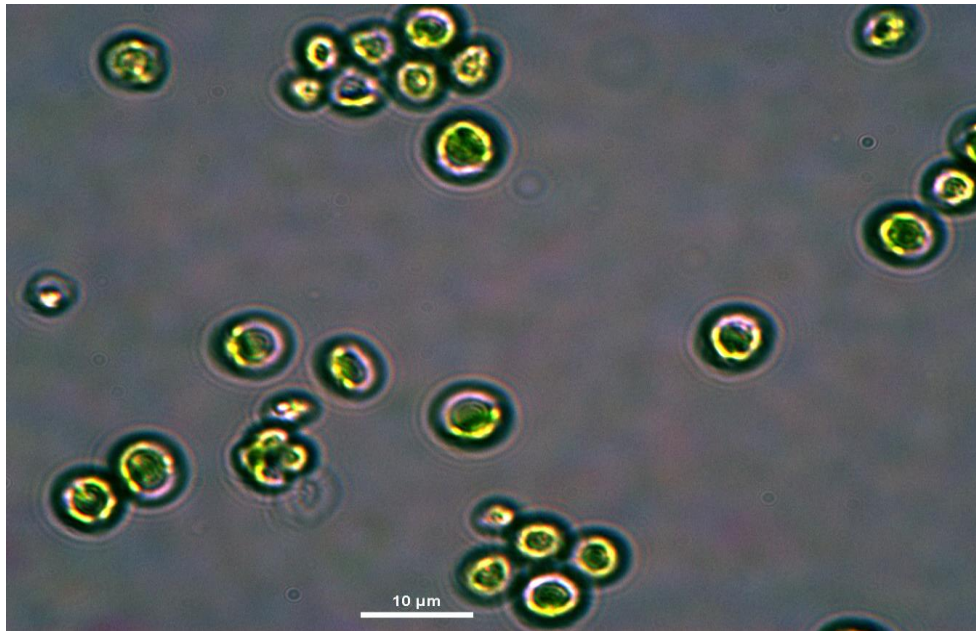
aerated or agitated. The algae was grown in Bristol medium ( $\text{NaNO}_3$  (25 mg/L),  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$  (2.5 mg/L),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (7.5 mg/L),  $\text{K}_2\text{HPO}_4$  (7.5 mg/L),  $\text{KH}_2\text{PO}_4$  (17.5 mg/L),  $\text{NaCl}$  (2.5 mg/L)) for 7 days. An aliquot of the culture suspension was inoculated into fresh medium when needed.

Jars were prepared with one liter of Bristol Medium with various nitrogen to phosphorus ratios in the form of nitrate and phosphate (mg/L) and inoculated with 2 mL of cultivated *Chlorella vulgaris* growing in Bristol medium. Wastewater effluent gathered from the secondary clarifier at the Noland and Westside wastewater treatment plants in northwest Arkansas (Fayetteville, AR) were also inoculated and studied. For a period of eight days, two 8 mL samples were filtered through Whatman<sup>®</sup> glass microfiber filters, Grade 934-AH (1.5  $\mu\text{m}$  retention) contained in advantec polypropylene filter holders using a syringe. Algal density was also checked daily at a 680 nm wavelength on a spectrophotometer. The nutrient concentration (nitrate and phosphate) of each sample was determined on a Metrohm Ion Chromatography System (Metrohm USA, Riverview, Florida). Light microscopy was conducted using a Nikon Eclipse Ni-E 100x microscope (Nikon, Melville, New York).

## **Results and Discussion**

The light microscopy picture of *Chlorella vulgaris* is shown in Figure 1. The diameter of algal cell is between 2 to 6  $\mu\text{m}$ . The growth of *Chlorella vulgaris* in Bristol medium at optimal condition is shown in Figure 2. In original Bristol medium, the nitrate to phosphate ratio is measured at 20:45. When varying the nitrate to phosphate ratios to 30:98, 36:34 and 3:28, *Chlorella vulgaris* followed the same growth pattern: a lag phase

of three days occurred at the beginning of algal inoculation, followed by a two day exponential growth phase, ultimately leading to a plateau limited by the density of *Chlorella vulgaris* in the flask. Results indicate that when phosphate is abundant, different nitrate levels do not have a significant impact on *Chlorella* growth.



Figures 1: Photos of *Chlorella vulgaris* under microscope at 100x magnification.

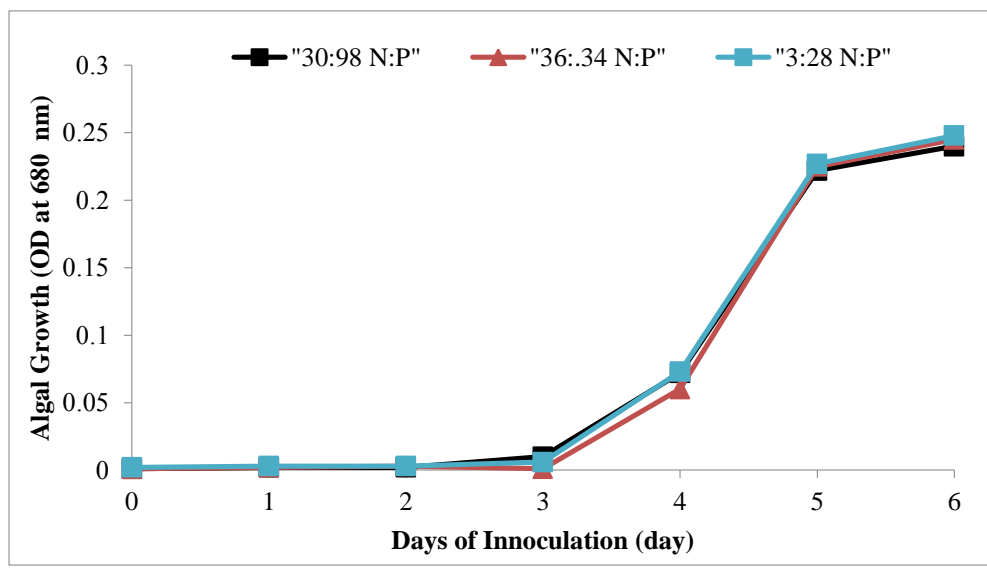


Figure 2: Algal Growth for *Chlorella vulgaris* in Bristol medium with various N:P ratios.

A nutrient reduction analysis was conducted on the Bristol medium (N:P ratio of 20:45) inoculated with *Chlorella vulgaris*. Compared with no change in the control sample (without *Chlorella vulgaris*), the inoculated samples showed an average 99% and 82% percent reduction of nitrate and phosphate, respectively, in five days (Figures 3 and 4). This showed promise for significant algal uptake of both phosphate and nitrate when neither nutrient is limiting.

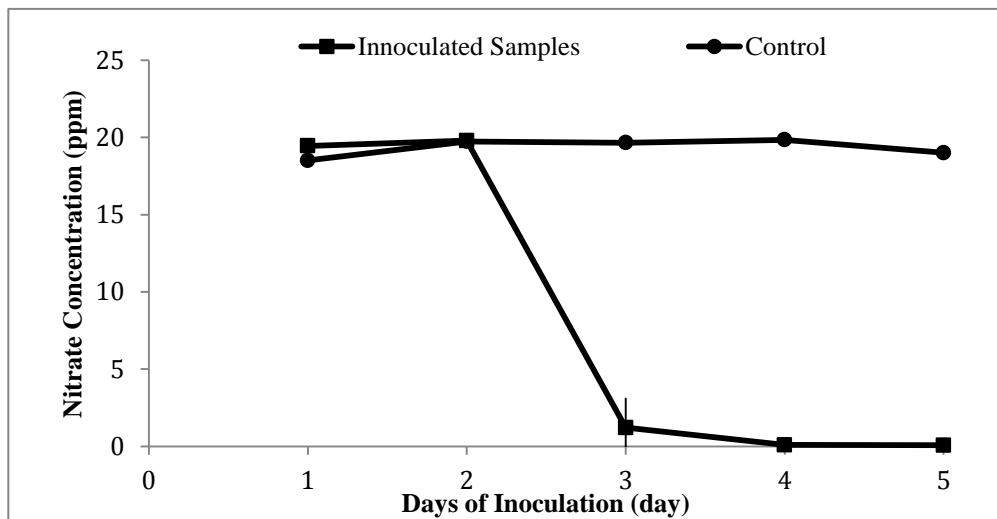


Figure 3: Nitrate reduction of *Chlorella vulgaris* in Bristol medium (N:P 20:45).

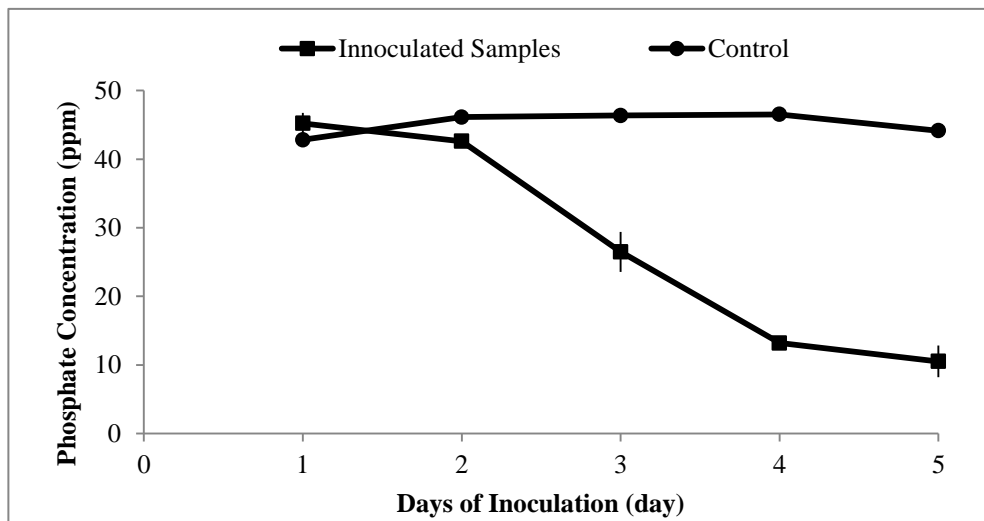


Figure 4: Phosphate reduction of *Chlorella vulgaris* in Bristol medium (N:P 20:45).

To further examine the impact of N:P ratio, Bristol medium with various nitrate to phosphate ratios was prepared and inoculated with *Chlorella*. A summary of the nitrate and phosphate reduction using *Chlorella vulgaris* at each N:P ratio are shown in Figures 5 and 6. Although previous experiments showed sufficient removal of both nitrate and phosphate at a 20:45 N:P ratio, results from these experiments showed significantly less nitrate reduction (0% - 41%) from *Chlorella vulgaris* but consistently high phosphate reduction (62% - 100%). It suggests when phosphate is not the limiting nutrient, the ratios of N:P could affect *Chlorella Vulgaris*' ability to remove these nutrients from wastewater. Results from Bristol medium with N:P ratios at very low concentrations of phosphate were not shown in this study. The planned experiments failed to consider the phosphorus concentration in peptone, one of the ingredients used in Bristol Medium, resulting in no successful experiments with limiting phosphate.

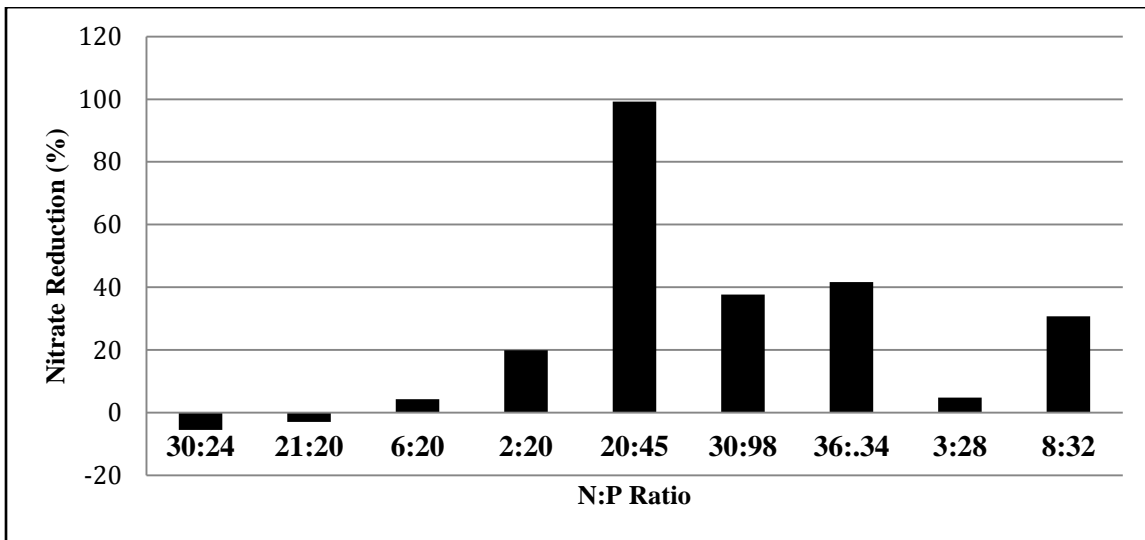


Figure 5: Nitrate reduction of *Chlorella vulgaris* in Bristol medium and wastewater effluent with various N:P ratios.

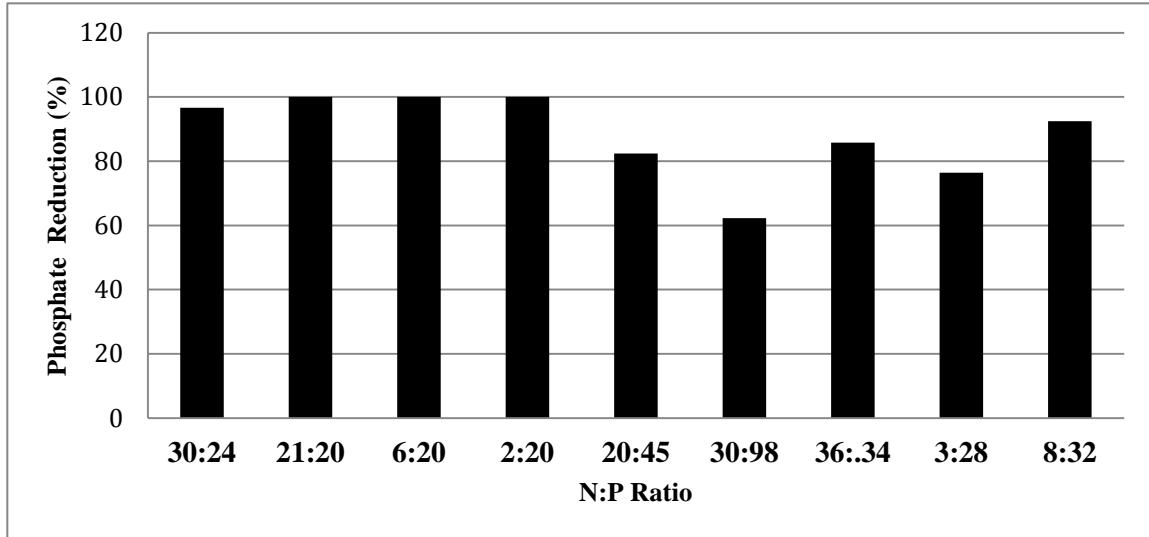


Figure 6: Phosphate reduction of *Chlorella vulgaris* in Bristol medium with various N:P ratios.

With the results from modified Bristol medium, *Chlorella vulgaris* was inoculated in real wastewater from local wastewater treatment plants. Secondary effluent taken from the secondary clarifier was used in this study to minimize the effect of indigenous microorganisms. Nutrient reduction results are shown in Figure 7. In Noland wastewater, although a 34% reduction in nitrate was observed, there is no difference in the control experiment and inoculated sample, suggesting the nutrient was not reduced by *Chlorella*; In Westside wastewater, 90% reduction of nitrate was observed, however, very minimum growth of algae was shown during the experiment period. The effluent from both plants had minimal initial phosphate concentrations (below 0.1 ppm). It is likely the limited or no algal growth was due to the lack of phosphate in these wastewater effluents. It shows that phosphorus is the limiting nutrient for *Chlorella vulgaris*.

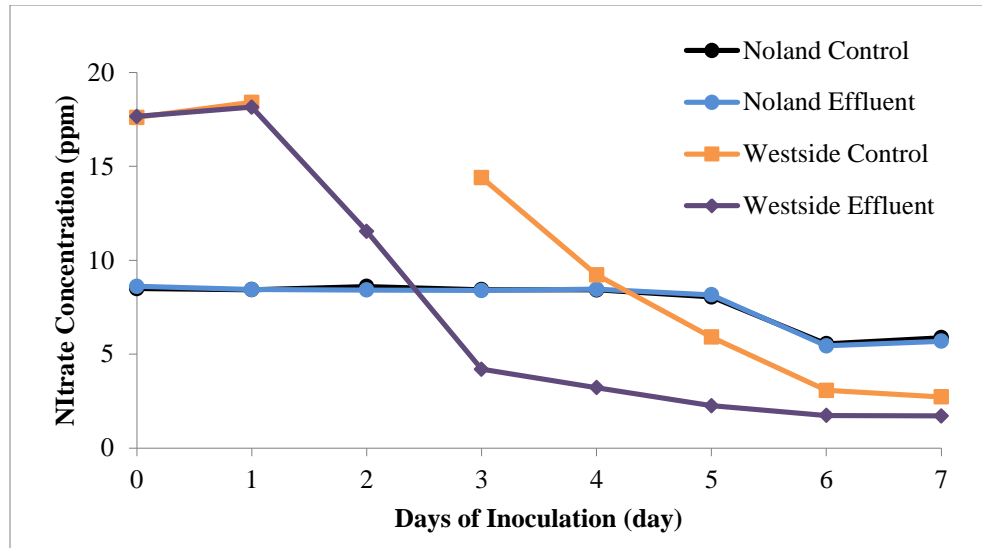


Figure 7: Nitrate reduction of *Chlorella vulgaris* in wastewater effluent from Noland and Westside Wastewater Treatment Plants (Westside Control day 2 data point inaccessible due to ion chromatograph error).

A comparison of nutrient reduction from *Chlorella vulgaris* with a previous study is shown in Table 1. *Chlorella* was grown in wastewater effluent with a N:P ratio of 19:1 that resulted in a 27.5% nitrogen reduction (Wang et al., 2012). This is similar to the Westside plant's phosphate limited effluent (N:P 18:0.1) in this study, however, a 90% removal was observed. This could be due to a number of factors regarding the composition of the wastewater effluent and illustrates the need for further experimentation on this subject. Because both studies yielded variable nitrate reduction, it can be observed that the amount of nitrogen and phosphorus in wastewater can affect the efficiency of nutrient removal using *Chlorella vulgaris*.

Table 1: Nitrogen and phosphorus removal from previous work (Wang et al., 2012)

Total Nitrogen (mg/L)		Total Phosphorus (mg/L)		N:P Ratio	Percent Reduction	
Intital Value	Terminal Value	Intital Value	Terminal Value		Total Nitrogen	Total Phosporus
38.03	15.65	1.83	0.053	38:2	58.8	97.1
29.45	16.81	0.57	0.046	29:0.5	42.9	91.9
19.01	13.79	0.92	0.065	19:1	27.5	92.9
14.73	12.22	0.29	0.05	15:0.5	17.0	82.8

### **Conclusions and Future Work**

This study demonstrated the importance of nitrogen to phosphorus ratio in algal nutrient reduction from wastewater. *Chlorella vulgaris* has the capability of reducing nitrate and phosphate effectively, and phosphate is the limiting nutrients for its growth. Different N:P ratio lead to different levels of nutrient reduction, and phosphate limited environment leads to little or no growth of *Chlorella vulgaris*. This result suggests although *Chlorella vulgaris* can reduce nitrate and phosphate effectively, algal nutrient reduction might not be suitable for all tertiary wastewater treatment, especially the wastewater with limiting concentration of phosphate. Future studies are needed to investigate the role of indigenous microorganisms in wastewater effluent and the synergistic effect between algae and these microorganisms.



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