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NITROGEN AND PHOSPHORUS BIOMASS-KINETIC MODEL FOR

***CHLORELLA VULGARIS* IN A BIOFUEL PRODUCTION SCHEME**

THESIS

William M. Rowley, Major, USMC

AFIT/GES/ENV/10-M04

DEPARTMENT OF THE AIR FORCE
AIR UNIVERSITY

AIR FORCE INSTITUTE OF TECHNOLOGY

Wright-Patterson Air Force Base, Ohio

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Presented to the Faculty

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Air Education and Training Command

In Partial Fulfillment of the Requirements for the

Degree of Master of Science in Environmental Engineering and Science

William M. Rowley, BS

Major, USMC

March 2010

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NITROGEN AND PHOSPHORUS BIOMASS-KINETIC MODEL FOR *CHLORELLA VULGARIS* IN A BIOFUEL PRODUCTION SCHEME

William M. Rowley, BS
Major, USMC

Approved:

______________/signed/__________________	15 Mar 10
Dr. Charles A. Bleckmann (Chairman)	date
______________/signed/__________________	15 Mar 10
Dr. Sukh S. Sidhu (Member) University Dayton Research Institute	date
______________/signed/__________________	15 Mar 10
Dr. Jerome C. Servaites (Member) University Dayton Research Institute	date

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Abstract

Chlorella vulgaris was cultured in microbioreactors using Bold's Basal medium at varying nitrogen and phosphorus concentrations to define nitrogen and phosphorus utilization standards. Nutrient concentration was varied between 137 mg/L to 7.33 mg/L NO3-N and between 55.2 mg/L to11.0 mg/L PO_4-P in five test scenarios. All were grown under a constant photoperiod at 22±2 °C and a mixture of 4 to 10% carbon dioxide/air. Maximum yield and growth rate occurred with the highest initial nitrogen and phosphorus concentrations. A statistically significant difference in biomass was found among all test levels at the end of the eight day growth period. Applying both Liebig's Law of the Minimum and the Blackman Limitation, it was determined that nitrogen was the limiting factor over the range of concentrations tested. Michaelis-Menten biokinetic coefficients (k), the reaction rate constant; the half saturation constant (K_m); and Y_x, the yield coefficients were also determined. To maximize *C. vulgaris* growth initial N concentration values should be 137 mg/L and should not be allowed to fall below 69 mg/L. No equivalent recommendation for P was determined. Yield coefficient calculations suggested that the N:P ratio should be at least 3:1. This study was conducted as a part of the ongoing advanced jet fuel project at the University of Dayton Research Institute and a part of the military objective to reduce the carbon footprint of jet fuel production.

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W. Morgan Rowley

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I. Introduction

On January 1, 2010 the U. S. Environmental Protection agency (EPA) will require large emitters of carbon dioxide to begin collecting greenhouse gas data (USEPA, 2009a). Facilities that emit 25,000 metric tons or more of CO_2 equivalent per year will be required to report greenhouse gas emissions data to EPA annually. This new program will cover approximately 85 percent of the nation's greenhouse gas emissions and apply to roughly 10,000 facilities. The data will require businesses to track their own emissions, compare them to similar facilities, and identify cost effective ways to reduce emissions in the future. This includes any facility producing jet fuel for the military. The EPA has also, under the Energy Independence and Security Act of 2007, become responsible for the regulations that ensure gasoline sold in the United States contains a minimum volume of renewable fuel (USEPA, 2009b). Furthermore, Section 526 of this act states that no Federal agency shall procure synthetic fuel unless the greenhouse gas emissions associated are less than or equal to such emissions from conventional petroleum sources. Other countries are looking for ways to reduce CO_2 emissions to meet the sustainability goals of the Kyoto Protocol (Mata, Martins, & Caetano, 2010).

The Fischer-Tropsch process has been identified as an alternative method of producing a viable aviation fuel to replace JP-8. In 1923, two German researchers, Franz Fischer and Hans Tropsch, discovered a method to convert carbon based materials into petroleum products. This liquid synthetic fuel can be produced from coal, natural gas,

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and any other solid feedstock that contain carbon. These fuels burn cleaner than comparable JP-8. Most importantly, resources that are found in the United States, coal for example, can also produce Fischer-Tropsch derived fuels. In September 2006, the Air Force conducted a successful test of a B-52 Stratofortress using a 50/50 blend of Fischer-Tropsch fuel and JP-8. Although Fischer-Tropsch fuels burn cleaner than JP-8, the Fischer-Tropsch process generates twice the carbon dioxide during manufacture as that of petroleum based fuel (Blackwell, 2007).

Algae come in many forms (species) and are primary producers of organic material in aquatic and marine ecosystems. They are of importance because they produce oxygen and absorb carbon dioxide. They are considered photosynthetic, oxygenic autotrophs, because they use light energy to convert carbon dioxide into oxygen and energy. Depending on species and type of algae, this energy can be in the form of lipids as well as carbohydrates. It is the lipid that can be easily converted into a suitable industrial fuel source. Most of the world's fossil fuel and industrial carbon emissions have little value at best, and will take on large costs in the future both environmentally and monetarily. Growing algae, to not only sequester the carbon but to also provide other possible fuel (Melis & Happe, 2001) and food sources, warrants extensive research and development.

In their review of microalgae for biodiesel production, Mata *et al*. (2010) concluded that "a considerable investment in technological development and technical expertise is still needed ... and correct policies and strategies are still needed." The U.S. National Renewable Energy Laboratory (NREL) concluded in 1996, based on their Roswell studies, that bio-diesel production from algae was "technically feasible" at a

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low-cost, but large scale production still needs much long-term research. In 1987 at the 4th International Meeting of the *Societe pour l'Algologie Appliquee*, Gudin (1988) advised that at the present time hydrocarbons can be produced from algae, but to be used as fuel it would take "a very special environment." He felt that managing a monoculture through seasonal fluctuations and controlling microbial contamination would be difficult. He stressed that a good bioreactor design is based on knowledge of the target algae physiology to provide the "controlled cultivation" that is necessary for economic industrialization.

The algal cultivation unit, be it an open system or closed loop system or something in between, is the key to the cost-effective production. In addition to the algae species selection, Mata *et al*. (2010) summarizes the points that Maxwell and his colleagues made in 1985. The first consideration is the water, its chemistry, and availability. The second is the amount of land needed, who owns it, and the accompanying geological considerations. The climatic conditions of temperature regime, evaporation, and precipitation are the third ones. Lastly, is the ease of access/cost to carbon and mineral nutrients (N and P in particular). This makes the use of sewage effluent and CO_2 sequestration attractive.

Large-scale algal photobioreactors are a technological tool that is suited to sequester the carbon dioxide produced during this process. Furthermore, the resultant algal biomass may be "milked" for oil and possibly reintroduced into the Fischer-Tropsch process. Algal biomass, like other plant biomass, is potentially suitable for conversion to gasoline, biodiesel, ethanol, methane, and hydrogen fuels. Algae vary, depending on species, from 30 to 70% oil content by weight in biomass. This translates to 51,927 to

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121,104 kg biodiesel/ha year. Compare this to corn, which yields 44% seed oil by weight in biomass and 152 kg biodiesel/ha year. *Chlorella vulgaris*, the alga in this study, usually yields between 20 to 50% oil content by weight of dry biomass (Mata, Martins, & Caetano, 2010; Singh & Singh, 2010).

Just as properly fertilizing an agricultural crop or forest can result in enhanced plant growth, the most common effects of increased nitrogen and phosphorous supplies on aquatic ecosystems are increases in the abundance of algae and aquatic plants (Smith, Tilman, & Nekola, 1999). Phosphorous is most often limited in nature because it is effectively bound in sediment (Engblom, 1998). Phosphorous in the form of orthophosphate is generally considered the main limiting nutrient in freshwater aquatic ecosystems: that is, if all the phosphorous is used, autotrophic growth will cease, no matter how much nitrogen is available (Barsanti & Gualtieri, 2006). In nature, nitrogen is not necessarily limiting because bacteria are fixing nitrogen and supplying the algae with a constant nitrogen source. In a photobioreactor the nitrogen and phosphorous must be found in the media in which algae are grown.

"The successful growth of algae is more or less an art and a daily tightrope act with the aim of keeping the necessary prerequisites and various unpredictable events involved in algal mass cultivation in a sort of balance" (Becker, 1994). The development of a best management nitrogen and phosphorus standard for optimal algal growth will be one step in the reduction of the carbon footprint associated with jet fuel production and other carbon producing activities. Algae use in bioreactors designed for carbon sequestration and oil extraction need not be provided with excessive nutrient loading which can be become labor intensive and potentially cost prohibitive. The goal for

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photobioreactor technology is economic efficiency and ease of use. The current objective of this study is to define the nitrogen and phosphorus requirements of cultured *Chlorella vulgaris* in order to efficiently grow the algae in a carbon sequestering scheme with the biomass that can be used to produce biodiesel by answering the following questions:

1. What is the appropriate nitrogen range for optimal growth of *Chlorella vulgaris?*
2. What is the appropriate phosphorus range for optimal growth of *Chlorella vulgaris?*
3. Can growth rate be correlated with N or P- limitation and thus be used as an early indicator of nutrient limitation? Will the correlation be sufficiently strong to suggest usefulness in mid to large scale culture?
4. Can these experimentally obtained ranges function determined in a microbioreactor be applied to growth in large photobioreactors? Are these experimentally obtained ranges within the parameters of available sewage effluent?
5. Using the experimental data, can a distinction be made between optimal growth and satisfactory growth with respect to long term goals of CO_2 sequestration and bio-fuel/jet fuel production?
6. What is the biomass potential of *Chlorella vulgaris* in small scale culture under the nitrogen and phosphorus range limits tested in this study?
7. Does the experimentally determined kinetic nutrient uptake model recommend application to larger scale production?

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8. Is there an optimal biological formula suggested from this data?

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II. Literature Review

Chlorella vulgaris

"The term algae has no formal taxonomic standing" (Barsanti & Gualtieri, 2006), because algae are an artificial taxonomic assemblage. *Chlorella vulgaris* is classified in the Division Chlorophyta, Class Chlorophyceae. The Chlorophyta, one of the 10 recognized Algal Divisions, are commonly known as the green algae. They have green chloroplasts that are not masked by other pigments and both chlorophyll *a* and *b* are present. In addition they have β- and γ- carotene and several xanthophylls. These characteristics are very similar to higher plants and this similarity may be of significance when investigating green algae nutrient requirements. Starch is the polysaccharide storage product. Green algae as a group range in body type from non-motile single cells, to flagellates, and to colonial multicellular complexes.

Plant evolutionists believe that land plants evolved directly from a class of green algae, the Trentepohliophyceae. In addition to this class, Division Chlorophyta contains nine other Classes: Prasinophyceae, Ulvophyceae, Cladophorophyceae, Briopsidophyceae, Zygnematophyceae, Klebsormidiophyceae, Dasycladophyceae, Charophyceae, and Chlorophyceae. Most of the Order Chlorophyceae within the Class Chlorophyceae live in fresh water. There are about 355 genera that include 2650 species in the Order. The approximately 10 species of genus *Chlorella* are unicellular, coccoid (round) cells, typically two to12 µm. They live in freshwater or on soil and are easy to grow, making them useful in physiological and biochemical laboratory studies.

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Chlorella species reproduce by formation of autospores formed within the parent cell. Each autospore has its own cell wall (Hoek, Mann, & Jahns, 1995). The autospores are non-flagellate and are released when the parent or mother cell wall ruptures (Barsanti & Gualtieri, 2006). *Chlorella vulgaris* is a haploid (1n) organism. Results in a study looking at the toxicity of an atrazine herbicide found a critical threshold cell size for growth phase completion and cell division initiation. The end of the lag phase, referring to Monod's growth curve, corresponded to reaching that critical size (Rioboo, *et al.*, 2009). *C. vulgaris* began the logarithmic growth phase when cells initiated division.

Genetic diversity is a result of micromutations. *Chlorella* species are haploid organisms so there is no opportunity for genetic buffering due to recombination. However, they undergo rapid vegetative reproduction that results in exploitation of the advantageous micromutations. They are highly variable physiologically, thus necessitating study of individual lines for culture (Pickett-Heaps, 1975; Spoehr & Milner, 1948) (Pickett Heaps, 1975; Spoehr & Milner, 1948). Genetically engineered algae, transgenic algae, will in all probability be banned from outdoor cultivation systems. They would pose a serious threat to the natural ecosystem (Pulz & Gross, 2004).

The growth physiology relative to major nutrient elements N, P, K, Mg, and S of *Chlorella* species was studied in the late 50's using a synchronous culture technique. The life cycle of the algae was divided into seven stages. The first stage was the appearance of new daughter cells followed by (2) the appearance of photosynthetically, chlorophyll-rich active cells, then (3) intermediate stage with less chlorophyll cells, and continuing to lighter cells (7) just prior to cell division. Six to 6.5 daughter cells per mother cell was the norm in this study. Deficiency of each element produced growth retardation at some

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stage. Media N-free and/or P-free each resulted in the strongest growth slowdown. The average number of daughter cells for N-free medium was 2.4 and 3.5 daughter cells for the P-free medium. N-free daughter cells were very etiolated (pale), but the P-free daughter cells were normal in color (Hase, Morimura, & Tamiya, 1957). However, it should be noted with respect to number of daughter cells per mother cell, Rioboo *et al.* (2009) using 5-,6-carboxyfluorescein diacetate succinimidyl ester staining that allows an in-vivo look at cell division, determined the normal number of daughter cells for *Chlorella vulgaris* is four autospores.

That *Chlorella* is a good choice for biodiesel production is a conclusion reached by Mata *et al*. (2010) in their extensive review of microalgae and biodiesel production. They found lipid content measured as percent dry weight biomass ranged from 5.0% to 58.0%, lipid productivity as mg/L/day from 11.2 to 40.0, and biomass productivity as g/L/day from 0.02 to 0.20 for *C. vulgaris*. *C. vulgaris* is also reported to grow in heterotrophic and mixotrophic (combining auto- and heterotrophic) conditions as well as the typical autotrophic condition. *Chlorella* and *Spirulina* (blue-green algae) have both been grown in large-scale systems. Algae collections are now found worldwide. The University of Coimbra in Portugal, Gottingen University in Germany, University of Texas, The National Institute for Environmental Studies in Japan, and the CSIRO Collection of Living Microalgae in Australia all house large collections of algae cultures.

Studies looking at algae to be used for animal feed found those harvested in the late-logarithmic growth phase were 30-40% protein, 10-20% lipids, and 5-15% carbohydrates. At other growth phases, these percentages can vary considerably. Carbohydrate levels can double when nitrate is depleted (Barsanti & Gualtieri, 2006).

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Individual growth phases can be identified by individual cell morphology or by growth rate based on biomass accumulation.

Culturing

Culturing requirements are species specific, but some media are "broad" with respect to meeting the nutritional/culturing needs of groups of microalgae. Successful culturing entails formulating the medium and environmental conditions to meet the target algae's requirements for optimal growth. Temperature, light, pH (Goldman, *et al.,* 1982), salinity, and mixing, as well as nutrient quantity and quality are the parameters of interest to obtain optimal growth. Table 2.1 gives ranges for these.

Table 2.1. Culturing Parameter Ranges

Culturing Parameters*	**Range***	**Comment***	***Chlorella vulgaris*****
Temperature	16 – 27 ° C	> 35 ° C usually lethal	22.5 $\pm$ 2° C
Light	100 – 200 µE sec^{-1} m^{-2}	Overheating a problem	45 µmol $m^{-2}s^{-1}$
pH	7 - 9	8.2 – 8.7 often optimal	6.6 at start
Salinity	Variable	Slightly lower than native habitat	Variable
Mixing	Bubbling with air, roller table, or manual swirling		Air and CO_2 bubbling
* (Barsanti & Gualtieri, 2006)			
** Current study			

CO_2 bubbling can physically damage cells and, unless filtered with a 0.2 µm filter unit there is a chance of bacterial or viral contamination. Bubbling does increase the surface area exposure to CO_2 and removes the excess O_2 produced. If there is not sufficient algae

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biomass to utilize the CO_2, the higher concentration of CO_2 can lower the pH. Beijerinck and Bold Basal are two common media recommended for freshwater algae in Chlorophyceae (Barsanti & Gualtieri, 2006). Optimal growth requires optimal nutrient availability, temperature, and light intensity. Optimal in this case means most advantageous to the specific algae, since each species have specific growth requirements.

Photobioreactors are closed systems that typically do not allow exchange between the algal culture and atmosphere. One of the advantages in the bioreactor system is the decreased possibility of contamination as well as the reduced water loss. This system also lowers CO_2 loss when compared with open pond systems.

Nutrient Requirements

Nutrient deficiencies and excess nutrients, both, can cause physiological and morphological changes in microalgae. For example, the dinoflagellate *Ceratium cornutum* forms microgametes in response to deficiencies of N and P as well as to low temperatures and shortened day length. Nitrogen depletion in most species of dinoflagellates stimulates gamete formation. In the Order Volvocales (part of the Class Chlorophyceae) vegetative cells do not differentiate into gametes, the sex cells, when grown in N rich medium. The ammonium ion (NH_4^+) was found to be particularly critical. Hair-like structures are found on the upright filaments of the freshwater green algae *Stigeoclonium*. If there is a deficiency of phosphorus, of nitrate, or of iron, filament formation is stimulated. It has been suggested that these structures function in nutrient

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uptake (Hoek, Mann, & Jahns, 1995). This is just one example of the morphological changes observed with varying growth strictures.

Industrial and agricultural wastewater and secondary sewage treated effluent can be used as medium source of nitrogen and phosphorus. This tackles the matter of eutrophication in the aquatic environment where the wastewater is eventually returned. *C. vulgaris* has been reported to remove from three to eight mg/L of NH_4^+ at an average removal efficiency of 72% for nitrogen. The same study showed 28% removal efficiency for phosphorus, removing 1.5 to 3.5 mg/L of PO_4^{-3} (Aslan & Kapdan, 2006; Valderrama, *et al.,* 2002).

Moving from the laboratory to large scale is not just “doubling” the batch. It does not work for brewing and it does not work for growing algae. One problem is that the laboratory algae may have been grown under “unbalanced growth” conditions. It is essential to develop standards or standardized ranges that parallel the conditions that will exist in the larger scale cultivation unit in the lab. Range requirements for N, P, and C, quality and quantity of light, temperature, salinity, and mixing or turbulence with respect to a particular species must be carefully established before moving out of the lab. Mata and her colleagues (2010) referred to this as a species specific “optimal photo-biological formula” for a cultivation unit. This formula would provide all the necessary growth requirements for the algae for any size facility at any geographic location, an automated process. An example is using a “formula” to maintain the stationary phase of the growth curve when algae are grown for carbohydrates and phytoglycogen rather than protein.

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Phosphorus

Algae, fungi, and higher plants all require phosphate, P, as a macronutrient for growth and increase in biomass. It is the second most limiting nutrient, after nitrogen, for plant growth. In fact, all commercial plant fertilizers are labeled as to their N-P-K contents. Phosphorus makes up 0.2% of a plant's dry weight but it is critical to energy conversion and genetic transfer. Inorganic orthophosphate controls enzyme activity, metabolic pathways, and transport systems within the cell. Much of the research on phosphorus uptake mechanisms has been done on higher plants, but most researchers agree that there is a "broad" similarity in algae (Schachtman, Reid, & Ayling, 1998). In the natural environment bacteria facilitate the release of inorganic P from organic phosphate compounds. Most algae can tolerate P in the range of 50 μgL^{-1} to 20 mgL^{-1} (Becker, 1994).

The pH of the growth solution or culture medium determines the form in which P_i, inorganic phosphorus, exists. The pK for dissociation for orthophosphate: H_3PO_4 to $H_2PO_4^-$ is 2.1 and from $H_2PO_4^-$ to HPO_4^{-2} is 7.2. Uptake rates in higher plants are highest between pH 5.0 and 6.0. The uptake of P_i into the individual cell requires energy because of the high concentration within the cell cytoplasm. Both Na^+ and H^+ cotransport systems have been described in green algae and cyanobacteria (Schachtman, Reid, & Ayling, 1998). Plants have multiple transporters based on kinetic study results. The affinity for a particular element is estimated by the rate of uptake for different external concentrations of the ion containing that element. This existence, or potential existence,

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in green algae of multiple transport mechanisms would allow the algae to cope with changing environmental conditions.

Algae biomass accumulations, often referred to as growth, in P-limited cultures was 30 to 40% lower that the P- sufficient control medium in a study using *Chlorella vulgaris* (Kozlowska-Szerenos, Zielinski, & Maleszewski, 2000). This was the primary effect of insufficient or low phosphorus. The control medium of orthophosphate was 45.5 mg L^{-1} (1.47 mM), and the P-limited was 4.5 mg L^{-1} (0.147 mM). The cultures were grown in cotton plugged closed flasks with gas exchange enhanced by shaking. The light to dark ratio was 16 hours followed by eight hours dark. The *C. vulgaris* used only five to 17% of the orthophosphate in the control, but used almost the entire amount in the P-limited medium. By the fifth day of the eight day growth period, half of the P_i had been removed from the medium. The increased pH that occurred in the P-limited medium may have been due to photosynthetic uptake of inorganic C. Also, in the P-limited medium dissolved inorganic C concentration was five times that of the control suggesting an increase of carbonic anhydrase. Analysis of the phosphorus cell content of those grown in P-limited medium showed that the cells contained the same amount of inorganic orthophosphate as the control algae cells at the end of the eight day culture period. The P-limited cells did have “slightly” less organic phosphate. The results of this study found enhanced production, excretion, and metabolism of glycolate in the P-limited algae. They concluded that “involvement of glycolate metabolism in acclimation to low phosphorus supply improves regeneration of inorganic orthophosphate and protects chloroplasts against photoinhibitory damage…”

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Phosphate starved algae may accumulate large amounts of lipids with a simultaneous decrease of protein, chlorophyll, and nucleic acids. This is similar to the effects observed in nitrogen starved algae. When P is adequate, inorganic phosphates accumulate in the cell as acid labile polyphosphates and are then available under P deficiency (Becker, 1994). This suggests a possible inquiry into lipid production optimization by P manipulation.

In another study looking at P limitation using a growth medium designed to mimic the natural water of Lake Superior, the results indicated that growth rate gave a solid early indication of P limitation. *Chlorella* were grown in P concentrations of 25, 50, 100, 150, and 10,000 nM. For *Chlorella*, the growth rate was reduced from 0.85 d^{-1} for 10,000 nM-P to 0.22 d^{-1} for 25 nM-P. Growth rate was measured as *in vivo* fluorescence vs. time. Growth rates at 100 and 150 nM-P were similar to the control growth rates. Cell density measured as number of cells per ml, increased from 5.5×10^4 to 4.0×10^5 with the increasing P. Alkaline phosphatase, (APase), is a group of enzymes that catalyze the hydrolysis of dissolved organic phosphorus at alkaline pH. They can be important in the natural environment when inorganic P is limited. The APase activity dropped to close to 0.0 with P at 100 nM. The researchers concluded that culturing using the four levels ($1.0 \times 10^{+4}$ nM-P is the control) of P gave better results for estimating threshold P-limitations than the usual present and absent experiments. Measuring the growth rate and the induced APase activity, they concluded that the threshold P limitation occurred in the 100 nM (Ji & Sherrell, 2008).

A follow-up study (Kozlowska-Szerenos, Bialuk, & Maleszewski, 2004), looking at O_2 evolution in P-limited grown *C. vulgaris*, found that "acclimation to phosphate

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deficiency stress is manifested by markedly increased potential ability of photosynthetic utilization of light energy…” There was enhanced nitrate removal from the medium for the first five days and then it equaled the control at day eight. An increase in NH_4^+ in the culture medium was noted and that fell to the control values on day eight also. The energy, photosynethic active radiation, was used increasingly for nitrate reduction resulting in less efficient CO_2 assimilation and consequential conversion to biomass, as seen in the slower growth of the P-limited algae. In this study they did not find a reduction in photosynthetic mechanisms in the initial phase of phosphate deficiency as measured by O_2 evolution. They suggest that the P-limited algae showed signs of acclimation to phosphorus stress by a “sun-type like adaptation,” because phosphate deficient cells, using similar culture conditions relative to P, responded to increased radiance and increased CO_2 concentration much higher than the first conditions. The result was an O_2 evolution significantly higher than the control cells exposed to the same increased conditions. This may be important in short term fluctuations of macronutrients that could be seen in large scale culture scheme.

Nitrogen

Nitrate, ammonia, organic urea, and nitrite are the nitrogen forms utilized by most algae. Ammonia or urea require the least energy to metabolize. Becker (1994) reported that the nitrogen requirement for green algae is five to 59 mM, but this can be quite variable. Nitrate-N is reduced to ammonia by enzymatic reaction before assimilation as follows:

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$NO_3^- \longrightarrow NO_2^- \longrightarrow NH_4^+$. Most media cultures contain nitrates or ammonium as the N source (Ahmad & Hellebust, 1984). If both are present the algae will utilize the ammonia and the nitrate assimilation will be inhibited until all of the ammonium is depleted.

As said earlier, nitrogen limitation can shift the production from proteins and carbohydrates to lipids and while this may seem economically attractive, several considerations must be taken into account. It takes longer for N-deprived algae to produce the equivalent amount of lipid as those replete with nitrogen. Lipid concentrations in C. *vulgaris* grown under differing N concentration varied from 14.1% to 62.9 % (Table 2.2). Green algae, in general, grown at low levels of N will have between 45% to 70% cellular lipid content. Those lipids will be 16:0 and 18:1 fatty acids. At higher levels of N, the lipid content shifts to 20% and the lipids are polyunsaturated fatty acids (Piorreck, Baasch, & Pohl, 1984; Converti, *et al.*, 2009). An optimal system will be able to recognize these differences and adjust N within appropriate ranges for algal growth and economic efficiencies.

Table 2.2. Total lipids % of dry weight for *Chlorella vulgaris*

N concentration levels	**0.0003%**	**0.001%**	**0.003%**	**0.01%**	**0.03%**	**0.1%**
N source – NH_4Cl	52.8	41.8	20.2	14.1	18.8	n/a
N source – KNO_3	57.9	62.9	42.7	22.0	21.8	22.6
(Piorreck, Baasch, & Pohl, 1984)						

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Bilanovic *et al*. (2009) developed a comparative analysis of biomass production from published data along with biomass production from their own experimentation. Only the data for *C. vulgaris* which includes concentration data for CO_2 and N is in Table 2.3.

Table 2.3. *Chlorella vulgaris* Grown under Varying CO_2 and N Concentrations (Bilanovic, *et al.*, 2009)

Medium	CO_2 (%v/v)	N (mg/L)	Reference
DS	0.038	569	Hu & Westerhoff
N-8	0.018	27.88	Jeong *et al.*
N-8	0.012	27.88	Jeong *et al.*
N-8	0.006	27.88	Jeong *et al.*
N-8	0.038	27.88	Jeong *et al.*
M4N	10	569	Sung *et al.*
M4N	30	569	Sung *et al.*
M4N	50	569	Sung *et al.*
M4N	70	569	Sung *et al.*
MBBM	2	90	Bilanovic *et al.*
MNNM	5	90	Bilanovic *et al.*
MBM	0.038	85	Yue & Chen
MBM	5	85	Yue & Chen
MBM	10	85	Yue & Chen
MBM	15	85	Yue & Chen
MBM	20	85	Yue & Chen
MBM	30	85	Yue & Chen
MBM	50	85	Yue & Chen
MBM	70	85	Yue & Chen
M4N	10	569	Watanabe &Saiki

Using response surface methodology modeling technique, they analyzed the different C and N concentration effects on biomass production trying to find the concentration "regions" that correspond to maximum biomass and CO_2 sequestration. Using both

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literature data and their own data and converting biomass production to dry weight (g DW L^{-1}) as needed yielded the following biomass model after 24 hours of culture.

$$[B24]^{0.5} = 0.57957 - 0.019393 \times [CO_2] - 3.26235E\text{-}3 \times [N] + 2.71782E\text{-}4 \times [CO_2] \times [N] + 1.61392E\text{-}4 \times [CO_2] + 1.01663E\text{-}5 \times [N]^2 + 3.766040E\text{-}7 \times [CO_2]^2 \times [N] - 4.69034E\text{-}7 \times [CO_2] \times [N]^2 - 3.14492E\text{-}6 \times [CO_2]^3 - 6.62044E\text{-}9 \times [N]^3$$

With an F-value of 7.45, the model was considered significant. Using this model, Bilanovic *et al*. (2009) concluded that for maximum biomass production with maximal CO_2 sequestration freshwater algae should be grown within a range of N concentrations of 285 to 427 mg N L^{-1}. All of the CO_2 concentrations were sufficient for growth.

Carbon Dioxide Sequestering

Basic chemistry of CO_2 in water is described as the following: $CO_2 + H_2O \longleftrightarrow H_2CO_3 \longleftrightarrow H^+ + HCO_3^- \longleftrightarrow 2H^+ + CO_3^{2-}$. This equilibrium is pH dependent; with CO_2 the predominant form at lower pH below 7.0 and CO_3^{2-} predominant above pH 10.0. Rapid growth of algae can, with the assimilation of CO_2 as the C source, cause the pH to rise. The pKa of H_2CO_3 is 6.3 to 6.4. A rapid shift in pH can change the availability of nutrients.

Algae biomass is normally 50% C, so that at least 1.8 kg of CO_2 is required for 1 kg of algae. In 1994 the delivered cost of CO_2 was \$40 to \$60 per day (Becker, 1994), making algae production costly. The current emphasis on sequestering C from industrial waste gas is both economically and politically attractive. Delivery systems designed to bubble the CO_2 have the most potential because they increase the surface area exposure

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of the gas to the algae. Land plants increase their surface area exposure to CO_2 by increasing leaf size or as in the case of redwoods, one of the largest land plants, the number of small needles. But just like land plants damaged in high winds, if the bubbles are too strong they will fracture the single cell algae.

Bilanovic *et al*. (2009) summarize the "questions" with respect to atmospheric CO_2 mitigation facing countries today.

- Which technologies will be used to capture CO_2 from stationary sources?
- How will the captured CO_2 be immobilized/fixed/sequestered?
- Is there an economically viable way to sequester the CO_2? They estimate there is a need to remove at least 15 to17 trillion tons per year.
- Should we accept the CO_2 released from mobile sources as contributing additional atmospheric CO_2, or instead replace the internal combustion engine with fuel cells and other alternative modes of local transportation?

They estimate that 53,000 km^2 of microalgae reactors would be able to remove 2.5% of the yearly CO_2 emissions. The 53,000 km^2 reactor could produce 395.45 tons of biomass which would in turn result in at least 79.0 million tons of biodiesel. Municipal and industrial effluent treatment plants can potentially provide both the water and N and P nutrients for algal growth. Bilanovic *et al*. (2009) puts further emphasis on the promise of mitigation technological, both technically and financially. They consider microalgae sequestering of CO_2 one of the "major" optimistic technologies and the only one that "has a substantial income generating potential." Others agree and emphasize that there are in reality only two main mitigation strategies: chemical reaction based and biological

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(Wang, *et al.,* 2008). The chemical reaction based is energy consuming and requires disposal of the captured CO_2. Biological mitigation can produce biomass energy using the CO_2 fixation in photosynthesis (Pulz & Gross, 2004).

Concern for the increasing CO_2 level in the atmosphere is worldwide. Using Buitenzorg, a strain of *C. vulgaris,* cultured in Benneck medium in single and series reactor conformation, researchers measured the CO_2 inlet and outlet concentration. Using this difference, they defined a cultural CO_2 fixation rate and then used it to calculate a carbon dioxide transfer rate (CTR). Not too surprisingly, they found that increasing growth rate is "caused by the increasing of CO_2 fixation." This rough experimental study concluded the single reactor was more cost effective than the series reactor with respect to energy requirement cost (light) (Wijanarko, *et al*., 2008).

Korean researchers evaluated three microalgae, *Botryococcus braunii*, *Chlorella vulgaris*, and *Scenedesmus* sp. for their growth, carbon fixation ability, total lipid content and fatty acid profile to determine which organism to select for use with high levels of CO_2 for the production of biodiesel. The *Chlorella vulgaris* strain KCTC AG10032 was obtained from the Biological Resource center of the Korea Research Institute of Bioscience and Biotechnology. It was grown in a BG11 medium at 25±1° C with continuous illumination of 150 $\mu mol^{-2}\ s^{-1}$ and cultured for 14 days. The CO_2 gas source was flue gas of determined CO_2 concentration. The study found that the *C. vulgaris* grew in up to 10% CO_2 with no negative effects. However, these researchers concluded that *Scenedesmus* sp. was the best of the three with regards to CO_2 mitigation. It had the highest 14 day biomass production, 217.50 ±11.24 mg dw $L^{-1}d^{-1}$, as compared to *C. vulgaris*, 104 mg dw $L^{-1}d^{-1}$, and C-fixation ability. *B. braunii* was determined to be the

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most suitable for biodiesel because of its high lipid content especially oleic acid (Yoo, *et al.*, 2010).

Biodiesel Production

Biodiesel has been produced from algal lipid culturing *Chlorella protothecoides* heterotrophically. This species can grow both photoautotrophically and/or heterotrophically (Huang, *et al.*, 2010; Klausmeier, Litchman, & Levin, 2007). Corn powder was used as a carbon source and a crude lipid content of 55.2% was realized. This is about four times the lipid content of 14.57% that was realized when *C. protothecoides* was grown autotrophically. The high lipid content obtained heterotrophically suggested this production technique was an economically feasible method of producing biodiesel from algae. No measure of CO_2 mitigation was considered (Xu, Miao, & Wu, 2006). Earlier, Grant and Turner (1969) found that glucose is effective as a carbon (CO_2) source for algae but only when metabolized to CO_2.

Analytical Considerations

A value for biomass concentration is obtained by measuring the absorbance of the cell suspension at 540 nm. The regression equation used is $y = 0.2821x$, where y is the cell concentration in g L^{-1} and x is the absorbance of the algae suspension at 540 nm. R^2 for the regression is 0.996, $P < 0.05$ (Xu, Miao, & Wu, 2006).

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Others have looked at modeling the uptake of two essential nutrients. In the case of algae, nitrogen and phosphorus are considered the essential macronutrients. Silicon is the third essential, but not for freshwater green algae. Mathematical models are often used to describe the relationship between macronutrient use and growth. This can be helpful when designing growth systems just as the agriculturist plans the crop fertilizer regime. Much discussion exists in the literature as to which model best defines algae growth requirements. The accuracy and reproducibility of growth varies both within and between laboratories.

Table 2.4. Monod Growth Phases

Phase	**Growth**	**Growth Rate**	**Description**
1	Lag	Zero	Physiological adaptation
2	Acceleration	Increasing	Continuously increasing growth rate, μ
3	Exponential	Constant	Cell density increases as a function of time $N_2 = N_1 \times e^{\mu}$, N_i cell number at times 1 and 2
4	Retardation	Decreasing	Changing condition effects manifest
5	Stationary	Zero	Exhaustion of nutrient(s) or light limiting Cell concentration remains constant at its maximum
6	Decline	Negative	Catabolite accumulation
Adapted from Barsanti and Gualtieri, 2006.			

Bacterial growth curves were first described by Monod in 1949 as having six distinct phases. He labeled them lag, acceleration, exponential, retardation, stationary, and decline (see Table2.4). The Monod model is graphed with the log of the bacterial density on the y-axis and time on the x-axis. The growth equation for this model is: $\mu = \mu_{max} S (K_s + S)^{-1}$, where μ is specific growth rate, μ_{max} is maximum specific growth rate, S is substrate concentrate, and Ks is the Monod or "half velocity constant." The usual Monod growth curve is seen in Figure 2.1.

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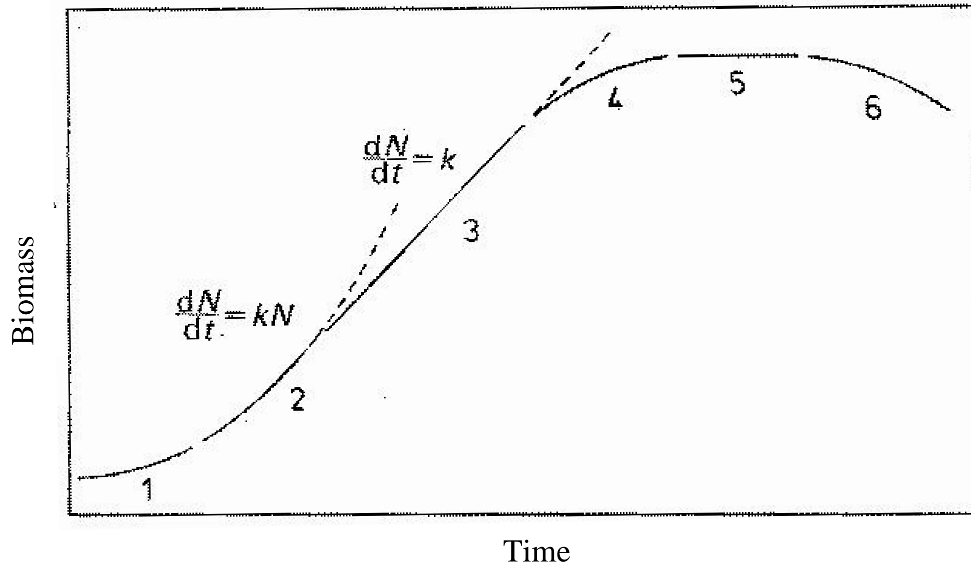
Figure 2.1 Monod Growth Curve (Becker, 1994)



One hundred samples of the green algae *Scenedesmus quadricauda* were analyzed in various media to investigate the reliability of growth curves for water sample analysis of eutrophication cases in the Netherlands. The data showed that maximal biomass, measured as OD_{750}, was the "most accurately determined property of growth curve." Rate measurements are limited by frequency of data collection relative to growth changes. Growth kinetics in surface water exhibited all phases, while those grown on the artificial medium only exhibited exponential and stationary phases typically (Bolier & Donze, 1989).

Results studying *S. obliquus* observed no lag or adaptation phase, confirming what Bolier and Donze (1989) had described in artificial media. The first phase was exponential followed by a linear phase. Specific growth rate was determined for the exponential phase ($\mu = 1/C \times dC/dt$) and biomass productivity for the linear phase ($P_B = dC/dt$). A biomass/P yield coefficient was calculated.

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Daily growth can be based on chlorophyll a fluorescence at 660-700nm and calculated using the formula:

$$DGR = \frac{[\ln(N_t) - \ln(N_0)]}{\ln 2\,(t - t_0)}$$

where N_t is cell density given as cells ml^{-1} and t is time in days. Rioboo *et al*. (2009) found *C. vulgaris* growth in their control grown in Bristol medium at 1.92±0.11 DGR for 24-48 hour growth period and 0.04±0.00 DGR for 72-96 hour. Cell viability was 99.99% and 99.79% respectively.

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III. Research Methodology

Algae Culture Procedure

The alga of choice for this study was *Chlorella vulgaris*. Stock cultures were maintained by the algae lab at University of Dayton Research Institute, Department of Environmental Engineering, Dayton, Ohio. The original culture strain is Carolina 152075 (Carolina Biological Supply Company, 2700 York Road, Burlington, NC). It comes as a bacteria-free or axenic living culture and is kept on agar slants. The inoculum for all experimental treatments was prepared in an identical manner. Cultivated cells were centrifuged at 780 xg for ten minutes. The resulting algae pellet was rinsed with distilled water and centrifuged again for ten minutes to obtain an inoculate free of lysed or damaged cells. Additionally, this helped to remove any excess N and P contamination. The resultant clean pellet was used to inoculate one microbioreactor.

The microbioreactor used in all treatments and control in this study was a 250 ml Erlenmeyer flask. Each flask was capped with a rubber stopper. Two hoses were inserted through each stopper. One hose supplied a constant mixture of air and carbon dioxide at a rate of approximately 1.0 liter per minute with a 4-10% addition of CO_2 measured with a Restek 6000 flow meter (S/N-983532). The air and CO_2 were from separate sources that were combined before entering the flask. This was the carbon source for the algae and provided continuous mixing as well. The second hose was used as a gas vent and was capped with sterile cotton to avoid contamination of the reactor flask (see Figure 3.1). All cultures were held at a room temperature of 22±2 degrees Celsius and under a constant photoperiod. Light intensity was approximately 45 μmol

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$m^{-2}s^{-1}$ provided by a single soft-white fluorescent light bulb positioned beneath Plexiglas. Light intensity was measured using a LI-COR Light Meter model # LI-250A, serial # LM2-2084 digital light meter. pH was measured every 48 hours using a Mettler Toledo Seven Easy pH meter.

Figure 3.1 Microbioreactor

Bold's Basal Medium 4N (four times the nitrogen) was the stock solution (Table 3.1). Bold's Basal Medium (BBM) 4N was used for treatment one. All other treatments varied, based on N and P content. One liter of media was prepared by placing the individual components of Table 3.1 in a clean large vessel and adding distilled water until the volume reached one liter. Media (175 mL) was then transferred into 250 ml Erlenmeyer flasks to be autoclaved prior to inoculation. The flask volumes were maintained daily throughout each experiment by the addition of distilled water.

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Six microbioreactors flasks were prepared for each treatment. Three were inoculated with *C. vulgaris* and three were not inoculated. The three microbioreactors containing only autoclaved media functioned as controls. Each treatment was grown for eight days regardless if nutrients were depleted.

Table 3.1 Bold's Basal Medium 4N (Bold 1949, Bischoff and Bold 1963)

Component	**Stock gL^{-1} dH_2O**	**Quantity used/L**	**Conc. in base media in moles (M)**
$NaNO_3$	100.00	10 ml	1.18×10^{-2}
$CaCl_2 \cdot 2H_2O$	2.50	10 ml	1.70×10^{-4}
$MgSO_4 \cdot 7H_2O$	7.50	10 ml	3.04×10^{-4}
K_2HPO_4	7.50	10 ml	4.31×10^{-4}
KH_2PO_4	17.50	10 ml	1.29×10^{-3}
NaCl	2.50	10 ml	4.28×10^{-4}
Alkaline EDTA Soln.		1 ml	
EDTA	50.00		1.71×10^{-4}
KOH	31.00		5.53×10^{-4}
Acidified Iron Soln.		1 ml	
$FeSO_4\ 7H_2O$	4.98		1.79×10^{-5}
H_2SO_4		1 ml	
Boron Soln.		1 ml	
H_3BO_3	11.42		1.85×10^{-4}
Trace Metal Soln.		1 ml	
$ZnSO_4 \cdot 7H_2O$	8.82		3.07×10^{-5}
$MnCl_2 \cdot 4H_2O$	1.44		7.28×10^{-6}
MoO_3	0.71		4.93×10^{-6}
$CuSO_4\ 5H_2O$	1.57		6.29×10^{-6}
$Co(NO_3)_2 \cdot 6H_2O$	0.49		1.68×10^{-6}

Experimental Design

This study used five treatments, three test replicates within each treatment, and three control replicates within each treatment, to examine the uptake rate of both NO_3-N and PO_4-P from the nutrient media (Reisner & Thompson, 1955). The five treatments varied relative to N and P content in media with all other media components held

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constant. Temperature and light intensity were also held constant throughout all treatments.

Table 3.2 Experimental Treatments

	Media
Treatment 1	BBM 4N
Treatment 2	BBM
Treatment 3	BBM 75% N & P
Treatment 4	BBM 50% N & P
Treatment 5	BBM 20% N & P

Data Collection

All P, N, and pH data were collected at 48 hour intervals. Absorbance was measured at 24 hour intervals. Before sample collection, each bioreactor was gravimetrically verified for correct volume and corrected, if needed, with addition of distilled water. Any change in pH was also noted. Data from both treatment and control flasks were collected and recorded.

***C. vulgaris* Growth**

C. vulgaris growth was determined by measuring absorbance using a Perkin-Elmer spectrophotometer (Model #LAMBDA-3B, Serial No. 69430) set at 550 nm, the University of Dayton Research Institute Environmental Engineering Lab's standard practice. A one ml sample was extracted from each flask and the light intensity entering and exiting the sample was measured. The percent transmittance (%T), the ratio of the

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intensity of the exiting light to the entering light, was recorded. The percent transmittance was related to the absorbance by: A = 2.00 – [log (%T)]. When T = 50%, then absorbance is 0.030. Algae cell count, the growth, is correlated to the absorbance using Beer's Law. Beer's Law states that absorbance is directly proportional to the concentration of a solution, and when absorbance versus concentration is plotted, a straight line regression relation results. This regression was used to determine the concentration of the algal solution.

NO_3-N Uptake

Nitrogen uptake was measured as nitrate. A five ml sample of algae was centrifuged for ten minutes at 780 xg to separate the algae from the media. The clear supernatant was measured for NO_3-N using a Hach DR-890 colorimeter and the cadmium reduction method (Method 8048). A Hach Nitra Ver 5 Nitrate reagent powder pillow was added to the appropriate dilution of the supernatant and distilled water. Final solution volume was equal to ten ml in all tests. The sample was shaken for one minute and allowed to rest for five minutes permitting the reaction to complete.

A blank was prepared with the same amount of sample as was used for testing purposes. Distilled water was used as the diluting agent; no reagent was used. This blank was used to zero the colorimeter prior to any testing of samples. The cadmium in the reagent powder reduced any nitrates present to nitrite ions which, in turn, reacted in the acidic solution to form an amber product. The colorimeter measurement of the treatment sample was converted to mg/L NO_3-N.

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PO_4-P Uptake

Phosphorus uptake was measured as orthophosphate, PO_4-P. Again, as with the N data collection, a five ml sample was collected and centrifuged for ten minutes at 780 xg. The clear supernatant was measured for PO_4 using the Hach DR-890 colorimeter and the molybdate-ascorbic acid method (Method8048). A Hach Phos Ver 3 Phosphate reagent powder pillow was added to the appropriate dilution of the supernatant and distilled water. Final solution volume was equal to ten ml in all tests. The sample mixture was shaken for 15 seconds and left to sit for two minutes for the reaction to complete. A blank was prepared in the same manner as for nitrate and the colorimeter was zeroed, again prior to any testing. Orthophosphate reacted with molybdate in the reagent in the acid medium to produce a phospho-molybdate complex. The complex was reduced by ascorbic acid and formed a blue color. This blue color indicated the presence of PO_4 and the intensity is converted to mg/L PO_4. PO_4 was converted to P by multiplying PO_4 by 0.3261.

Method Performance

Method Detection Limits (MDL) were calculated in accordance with the Code of Federal Regulations (CFR) (40 CFR 136, 1993). Both the Hach cadmium reduction method and the Hach ascorbic acid method have "estimated detection limits" (EDL) published by Hach. These must be tested prior to use. Each was tested by preparing an analyte that was from one to five times that of the EDL. Calculating the MDL was accomplished by applying the formula: MDL = Student's t (s). The Student's t value at the 99% confidence interval is multiplied by s, the standard deviation of the average

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concentrations. The MDL was calculated experimentally to be 0.3 mg/L for NO_3-N and 0.08 mg/L for PO_4-P, which is slightly higher than the EDL published by Hach.

Lastly, a standard curve was generated for each method. Known amounts of research grade KH_2PO_4 and $NaNO_3$ dissolved in distilled water were used as the analytes in varying concentrations. Each known concentration was then measured using the procedures outlined in detail above. Instrument measured P and N concentrations were then plotted against calculated concentrations to obtain a standard curve. Standard curves are presented in Appendix A.

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IV. Results and Analysis

Biomass

Biomass was measured initially and then daily, in order to understand the relationship between growth and nutrient availability, particularly nitrogen usage in *Chlorella vulgaris* in a small batch culture. These time series data were used to estimate growth rates for each nutrient level. Raw data was collected as absorbance at 550 nm according to procedures outlined in the methodology section. Absorbance (x) was positively linearly correlated with biomass (y), defined as $y = 0.1613x$, with a coefficient of determination, R^2, of 0.995 (Figure 4.1). The raw data was converted to g/L biomass.

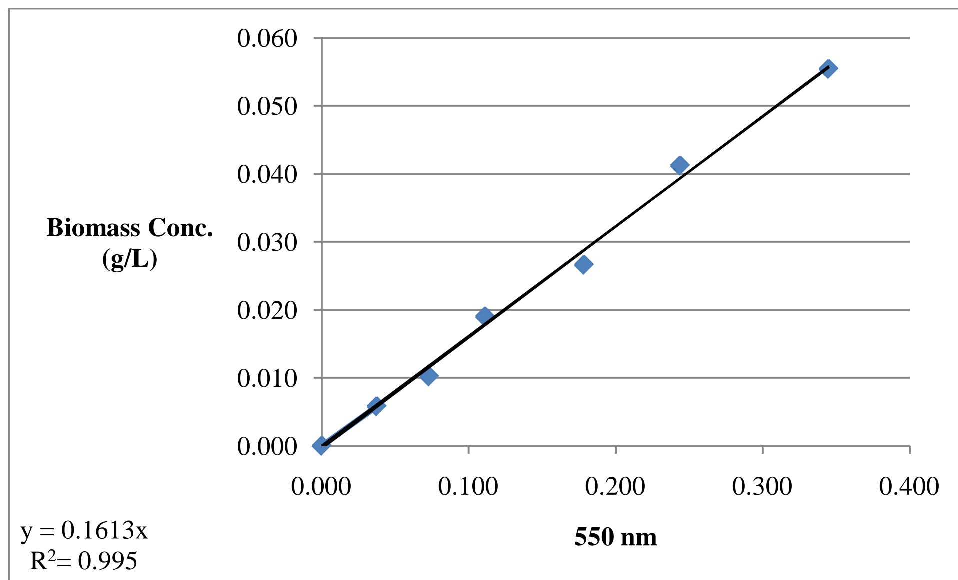

Figure 4.1. Biomass Concentration vs. Absorbance

The condition of steady state growth, also termed balanced growth, occurs during the exponential phase of growth in batch cultures (Wood, Everroad, & and Wingard,

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2005). The rate of increase of biomass, which is the proxy measurement of cell number used throughout this study, was plotted against time for each nutrient level. Nutrient levels were based on Bold's Basal Medium. A log-linear plot for each level was generated and a straight-line was visually fitted. This was used to identify the exponential growth phase and to find the onset of the stationary growth phase (Figure 4.2). All nutrient levels appeared to have a period of exponential growth from day one to day five. This also had the greatest net biomass increase.

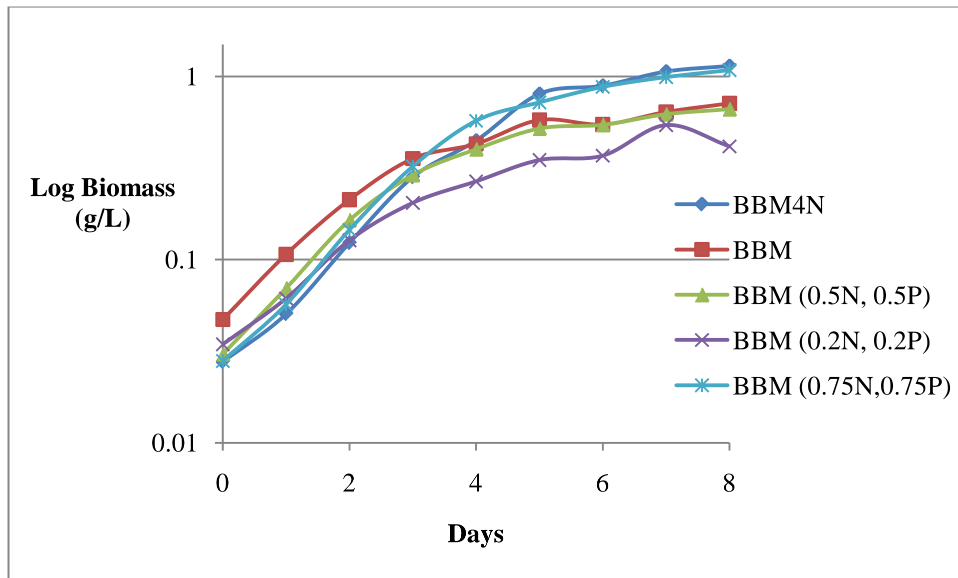

Figure 4.2 Log of Biomass vs. Days

Exponential population growth rate, r; division per day, k; and population doubling time, T_2, were determined for each level of nutrients. Exponential growth rate, r, is a proportional rate of change. In literature it may also be referred to as intrinsic rate

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of increase, Malthusian parameter, or instantaneous rate of increase and is expressed as a per time unit (MacIntyre & Cullen, 2005). In this study, r is expressed per day. Equation 1 is solved for all experimental levels. N_t is the biomass at day five, N_0 is the biomass at day one, and Δt is four days for all levels.

Equation (1) $$r = \frac{\ln(\frac{N_t}{N_0})}{\Delta t} = \frac{(\ln N_t - \ln N_0)}{\Delta t}$$

The calculated r is equal to the specific growth rate (μ), where r = μ - m, because mortality (m) is considered zero during the exponential phase of growth.

The unit for t is days, so a doubling per day, k, can be computed using Equation 2 or Equation 3. N, the biomass, is used as a cell count proxy. Using a four day period with time increments of days is sufficient to remove errors. Doubling time can be calculated using Equation 4. Note that because the algae are in the exponential growth phase which is continuous in nature, a cell division per day does not equal a growth of one per day. Study results are found in Table 4.1

Equation (2) $$k = r / \ln 2 = r / 0.6931$$

Equation (3) $$k = \log 2(N_t / N_0) / \Delta t$$

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Equation (4) $$T_2 = 0.6931 / r$$

Table 4.1. Exponential growth rate, divisions per day, and doubling time for days 1-5.

	Days 1-5	BBM 4N	BBM 1N	BBM (0.75N, 0.75P)	BBM (0.5N, 0.5P)	BBM (0.2N, 0.2P)
Inst rate of increase	r	0.69	0.42	0.64	0.50	0.43
Doubling per day	k	1.00	0.61	0.92	0.72	0.63
Doubling time	T_2	1.00	1.64	1.09	1.38	1.59

Looking at just the data used for the growth rate calculations, an analysis of variance (ANOVA) of the biomass data was used to test the null hypothesis (H_o) that the biomass means for each level are equal to each other, $H_o = \mu_1 = \mu_2 = \ldots\ldots = \mu_5$. The research hypothesis was that there would be a difference between the mean biomass for the five different nutrient levels. The results rejected the null hypothesis for day one and day five and the research hypothesis was accepted, i.e., a difference exists (Table 4.2 and 4.3).

Table 4.2. Biomass Analysis of Variance - Day 1

Source of Variation	SS	df	MS	F	P-value	F-critical
Between	0.002644	4	0.000661	12.12041	0.000752	3.47805
Within	0.000545	10	5.45E-05			
Total	0.003189	14				

Table 4.3. Biomass Analysis of Variance - Day 5

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Source of Variation	SS	df	MS	F	P-value	F-critical
Between	0.394670	4	0.098668	17.00787	0.000185	3.47805
Within	0.058013	10	0.005801			
Total	0.452683	14				

Tukey's honestly significant difference method (HSD) test was then done. Using this test allows the probability of making a Type I error (alpha = 0.05 or alpha =0.01) to remain the specified level rather than becoming cumulative. Tukey's is therefore a more powerful test. This test is also known as the T-method and it uses the minimum significant range (MSR) as a critical difference value, Equation 5. The MSR can be used to test the differences between any pair of means for equal sample sizes when a difference is found using an ANOVA (Sokal & Roth, 1969). The null hypothesis of no difference is tested for each pair-wise comparison (Table 4.4).

Equation (5) $$MSR = Q_{\alpha[k,\,y]}\sqrt{\frac{MS_{within}}{n}}$$

Table 4.4. Critical Differences Between Paired Means – Day 1

	4-BBM	BBM	0.75-BBM	0.50-BBM	0.20BBM
4-BBM					
BBM	0.0369 **				
0.75 -BBM	0.0061	0.0308 **			
0.50-BBM	0.0165	0.0205 *	0.0104		
0.20-BBM	0.0040	0.0329 **	0.0021	0.0125	
CD $_{\alpha\,0.05}$ = 0.0185, CD $_{\alpha\,0.01}$ = 0.0246 * Significantly different, ** Highly significantly different					

Table 4.5. Critical Differences Between Paired Means – Day 5

	4-BBM	BBM	0.75-BBM	0.50-BBM	0.2BBM
4-BBM					

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BBM	0.2470 *				
0.75 -BBM	0.0836	0.1634			
0.50-BBM	0.2883 **	0.0413	0.2047		
0.20-BBM	0.4634 **	0.2164 *	0.3798 **	0.1751	
$CD_{\alpha\ 0.05} = 0.1904$, $CD_{\alpha\ 0.01} = 0.2537$ * Significantly different, ** Highly significantly different					

NO_3-N Removal

Media NO_3-N concentrations were measured initially and then every 48 hours, in order to determine the NO_3-N uptake rates by *C. vulgaris*. Experimental data showed that the removal efficiencies for NO_3-N were 50.8% for BBM4N, 95.2% for BBM, and anything less had a removal efficiency of 100%. Removal efficiency was calculated with the following equation:

Equation (6)

$$\frac{([S_0]-[S_f])*100}{[S_0]} = \text{removal efficiency}$$

where $[S_0]$ is the initial substrate concentration (in this case NO_3-N) in mg/L and $[S_f]$ is the final substrate concentration after day eight. Complete NO_3-N removal occurred when the initial media concentration was less than 35 mg/L NO_3-N. Furthermore, initial NO_3-N concentration of 7.33 mg/L was completely depleted within 48 hours. The decrease in concentration of NO_3-N is attributed to uptake by *C. vulgaris* in all instances. Removal efficiency rates are important when using wastewater as media which requires a percent removal of NO_3, for example.

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NO_3-N removal rates (mg/L/d) were calculated by plotting NO_3-N concentration versus time and performing a regression of data points. NO_3-N removal rates varied from 8.52 mg/L/d to 4.74 mg/L/d (Table 4.6). This agrees with results reported by others of 10.5 mg/L/d to 5.44 mg/L/d (Aslan and Kapdan, 2006).

Table 4.6. NO_3-N Depletion Rates

Media	NO_3-N mg/L/d depletion rate	R^2
BBM4N	8.52	0.92
BBM	7.98	0.98
BBM (0.75N, 0.75P)	6.04	0.97
BBM (0.5B, 0.5P)	4.74	0.99
BBM (0.2N, 0.2P)	3.55	NA

NA = not applicable

Experimental results demonstrated that removal rates increased with increasing NO_3-N concentrations.

At all nutrient levels, phosphorus never depleted below detectable limits nor became limiting. Growth was depressed when BBM was reduced to 0.2N and concurrently the biomass yield was restricted. This is evident in Figure 4.3, also noting the decrease in rate of growth.

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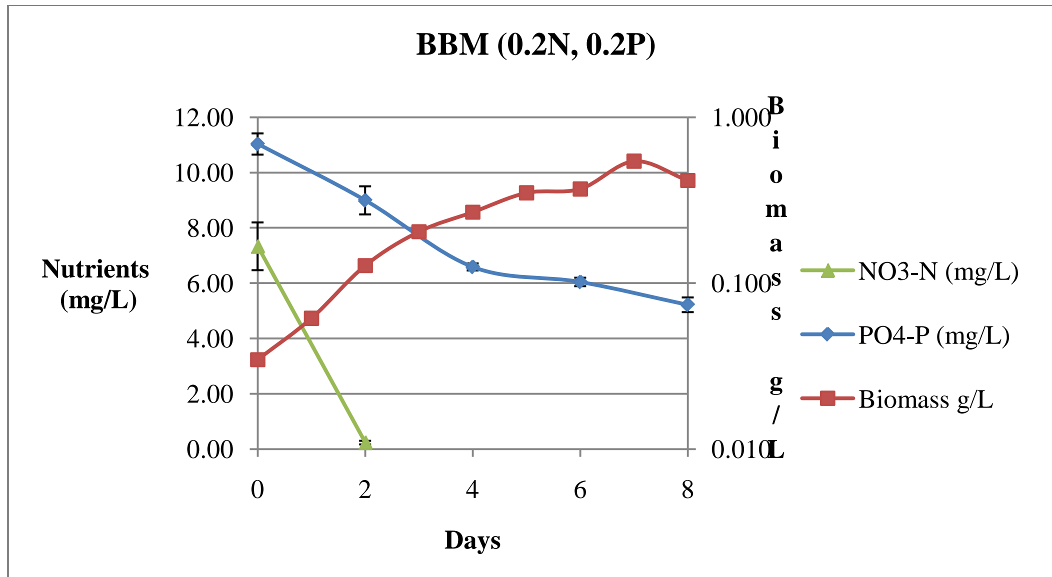

Figure 4.3. Effect of N limitation on growth.

Yield rate was calculated for all levels of NO_3-N using the following equation

Equation (9) $$\frac{Biomass_f - Biomass_i}{[S_0]-[S_f]} = \text{Yield rate (g biomass/mg } NO_3\text{-N)}$$

where total biomass is divided by total substrate removed. The study results showed a trend toward more efficient use of NO_3-N as N was depleted. Figure 4.4, graphically depicts this trend.

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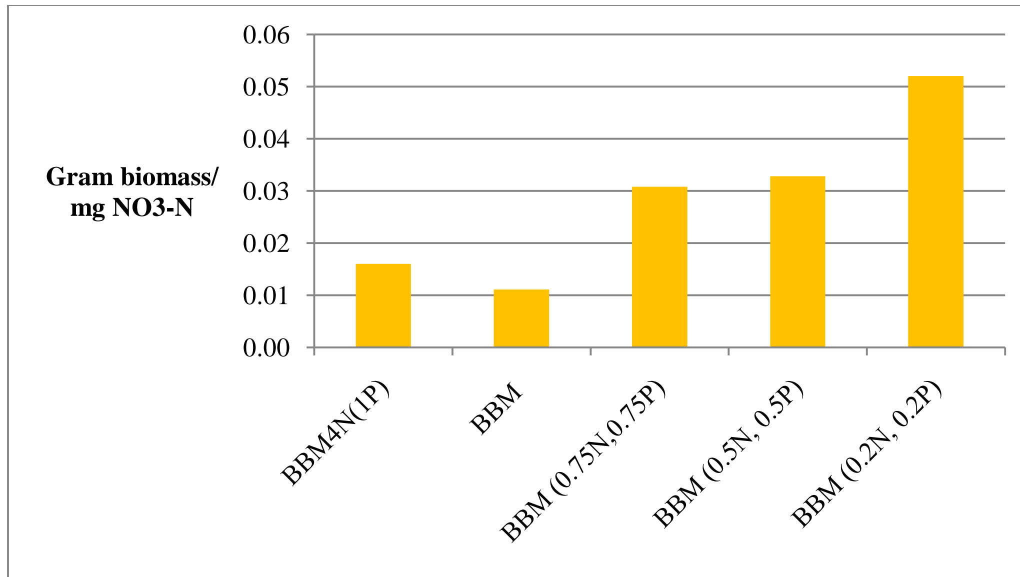

Figure 4.4. Grams biomass produced per mg NO_3-N consumed over eight days.

These results illustrate that growth of *C. vulgaris* continues even after NO_3-N was depleted below detectable limits. Although the algae generally demonstrated a higher Nitrogen Use Efficiency (gram biomass/mg N, Figure 4.4) at low levels of N, total biomass (g/L) produced was significantly higher at elevated levels of initial nitrogen concentration.

PO_4-P Removal

Medium PO_4-P concentrations were measured initially and then every 48 hours, in order to determine the PO_4-P uptake rates by *C. vulgaris*. Experimental data showed that the removal efficiencies for PO_4-P were 30.3% for BBM4N, 22.0% for BBM, 43.6% for BBM (0.75P), 29.5% for BBM (.5P), and 52.7% for BBM (0.2P). Unlike nitrogen removal, complete PO_4-P removal never occurred.

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PO_4-P removal rates (mg/L/d) were calculated by plotting PO_4-P concentration versus time and performing a regression of data points. PO_4-P removal rates are 2.08 mg/L/d to 0.73 mg/L/d (Table 4.7). Removal rates reported in the literature for *C. vulgaris* are 2.0 mg/L/d to 1.30 mg/L/d (Aslan and Kapdan, 2006).

Table 4.7. PO_4-P Depletion Rates

Media	PO_4-P removal rate (mg/L/d)	R^2
BBM4N	2.08	0.98
BBM	1.32	0.96
BBM (0.75N, 0.75P)	2.62	0.99
BBM (0.5B, 0.5P)	1.01	0.97
BBM (0.2N, 0.2P)	0.73	0.93

Experimental results demonstrated that removal rates did not necessarily increase with increasing PO_4-P**.**

Yield rate was calculated for all levels of PO_4-P. Experimental results showed that no general trend toward more efficient use of PO_4-P per gram of biomass exists in this case. This is illustrated in Figure 4.5. In fact, a trend toward less efficient use of P exists at high nitrogen levels.

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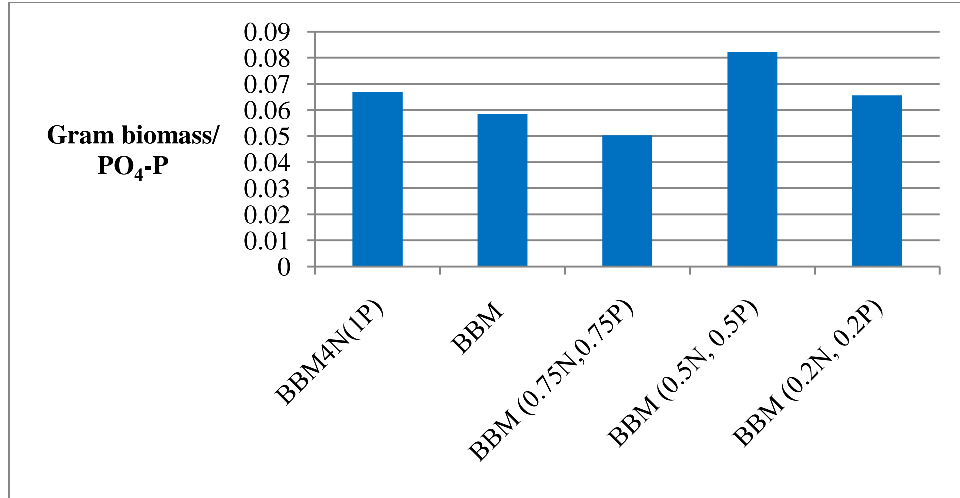

Figure 4.5. Grams of biomass produced per mg PO_4-P consumed over eight days.

Biokinetic Coefficients

The Michaelis-Menten kinetic relationship is used to determine saturation constants and reaction rate coefficients, K_m and k. This logistic model was found to give a "more consistent and accurate description" of algal growth than the Monod or exponential model (Stringfellow, Borglin, & Hanlon, 2006). Aslan and Kapdan (2006) outline this method and equations are used as presented by them for comparison of methods and algae substrate (nutrient) utilization.

Equation (8)

$$R = \frac{R_{max}[S]}{K_m + [S]}$$

Where R, which is the substrate removal rate or velocity of the reaction, is calculated using Equation 8. R_{max} is the maximum substrate removal rate and [S] is the substrate

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concentration. The velocity of the reaction R as a function of [S] is calculated and plotted. The initial substrate concentrations are known in batch culture and the initial substrate removal rate is determined experimentally and so the following form of the equation is used, Equation 9

Equation (9) $$R_{S0} = \frac{R_{mo}[S_0]}{K_m + [S_0]}$$

Where $R_{mo} = k * X_0$ is the maximum initial rate of substrate removal.

Equation (10) $$R_{S0} = \frac{kX_0[S_0]}{K_m + [S_0]}$$

Equation 10 is rewritten, where, k is the reaction rate constant per day and X_0 is the initial biomass concentration of the *C. vulgaris* as Equation 10. Then the specific rate of substrate removal (R_{Xi}) can be calculated by dividing both sides of Equation 10 by the initial biomass concentration shown in Equation 11.

Equation (11) $$R_{xi} = \frac{R_{S0}}{X_0} = \frac{k[S_0]}{K_m + [S_0]}$$

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A Lineweaver-Burk transformation was used to discern the two parameters k and K_m.

Equation (12) $$\frac{1}{R_{xi}} = \frac{K_m}{k}\frac{1}{[S_0]} + \frac{1}{k}$$

The plot of $\frac{1}{R_{xi}}$ as a function of $\frac{1}{[S_0]}$ provided a linearized function. A regression line was fitted to find the equation of the straight line for this transformed data (Figure 4.6). The fitted line yields a slope of $\frac{K_m}{k}$ and a y-intercept of $\frac{1}{k}$.

Both K_m and k were calculated from experimental data (Figure 4.6) using the steps outlined above. The kinetic coefficients for removal of NO_3-N by *C. vulgaris* were as follows: reaction rate constant (k) of 8.47 mg NO_3-N/g biomass/day and a saturation constant (K_m) of 19.4 mg/L.

Data from PO_4-P removal rates did not present a fitted regression line using the procedures outlined above. No manipulation of experimental PO_4-P data fit any kinetic model sufficiently, (Figure 4.7). Nonetheless, calculated kinetic coefficients (from best fit regression line) for removal of PO_4-P by *C. vulgaris* were as follows: reaction rate constant (k) of 2.05 mg PO_4-P/g biomass/day and a saturation constant (K_m) of 1.61 mg/L. This suggests that PO_4-P throughout all experiments was above saturation.

The N and P substrate removal rates in this study were in the same range as those reported by Aslan and Kapdan (2006), so it is appropriate to be confident in the validity of the biokinetic coefficients obtained here. A direct comparison cannot be made because they used chlorophyll a as biomass and this study uses biomass at 550 nm.

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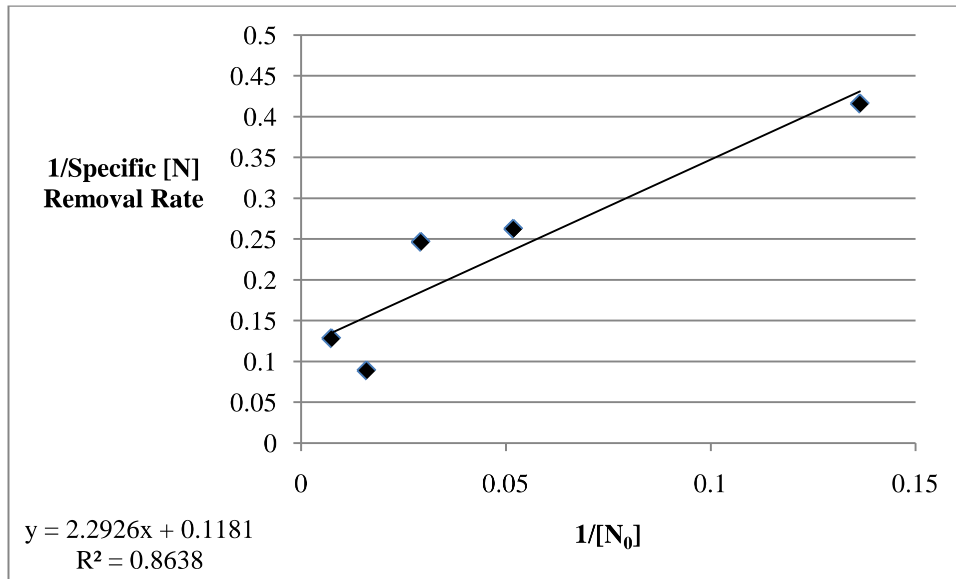

Figure 4.6. Inverse specific substrate removal rate vs. inverse of [NO_3-N] initial.

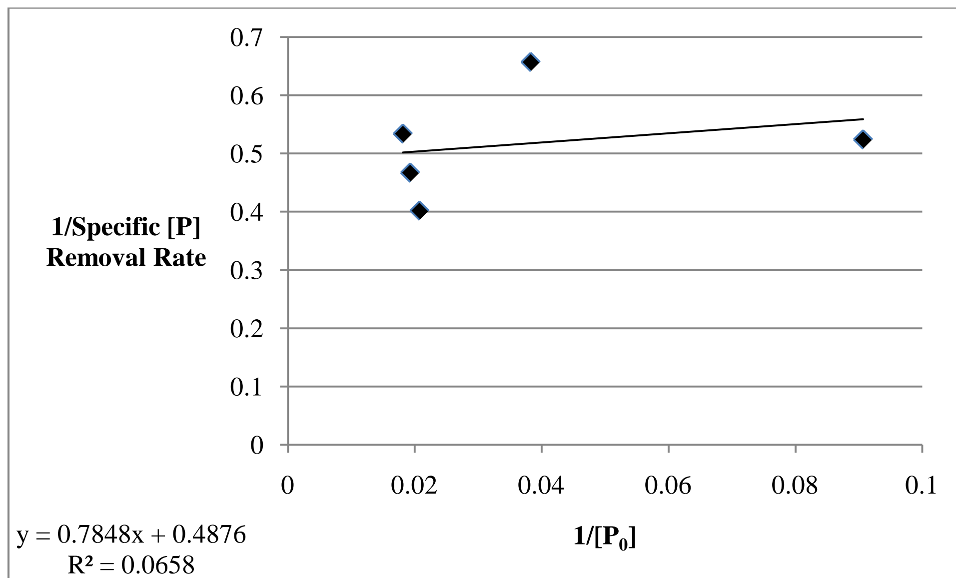

Figure 4.7. Inverse specific substrate removal rate vs. inverse of [PO_4-P] initial.

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Yield coefficients for NO_3-N and PO_4-P were calculated with the following equation:

Equation (13) $$Biomass_f - Biomass_i = Y_x([S_0]-[S_f])$$

For all NO_3-N and PO_4-P treatments, biomass final was subtracted from biomass initial and substrate usage was calculated by subtracting initial substrate concentration from final substrate concentration. After all points were calculated, plots of biomass produced versus substrate depleted were created (Figures 4.8 and 4.9). The slope of this relationship provides a yield coefficient for NO_3-N (Y_N) and PO_4-P (Y_P).

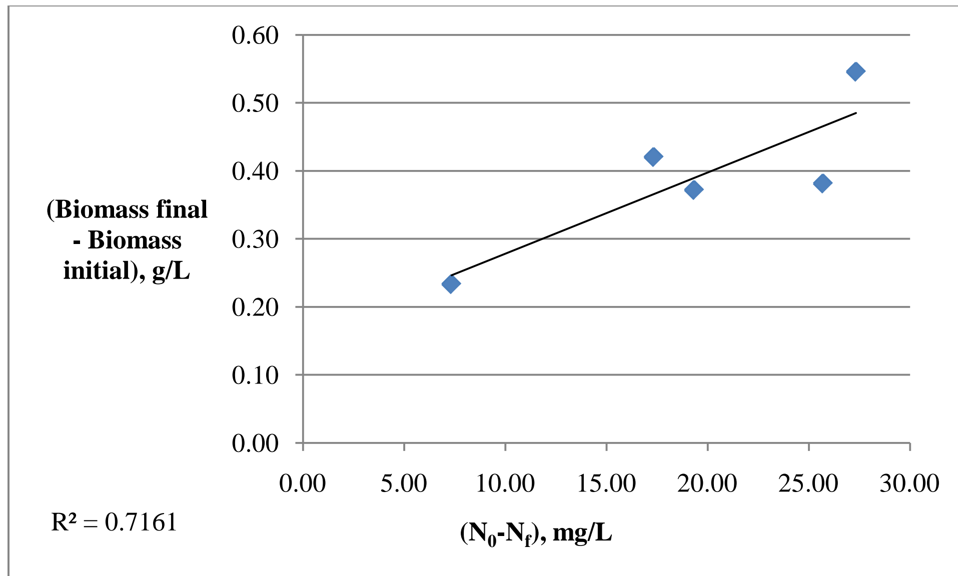

Figure 4.8. Coefficients (Y_N) NO_3-N

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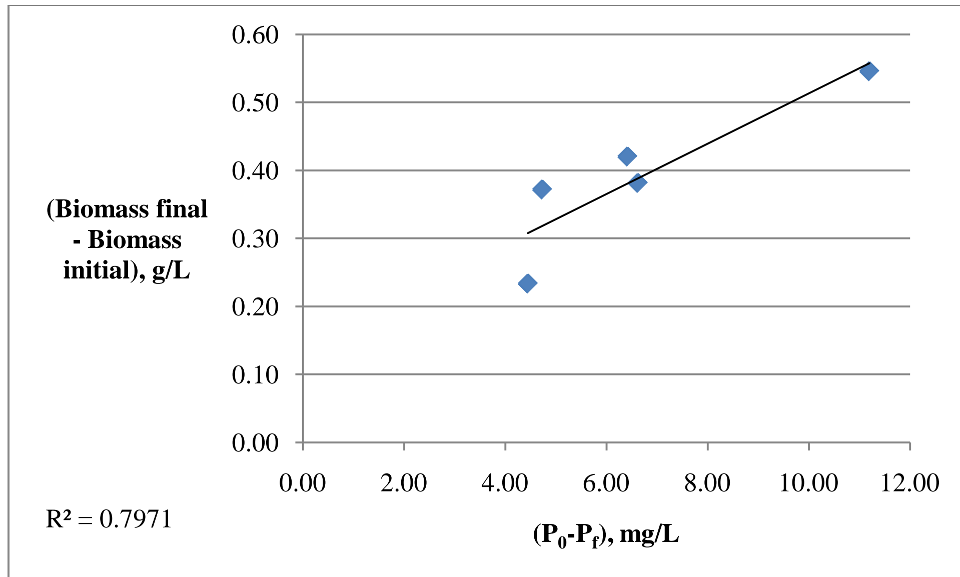

Figure 4.9. Coefficients (Y_P) PO_4-P

Calculated Y_N was 0.0119 g biomass/mg NO_3-N (R^2=0.72), and calculated Y_P was 0.037 g biomass/mg PO_4-P (R^2=0.80). This suggests that three times as much biomass is produced for every milligram of PO_4-P as compared with NO_3-N, or conversely, that *C. vulgaris* utilizes three times as much NO_3-N as PO_4-P for every gram of biomass produced.

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V. Discussion

In discussing the findings of this study, I will reflect back on the research questions and objectives given as the basis for this study. MacIntyre and Cullen (2005), remind us "experiments with cultures are and will remain central to our understanding of microalgal responses to environmental variability." This study does just that by looking at the response of *Chlorella vulgaris* to varying levels of the essential nutrients nitrogen and phosphorus, keeping in mind that the algae response is under genetic control. Extrapolating from the batch culture environment found in this study to large scale growth bioreactors is a long term goal of the University of Dayton Research Institute, Division of Energy and Environmental Engineering algae lab.

What is the biomass potential of *Chlorella vulgaris* in small scale culture under the nitrogen and phosphorus concentrations tested in this study?

The maximum volumetric biomass production was 1.118 g/L or 0.140 g /L /day reached with the highest N concentration. The minimum biomass production was 0.381 g/L or 0.048 g /L /day. Both results are within the ranged of published findings (Lee, *et al.,* 2010) and agree with Mata *et al.* (2010) reported biomass productivity for *C. vulgaris* as 0.02 to 0.20 g /L/day.

What is the appropriate nitrogen range for optimal growth of *Chlorella vulgaris?*

Optimal growth occurs when nutrients are not limiting during the entire growth period. This occurred at the highest level of N (Figure 4.2). Growth occurred at all other

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levels, but all used N to depletion by the end of the eight day growing period. Optimal growth can be described in terms of total biomass or rate of biomass accumulation. If it is evaluated in terms of total biomass or final yield, relating growth to nutrient availability, then Liebig's Law of the Minimum can be used to explain the appropriate N range. It states that the maximum final yield is determined by the availability of a single nutrient. At the beginning of the growth period, all N levels produce the same biomass because the "limiting" nutrient is abundant. At some point in time, the yields diverge and the resulting difference in production is attributable to the limiting nutrient, in this case N. BBM-4N final yield was 1.118 g/L, BBM-0.2N was 0.381 g/L, and they diverged on day two at yields of 0.124 g/L and 0.124 g/L respectively. Although they had very different initial N concentrations, both produced approximately the same biomass until N became limiting for one. BBM diverged on day four at yields approximately 0.435 g/L and BBM-0.5N diverged on day three. Corresponding N utilization for BBM-4N for day two is 8.66 mg/L and for BBM-0.2N is 7.10 mg/L. Table 5.1 summarizes this pattern. Graphically this takes the shape seen in Figure 5.1, where the difference in the biomass yield can be seen as the arrow filled space and is due to the availability of the limiting nutrient.

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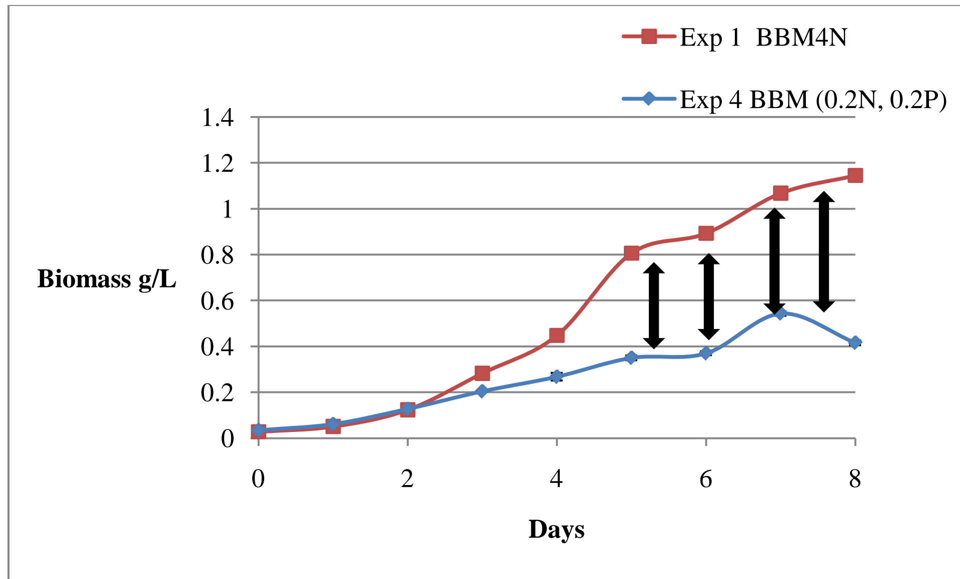

Figure 5.1. Representation of Liebig limitation of yield where the divergence of final yields is caused by an abundance of a limiting nutrient.

Table 5.1. Liebig's Law of the Minimum – Yield and Nitrogen Nutrient Usage Compared to BBM-4N

Nutrient Levels	Total Yield (g/L) at Day 8	Divergence Day	Yield (g/L) at Divergence from BBM-4N	N (mg/L) Utilized at Divergence	Total N (mg/L) Utilized
BBM-4N	1.118				69.73
BBM	0.667	4	0.429-0.448	17.33-25.70	60.00
BBM-0.75N	1.057	5 ++	0.723-0.809	34.06-39.66 ++++	34.33
BBM-0.5N	0.634	3	0.283-0.291	17.33-18.96 +++	19.33
BBM-0.2	0.381	2	0.124	7.10-8.66	7.33
++ BMM and BBM-0.75 diverge on day 3, +++ Used data from day 4, ++++Used data from day 6					

Tukey's HSD testing for differences between the paired nutrient levels and biomass produced confirms the identified days of divergence. One exception is the paired BBM-4 and BBM-0.75, though BBM-0.75 may be an outlier.

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Looking at just N with respect to Liebig's Law, the appropriate range suggested for N is 7.33 mg/L to 69.73 mg/L because Liebig's applies to the final yield. However, in order to maintain growth for a maximum final yield the "rate of N application" would necessarily be at or above 69.73 mg/L, but not higher than the initial concentration of 137.33 mg/L. The higher concentration was not toxic, but recommendation beyond the parameters of this study is inappropriate. The N requirement for green algae has been reported to vary from five to 59 mM (Becker, 1994). The study results for N requirement are in agreement with previous ones.

Bilanovic *et al.* (2009) reported maximum biomass production with an initial N concentration of 285 to 427 mg/L and a goal of maximizing CO_2 sequestration. The initial concentration in the current study was 137.33 mg/L. Other studies looking at lipid content of *C. vulgaris* found it to be lowest when grown at high levels of N (Piorreck, Baasch, & Pohl, 1984). These findings suggest future study before recommending a higher N application rate.

What is the appropriate phosphorus range for optimal growth of *Chlorella vulgaris?*

Using the same interpretation as the previous N with Liebig's Law of the Minimum and points of divergence but with P as the possible limiting nutrient; the pattern of usage is similar. While it would appear at first that both nutrients are utilized identically, all tested nutrient levels had P in the medium at the end of the eight day growth period. Contrast this to the N fully utilized (not detectable) by day two for BBM-

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0.2, by day four for BBM-0.5N, and by day six for BBM-0.75. Table 5.2 summarizes the pattern for P.

Table 5.2. Liebig's Law of the Minimum – Yield and Phosphorus Nutrient Usage Compared to BBM-4N

Nutrient Levels	Total Yield (g/L)	Divergence Day	Yield (g/L) at Divergence	P (mg/L) at Divergence	Total P (mg/L) Utilized
BBM-4N	1.118				16.740
BBM	0.667	4	0.429-0.448	6.41-6.62	11.414
BBM-0.75N	1.057	5 ++	0.723-0.809	10.54- 21.03 ++++	21.034
BBM-0.5N	0.634	3	0.283-0.291	6.41-6.62 +++	7.719
BBM-0.2	0.381	2	0.124	2.39 – 2.03	5.816
++ BMM and BBM-0.75 diverge on day 3, +++Used data from day 4, ++++ Used data from day 6					

Next, N, P, and biomass versus time were graphed together, Figures 5.2 and 5.3, to examine possible relationships. In the batch cultures used in this study, there was what is termed balanced nutrient-limited growth during exponential growth. The intent was to find when that limiting nutrient was wholly utilized. The study was designed to determine which nutrient and at what level that nutrient set the yield. The Liebig limiting nutrient is the one that declines to cellular minimum; here in this study it was defined as that nutrient that was no longer detectable in the growth medium. When nitrogen is low relative to phosphorus, the algae reduces the nitrogen allocation before the phosphorus requirement hits the minimum level. In other words, the biomass yield was nitrogen limited; the phosphorus never was wholly utilized.

The graph of BBM4N (Figure 5.2), shows that day five was when the algae were moving into the stationary phase of growth. Biomass was still accumulating, N and P

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utilization continued, and neither N nor P had been depleted (Figure 5.2). This contrasts with the same graph for BBM, where N was depleted by day eight. A line drawn on each graph at day five allows the nutrient condition in the medium to be examined relative to the end of the exponential phase and the transition into the stationary phase. The N available at this point is ~100 mg/L and the P is ~45 mg/L, and since they neither crossed each other nor were depleted, both were fully available to the algae and more N than P was available.

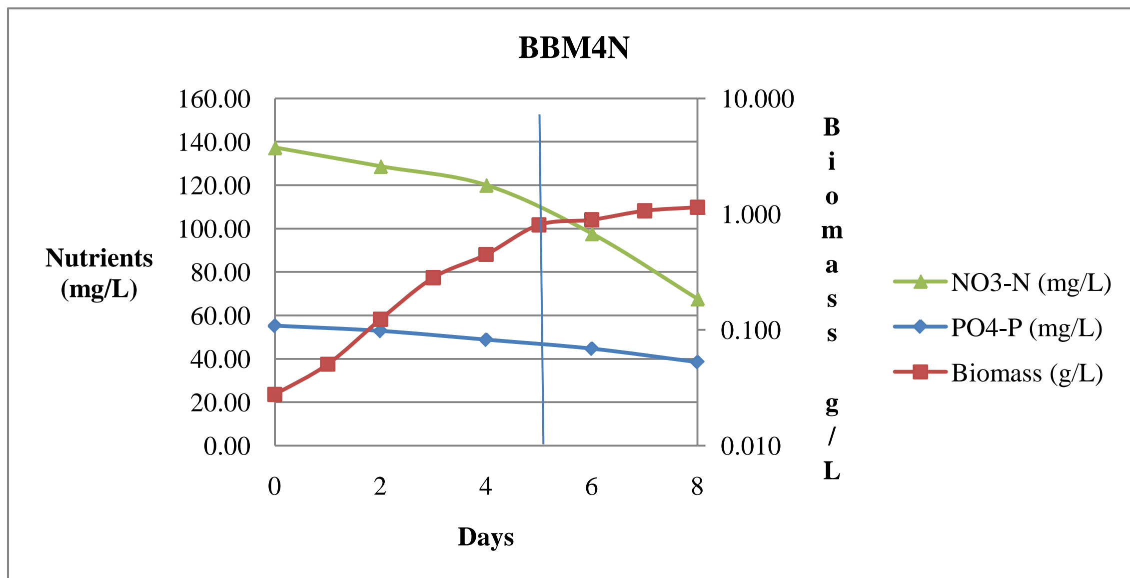

Figure 5.2. BBM4N Nutrient Utilization and Biomass (note log scale) vs. Days

The nutrient scenario for the BBM was different. By day five, the P was still readily available as seen by the slope or shape of the P line, while the N was rapidly approaching depletion (Figure 5.3). The intersection of the N and P lines marks the point at which the nutrients were available in equal concentrations. Green algae require more N than P. When P was present in higher concentrations than N, N became limited. This

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is often expressed as the Redfield ratio or critical ratio of N:P. It varies by genera, but N is always higher than P and usually 16:1 (MacIntyre and Cullen, 2005). This graphic form continues for the other nutrient levels, with P never going to depletion (Appendix C). The range of consumption of P was 5.82 mg/L to 21.03 mg/L. Because the *C. vulgaris* is N-limited under the conditions of this study, using Liebig's Law of the Minimum analysis of the P data here, no suggested range is found, but it was obvious that N needed to be higher than P.

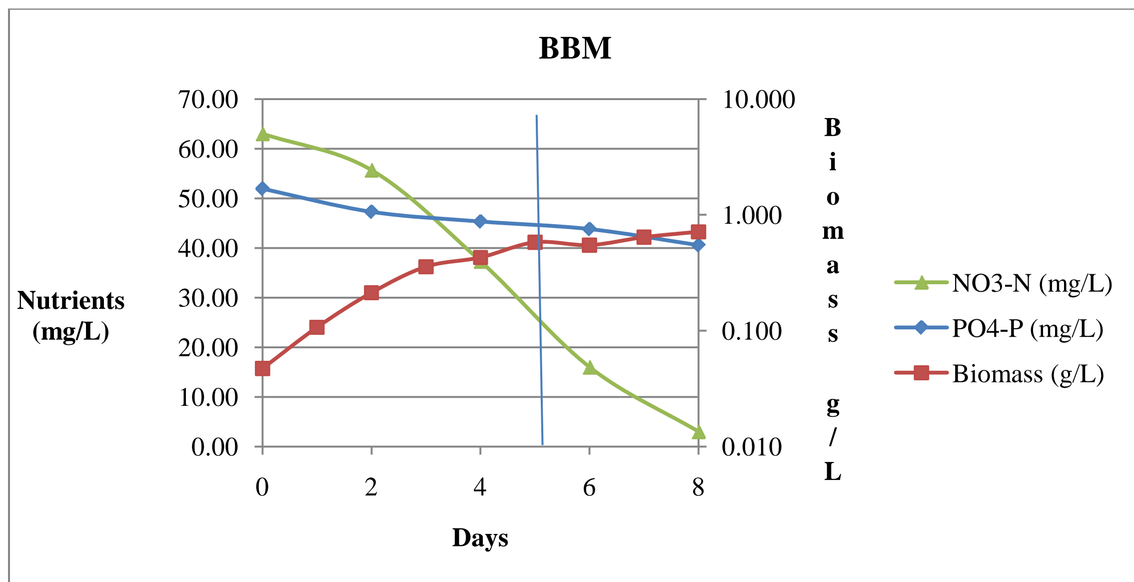

Figure 5.3. BBM Nutrient Utilization and Biomass (note log scale) vs. Days

Can growth rate be correlated with N-limitation and/or P- limitation and thus be used as an early indicator of nutrient limitation? Will the correlation be sufficiently strong to suggest usefulness in mid to large scale culture?

To answer these questions the rate of growth for the varying nutrient levels was reviewed. When the limiting nutrient is gone, the rate of growth declines, termed the Blackman Limitation. Note that the Blackman Limitation concept is based on simple

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kinetics and looks at the nutrient's effect on rate, while Liebig Limitation is based on final yield (MacIntyre and Cullen, 2005). The Blackman Limitation comparison asks how long it takes to reach a "particular" yield. The growth rate is determined by the limiting nutrient. For discussion, the yield chosen is the maximum biomass for the lowest N level (BBM-0.20), 0.381 g/L which required five days. The specific growth rate for this level, μ, or r in this study, at the end of five days was 0.435 g/L/day. Comparing that to the highest initial N level (BBM -4N) with a specific growth rate of 0.692 g/L/day at the end of five days, the effect of the limiting nutrient begins at the point of divergence on day two and is seen when the biomass yields are the same (at the arrow) in Figure 5.4. The distance at the arrow represents the difference in availability of the limiting nutrient. For BBM4N at day five there was 97.67 mg/L of N, and for BBM-0.20 there was no N available. The algae were in starvation mode. Note that the BBM4N continued growing and for the BBM -0.20 the growth was effectively stopped.

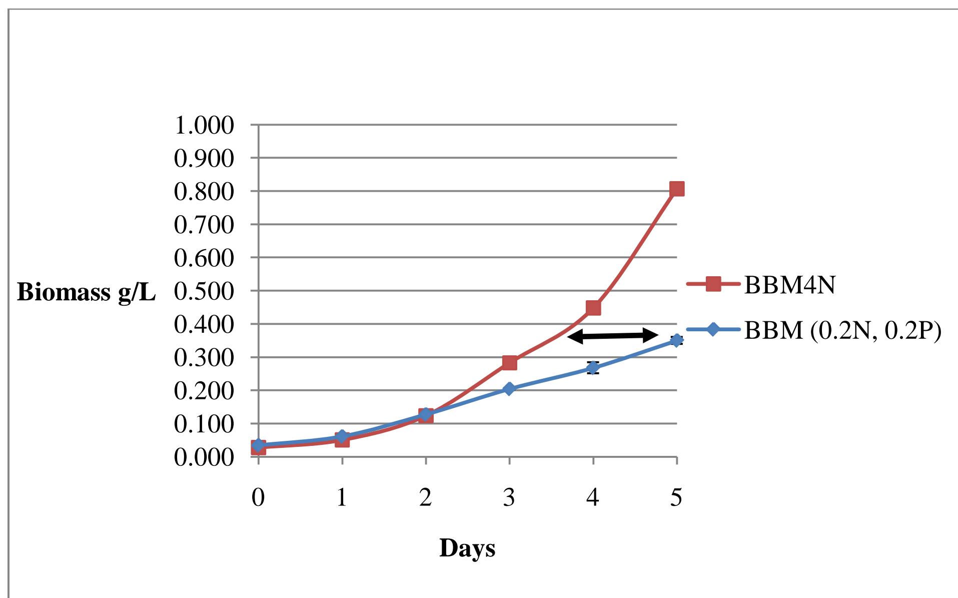

Figure 5.4. Representation of Blackman Limitation of Growth

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Because the biomass yield for the highest initial N level at day five was significantly different (see Tukey's HSD in Results section) from all lower levels except BBM-0.75, it follows that the specific growth rates (also termed intrinsic rate of increase) should be strong enough to be useful in larger bioreactors, mid to large scale. An intrinsic rate of increase of 0.692 would then require ~70 mg/L to ~140 mg/L of N.

Does the experimentally determined kinetic nutrient uptake model recommend application to larger scale production?

Kinetic nutrient uptake models differ from the Liebig and Blackman Limitation models that are based on exponential growth phase and algal response to limiting nutrients. Looking at the same data, but focusing on nutrient removal rates and yield coefficients, a kinetic nutrient uptake model was derived. The calculated Y_N was 0.0119 g biomass/mg NO_3-N (R^2=0.72). The calculated Y_P was 0.037 g biomass/mg PO_4-P (R^2=0.80). This suggests that three times as much biomass is produced for every milligram of PO_4-P as compared with NO_3-N, or conversely, that *C. vulgaris* requires at least three times as much NO_3-N as PO_4-P for every gram of biomass produced.

This study assumed that apparent k and apparent K_m calculated from the net uptake measurement reflect the influx carrier ability of *C. vulgaris*. It has been demonstrated that NH_4-N and PO_4-P uptake by *C. vulgaris* can be described by Michaelis-Menten kinetics (Aslan and Kapdan, 2006). Furthermore, Michaelis-Menten kinetics are used to describe nitrate uptake in higher plants; barley, corn, and rice are three examples (Hasegawa & Ichii, 1994).

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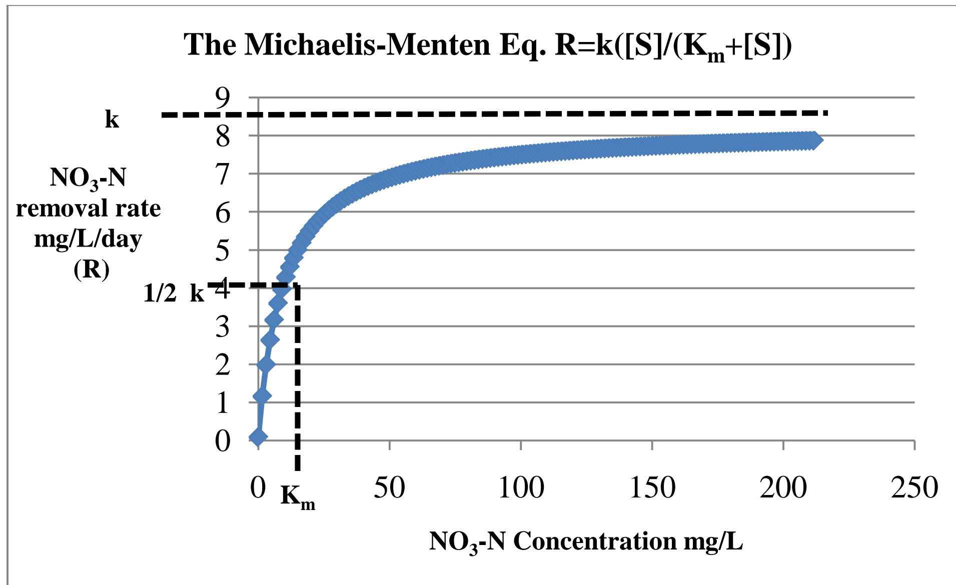

Figure 5.5. Modeled relationship between N concentration and N uptake rate of experimental data.

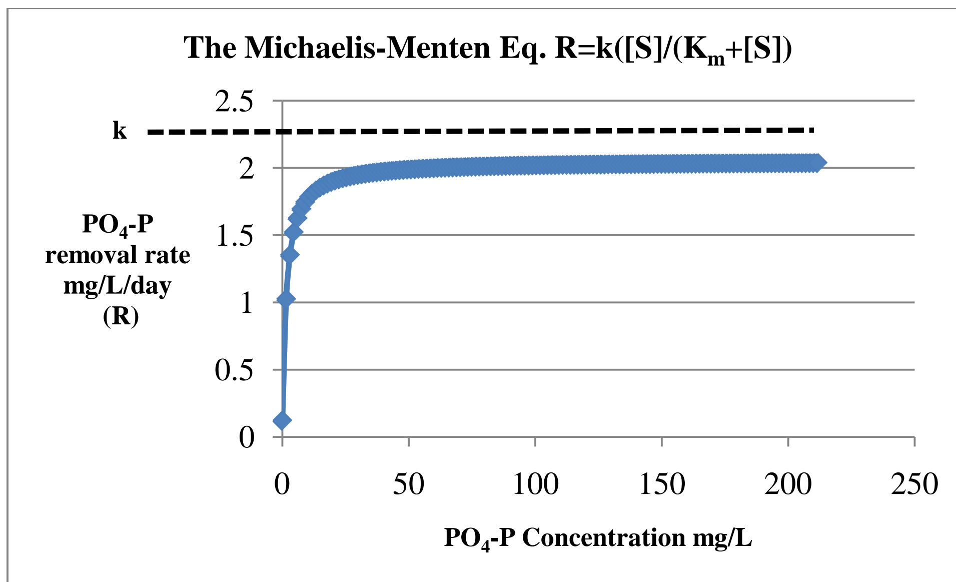

Figure 5.6. Modeled relationship between P concentration and P uptake rate of experimental data.

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This study established an affinity to nitrate, the reaction rate constant (k), of 8.47 mg NO_3-N/g biomass/day and a saturation constant or maximum uptake rate, (K_m) of 19.4 mg/L. The uptake rate of PO_4-P did not precisely fit Michaelis-Menten kinetics. Nonetheless, a best fit regression line did offer a reaction rate constant (k) of 2.05 mg PO_4-P/g biomass/day and a saturation constant (K_m) of 1.61 mg/L which is surprisingly in accord with what the experimental data would suggest. Figures 5.5 and 5.6 are the Michaelis-Menten models propagated with the calculated data K_m and k with varied substrate concentration. It is important to note that this model demonstrates a *nutrient limited uptake rate* and not a nutrient limited growth rate.

Aslan and Kapdan reported a k of 1.5 mg NH_4-N mg $^{-1}$ chl *a* day^{-1}, a K_m of 31.5 mg L^{-1} for ammonium nitrogen removal, and a k of 0.5 mg PO_4-P mg $^{-1}$ chl *a* day^{-1} and a K_m of 10.5 mg L^{-1}. These reported parameters differ somewhat from the results of the current study due to their measure of biomass or cell concentration as chlorophyll a.

The coefficients developed from the data will apply to the Michaelis-Menten model and be reasonably constant within a range of conditions and around the conditions used to generate the data for coefficient determination. The model developed here is a representation of a very complex system. Algal species selection, intraspecies differentiation, bioreactor size, media composition, light source, CO_2 concentration, temperature, and pH will all have effects on maximum uptake rate and reaction rate constants developed for N and P above. However, once biomass and substrate concentrations are measured the calculation steps outlined in the results section are easily applied to a larger scale.

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Is there an optimal biological formula suggested from this data?

Optimal growth in terms of biomass and optimal intrinsic growth rate for the overall study were both found in the BBM4N nutrient level. Growth was found to be nitrogen limited. The nitrogen concentration range suggested was 69.73 mg/L to 137.33 mg/L, based on utilization and study limitations. Using the yield kinetics model suggestion of N:P of 3:1, the phosphate concentration range would be 23 mg/L to 45 mg/L. The actual usage for P was 16.74 mg/L when 55.22 mg/L was available, so the yield model suggested concentration range is within study parameters. Kozlowska-Szerenos *et al.* (2000) found that *C. vulgaris* grown in medium with 45.5 mg/L P used five to 17% of the P, while those grown in medium with 4.5 mg/L used it all. Their range fits within that suggested here.

Can these experimentally obtained ranges function as standards? Will they target the algae's requirements for optimal growth? Will these standards fit within the parameters of available sewage effluent?

Wastewater, "sewage", can carry 34 to 48 mg/L of N in Mexico (Ruiz-Marin, Mendoza-Espinosa, & Stephenson 2010). Secondary sewage in a California system had a yearly mean of 5.0 mg/L N and 3.1 mg/L P (Craggs, *et al.*, 1996). In Spain, the secondary effluent was 28.1 mg/L N and 8.7 mg/L P (Martinez, *et al*., 2000). In the metro Atlanta area, the N influent load varies from 10.1 to 26.9 mg/L (Mines, Behrend, & Bell, 2004). The total N consumed in the current study ranged from 7.33 mg/L to 69.73 mg/L. This covers the wastewater load. BBM and BBM-0.75 levels most closely match these concentrations. Both fully utilized the initial N in the medium, BBM by day eight

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and BBM-0.75 by day six (Appendix C.1-C.5). This would suggest that either supplemental N would need to be added or the wastewater would need refreshing just prior to the limiting days. This should be a consideration in the design of any large scale operation. The results of this study indicate that meeting a discharge requirement of eight mg/L of N is feasible.

Looking at the local Dayton, Ohio wastewater (2009), Table 5.3, the influent contains sufficient N, 26 mg/L, to meet the growth requirements of the algae but not at optimal growth. A second source of N will be necessary (Gao, *et al.*, 2010; Gonzalez & Bashan, 2000; Klausmeier, Litchman, & Levin, 2007). The influent concentration most closely matches the initial BBM-0.05 medium and that reached N limitation on day six.

Table 5.3. Locally Available Waste Water, Dayton, OH

At 72 MGD	BOD	SS	NH3-N
Influent Concentration - mg/L	280	250	25
Primary Removal - %	32	63	N/A
Secondary Removal - %	85	85	N/A
AWT Influent - mg/L	40/45*	40/45*	26/26*
AWT Removal - %	80/45*	50/55*	92/70*
Effluent Filter Influent - mg/L		35	
Effluent Filter Removal - %		85	
Effluent Concentration - mg/L	12/30*	12/30*	2/8*
* = (summer/winter)			
(City of Dayton Water Department)			

Conclusion

In 1994 Becker wrote, "The successful growth of algae is more or less an art and a daily tightrope act with the aim of keeping the necessary prerequisites and various

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unpredictable events involved in algal mass cultivation in a sort of balance." The goal of this study was to define the nitrogen and phosphorus requirements of cultured *Chlorella vulgaris* in order to efficiently grow the algae in a carbon sequestering scheme by growing *C. vulgaris* under five different nutrient concentration regimes. I found the *C. vulgaris* to be nitrogen limited based on both nutrient removal rates and final biomass production.

Major conclusions include the following:

1. All nutrient levels appeared to have the longest period of exponential growth from day one to day five with corresponding population growth rate values decreasing with decreasing nutrients. Biomass means on day five and day eight were significantly different and biomass decreased as available N decreased.
2. Nitrogen removal rates ranged from 3.5 mg/L/day to 8.52 mg/L/day and P removal rates ranged from 0.73 mg/L/day to 2.08 mg/L/day. *C. vulgaris* can become N limited in as little as eight days using BBM. Phosphorus never became limiting when using BBM as a growth medium due to the relatively low N:P ratios found in BBM.
3. Batch kinetic coefficients of NO_3-N removal from experimental data were determined as k=8.47 mg NO_3-N/g biomass per day and K_m=19.4 mg/L. PO_4-P did not sufficiently fit models but is considered in both the Results and Discussion sections. The yield coefficient for NO_3-N was 0.0119 g biomass/mg NO_3-N, and the yield coefficient for PO_4-P was 0.037 g biomass/mg PO_4-P.
4. Nitrogen was a limiting factor applying both Liebig's law of the minimum and the Blackman Limitation. Suggested N values should fall between 69-137 mg/L N for an eight day growth cycle in order to achieve the greatest yield and/or highest rate of

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increase in biomass. No suggested range for P was discovered, but N should be greater than P and kinetic yield coefficient calculations suggest N:P should be at least 3 to 1.

5. Local Dayton, Ohio, wastewater contains sufficient N, 26 mg/L. This will meet the growth requirements of the alga but additional wastewater loading will be necessary to prevent N limitation. In addition, P availability and concentration of the wastewater will need to be explored in detail for it to provide optimal alga growth.

Most of the world's fossil fuel and industrial carbon emissions have little value at best, and will take on large costs in the future, both environmentally and monetarily. Algal photobioreactors are a technological tool that is suited to sequester this carbon dioxide. "Microalgae are a sustainable energy resource with great potential for CO_2 fixation" (Amin, 2009). This study is one component in the process.

Suggestions for Further Research

1. Bring the scheme outlined in this study to large-scale photobioreactors utilizing N and P concentrations suggested.
2. Apply methods outlined in this study to determine nutrient usage for other species of algae.
3. Refine study to determine optimal N:P ratios that will simplify growth of algae and minimize cost of nutrients.
4. Establish effects of N and P manipulation on algal lipid content relevant to biodiesel production.

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5. Test the suitability of wastewater, industrial, municipal, or agricultural, for algal growth.
6. Determine usability of varying flue gases as CO_2 source.
7. Cost analysis of a bioreactor CO_2 sequestration scheme.
8. Cost analysis of biofuel production from large photobioreactors.

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Appendix A. Standard Curves

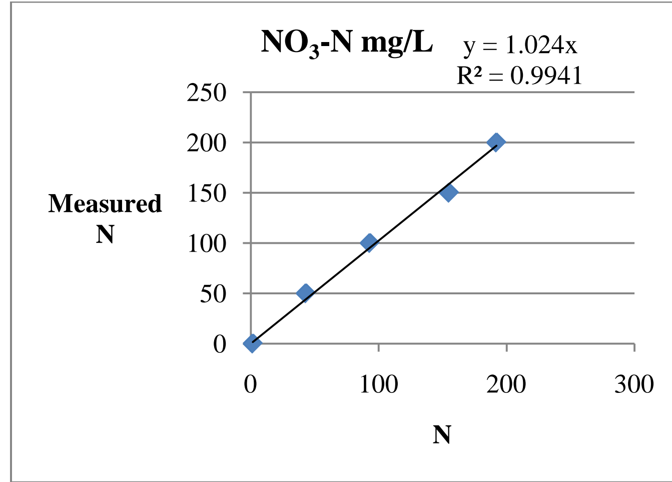

Figure A.1. Nitrate Standard Curve

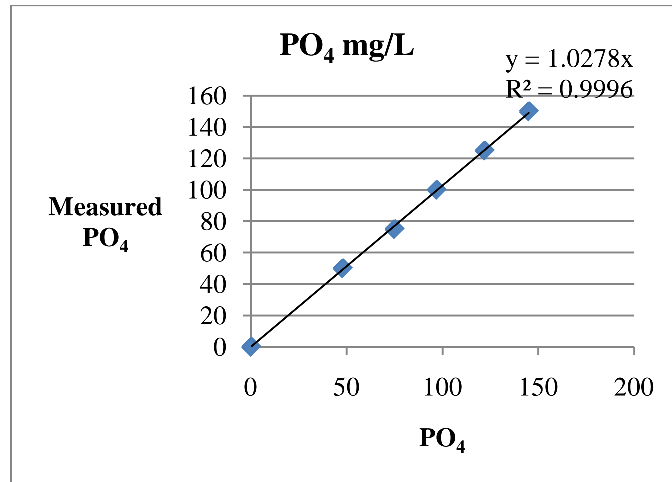

Figure A.2. Phosphate Standard Curve

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Appendix B. Biomass growth vs. time

Figures B.1-B.5. Biomass growth (± 95% confidence intervals) versus time of *C. vulgaris* cultured in variations of Bold's Basal Medium (BBM).

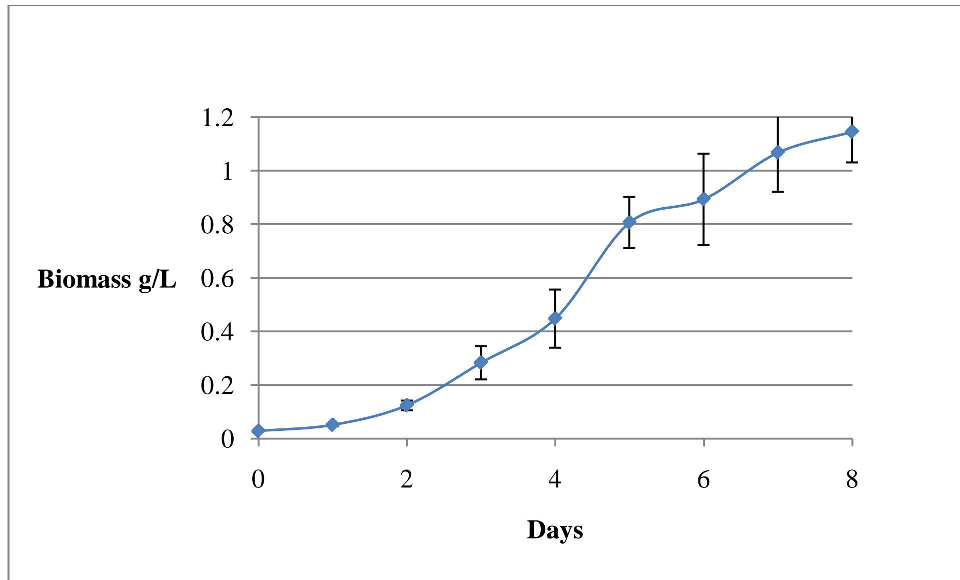

Figure B.1. BBM4N

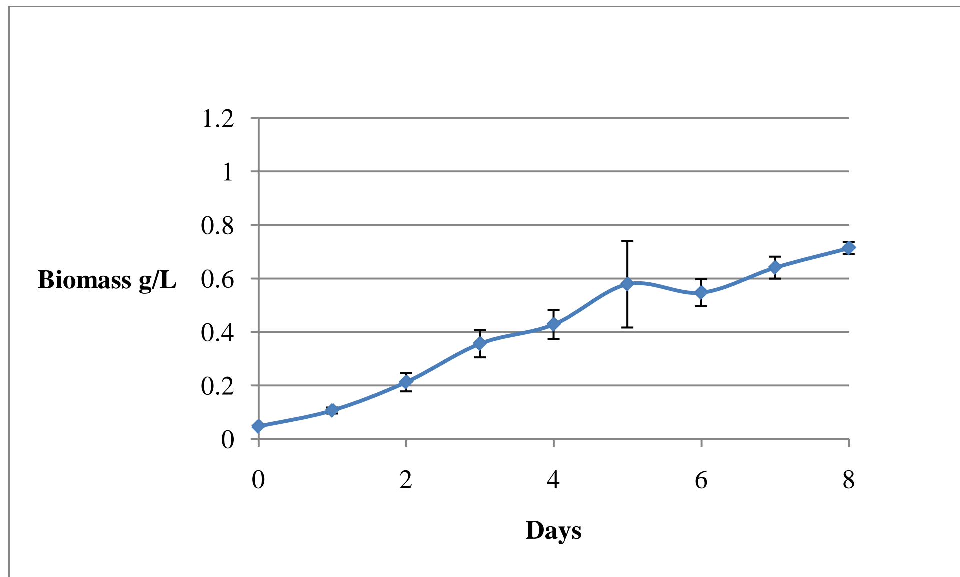

Figure B.2. BBM

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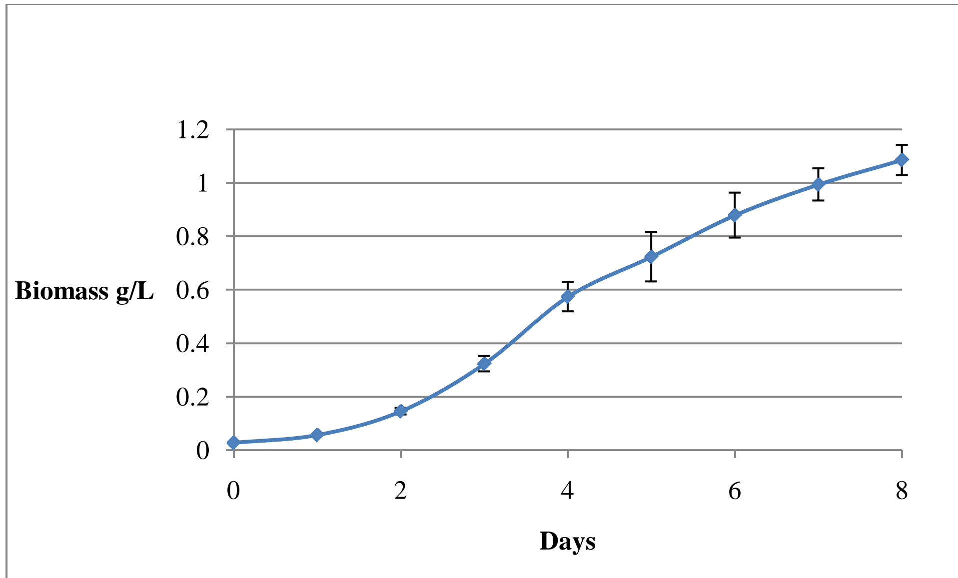

Figure B.3. BBM (0.75N, 0.75P)

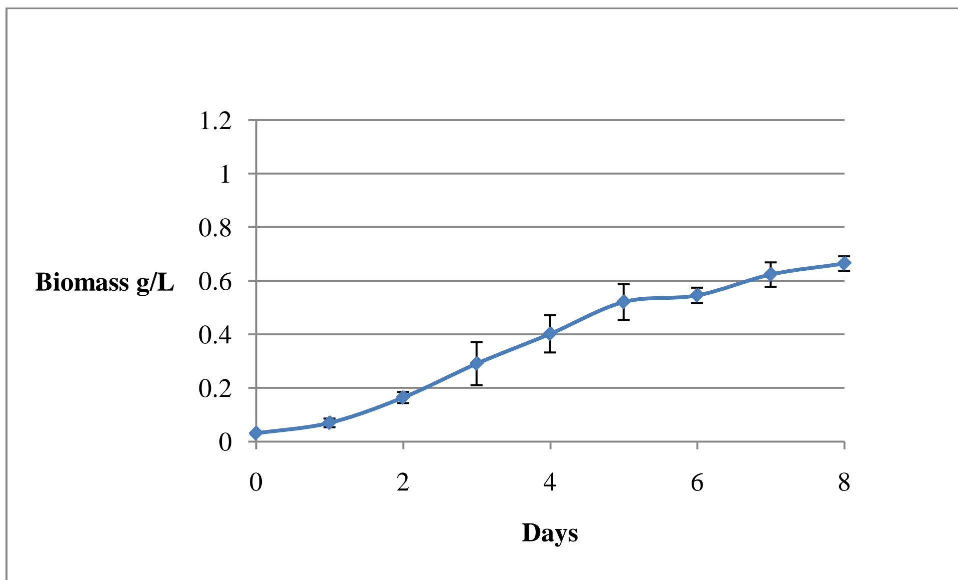

Figure B.4. BBM (0.5N, 0.5P)

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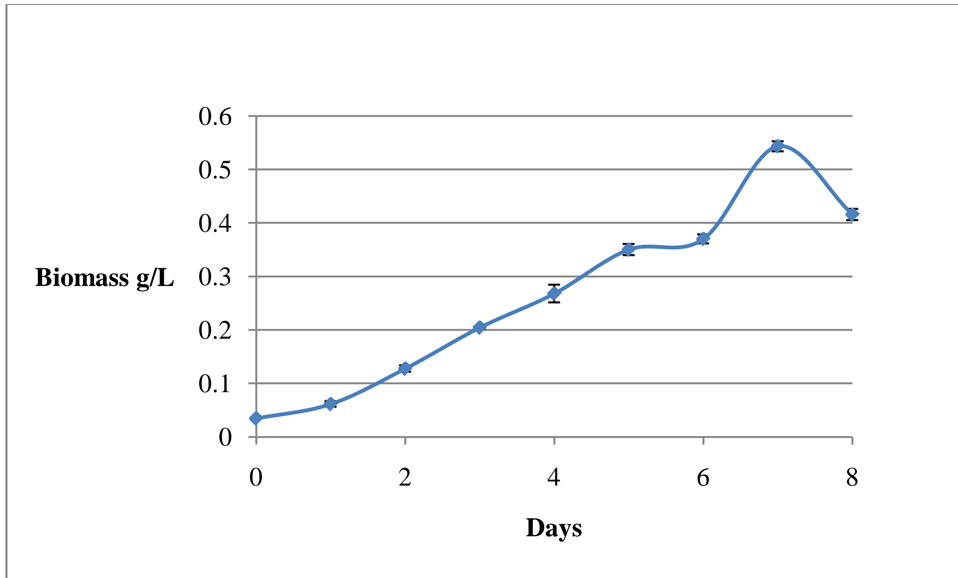
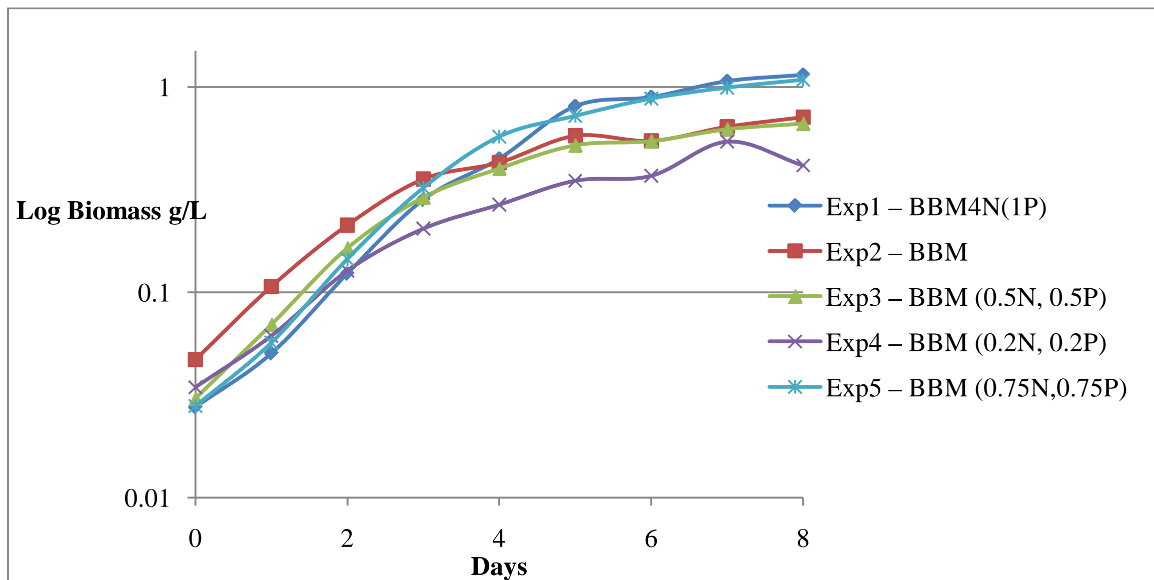


Figure B.5. BBM (0.2N, 0.2P)



Figures B.6. Log of biomass growth versus time of *C. vulgaris* cultured in variations of BBM.

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Appendix C. NO_3-N and PO_4-P Removal

Figures C.1-C.5 are summaries of NO_3-N removal with associated linear regression (error bars are ±90% confidence intervals).

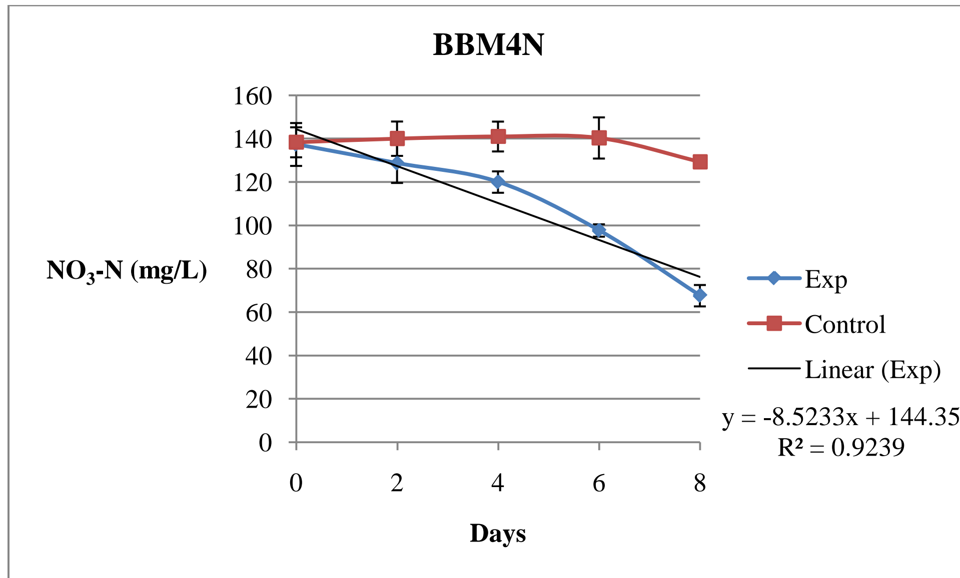

Figure C.1

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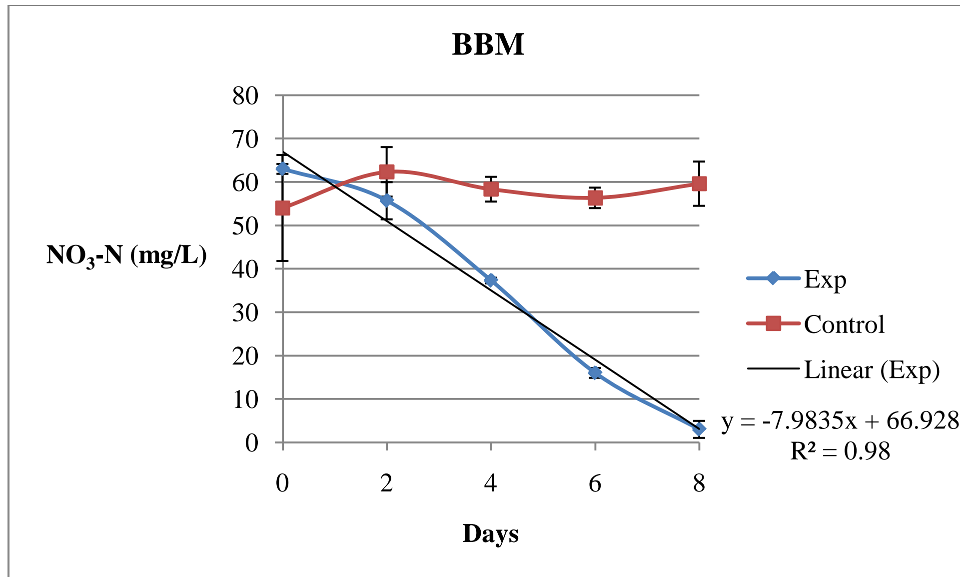

Figure C.2

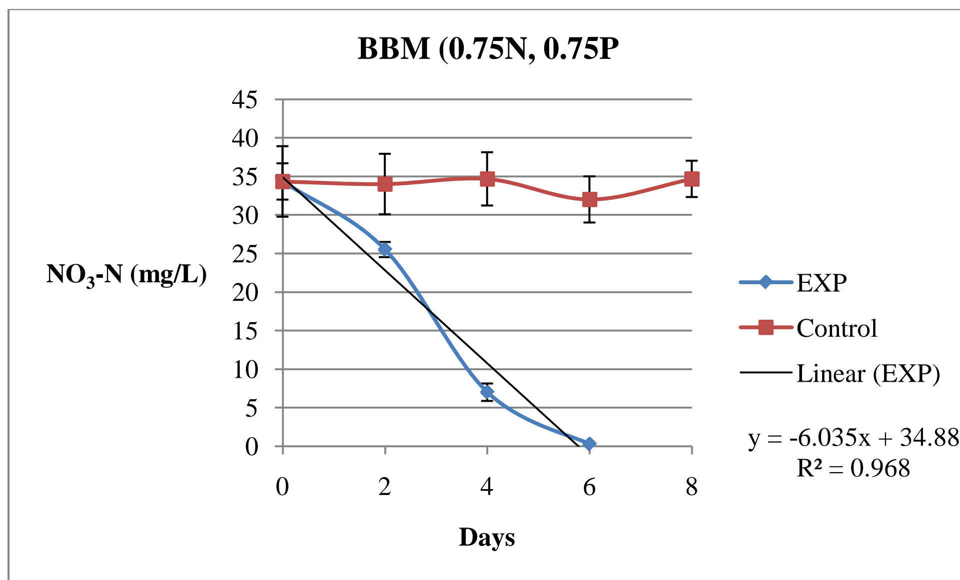

Figure C.3

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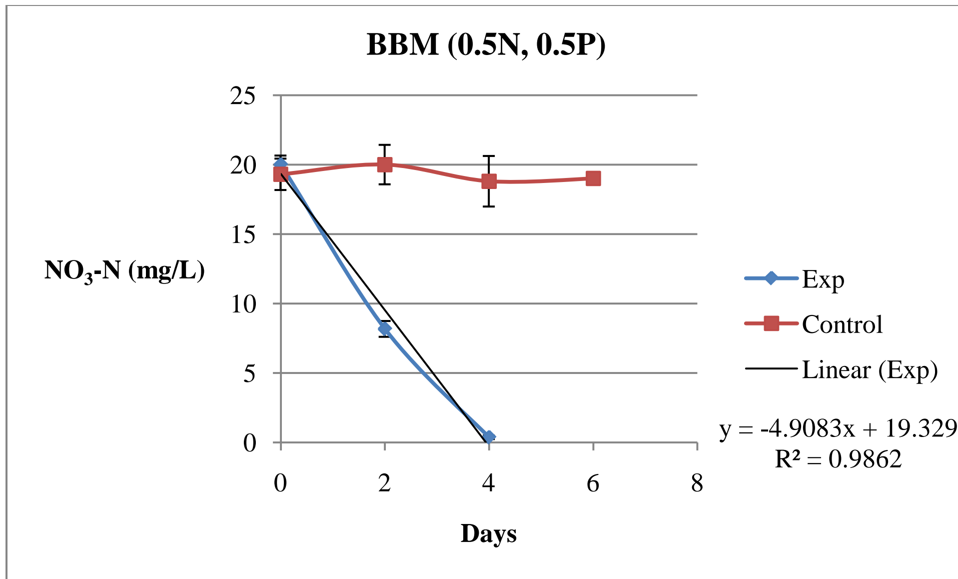

Figure C.4

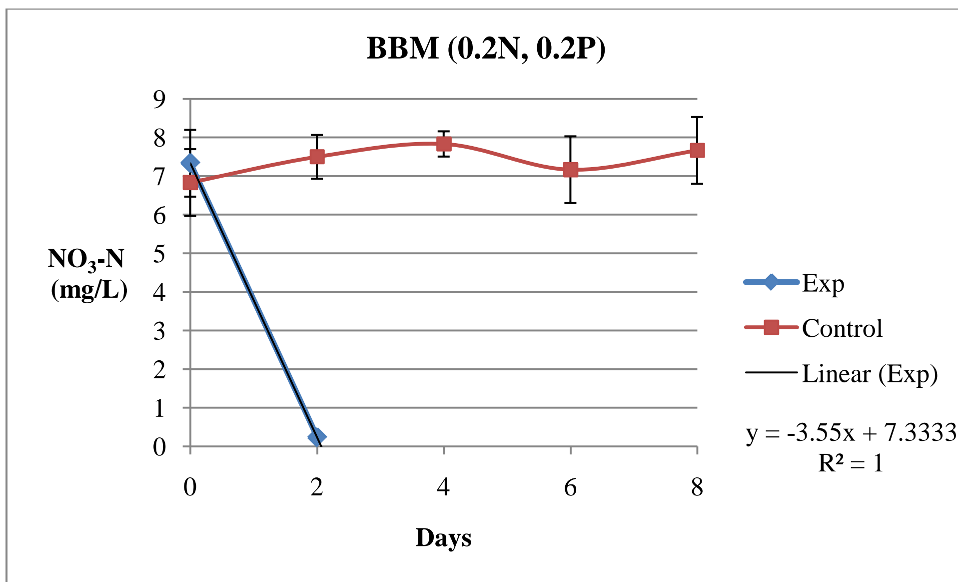

Figure C.5

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Figures C.6-C.9 are summaries of PO_4-P removal with associated linear regression (error bars are ±90% confidence intervals).

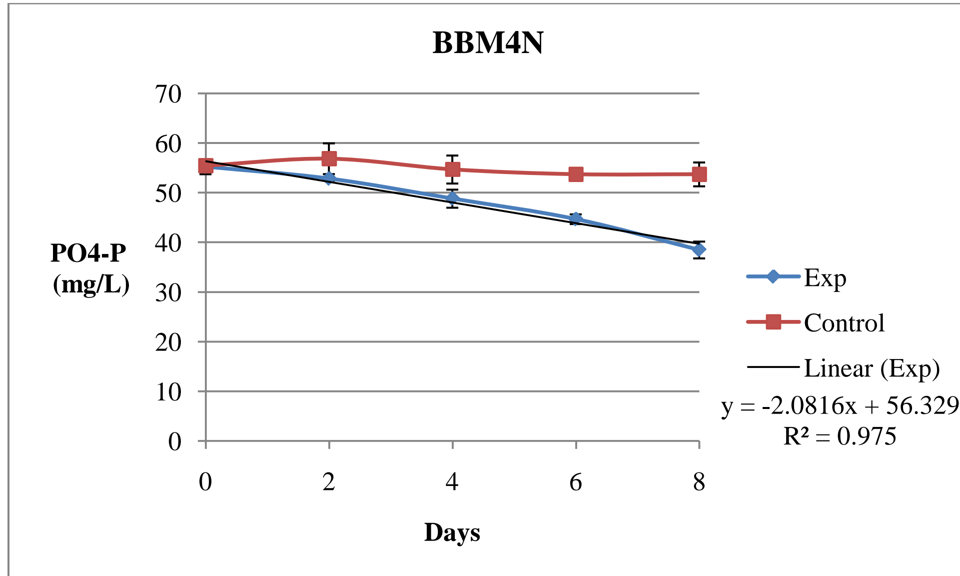

Figure C.6

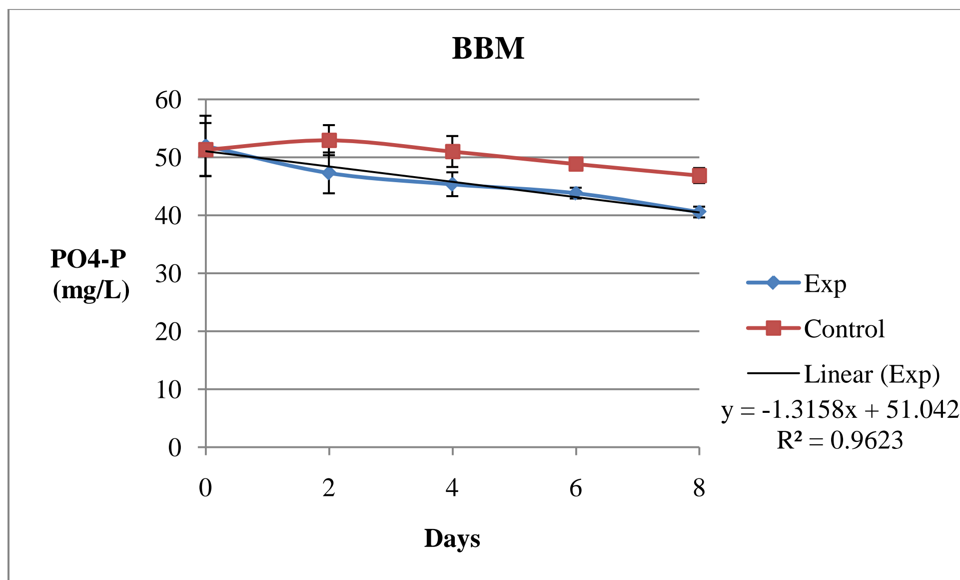

Figure C.7

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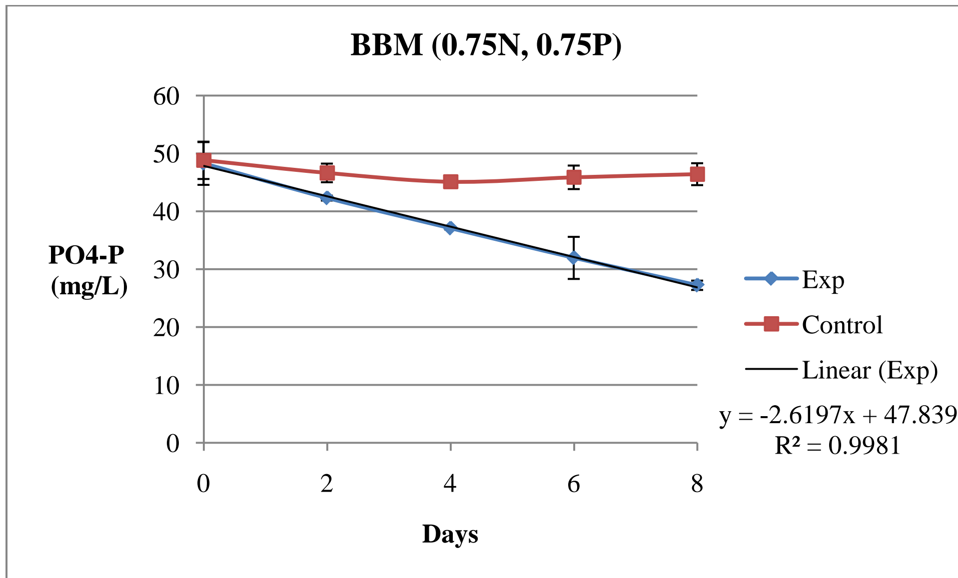

Figure C.7

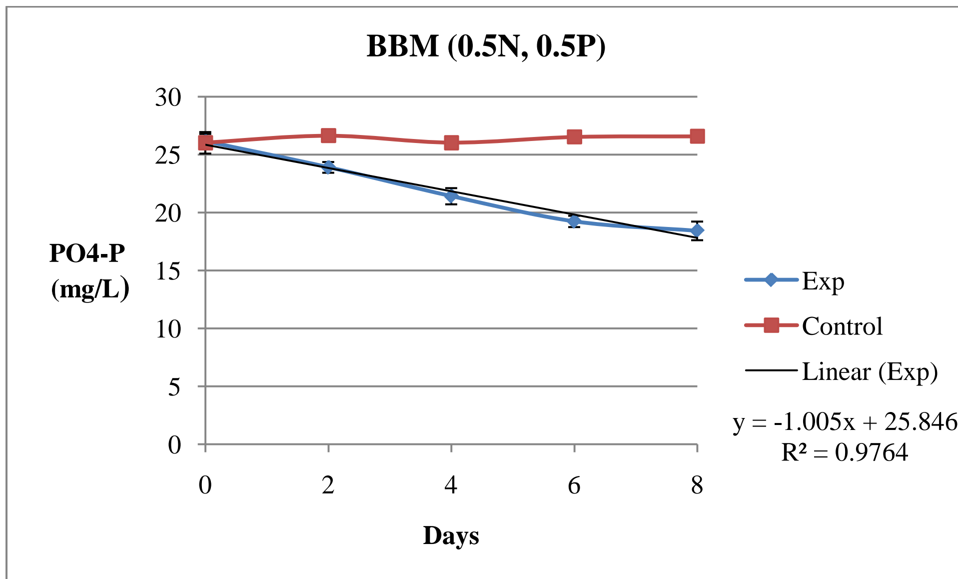

Figure C.8

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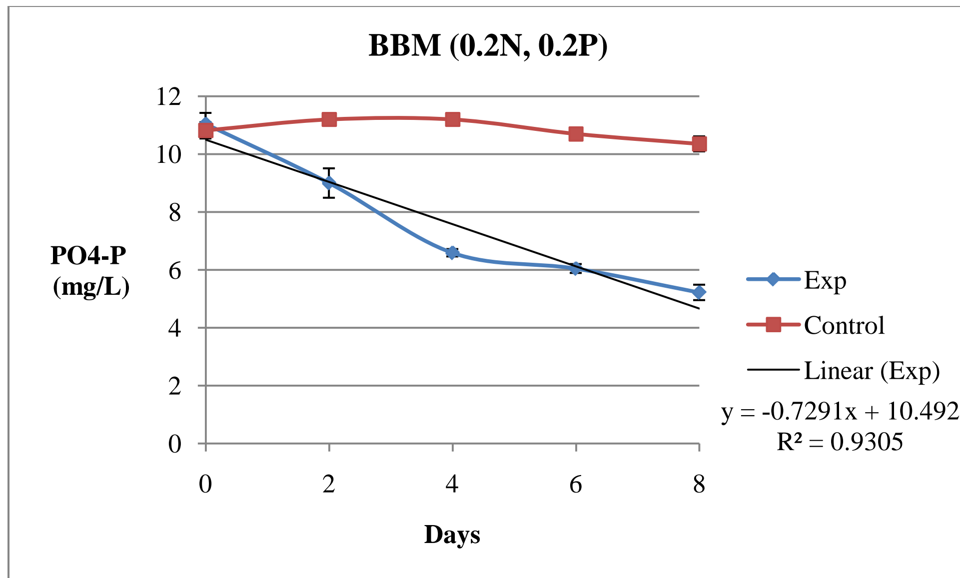

Figure C.9

Figures C.10-C.14. Graphs of NO_3-N and PO_4-P utilization through day 8 and log of growth with media containing different levels of N and P.

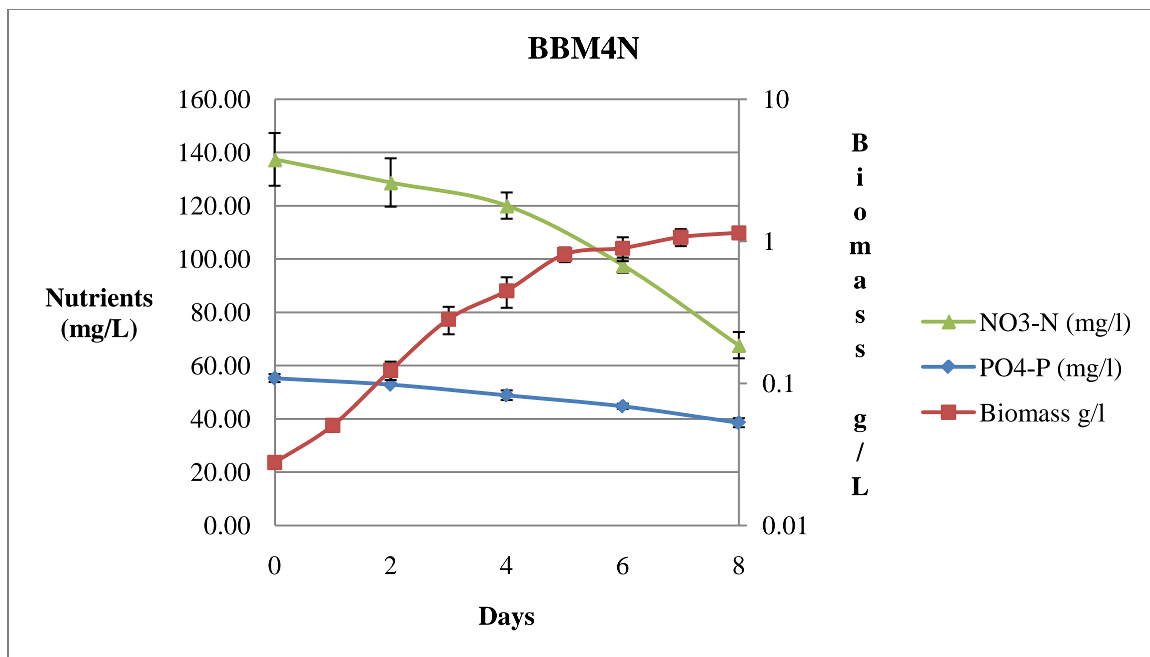

Figure C.10

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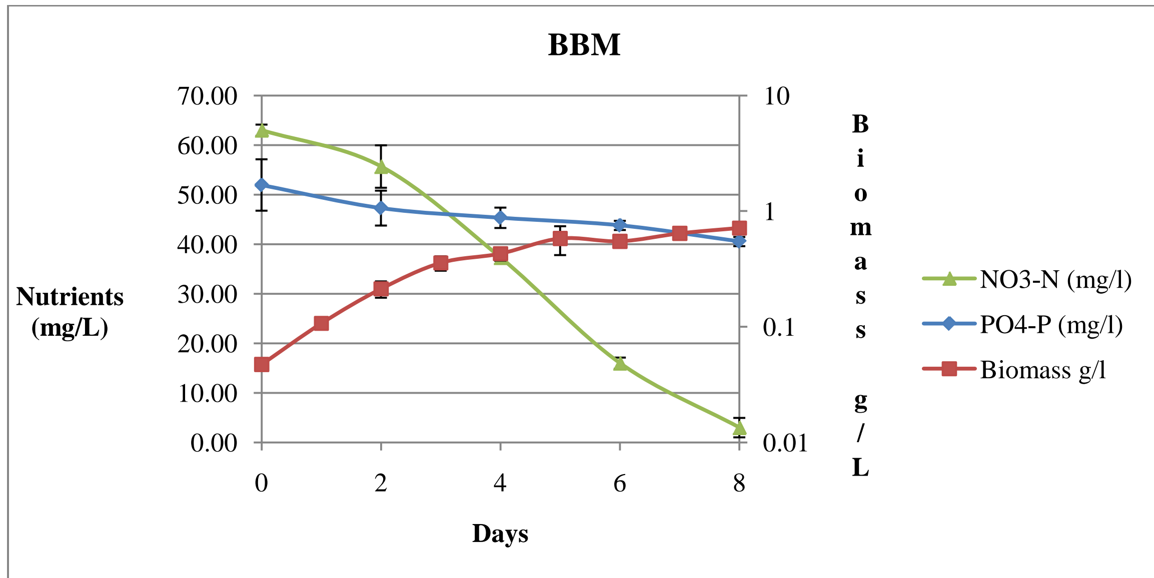

Figure C.11

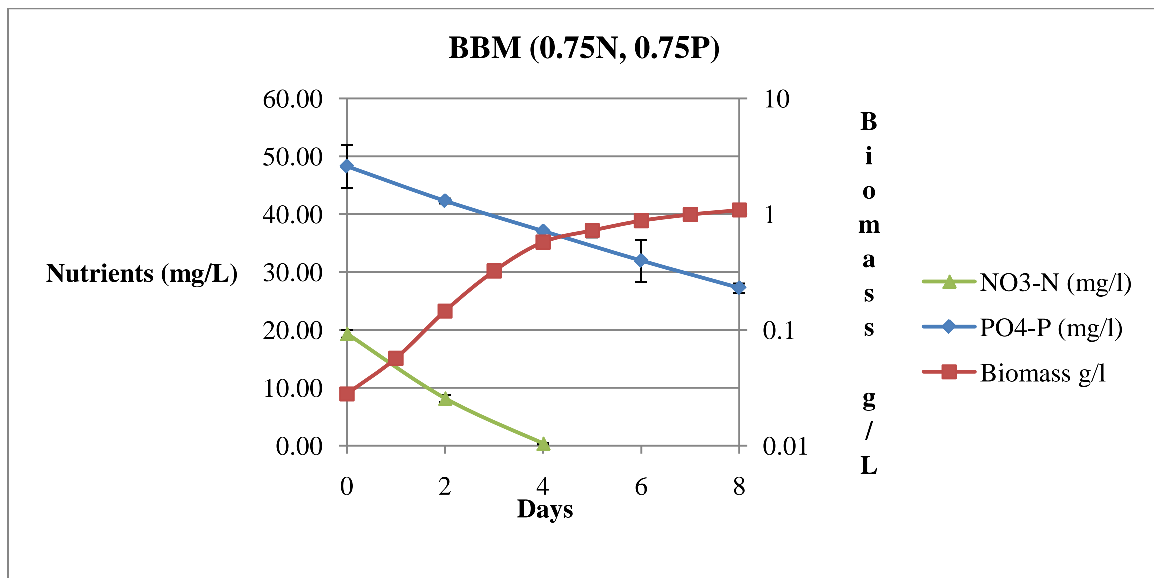

Figure C.12

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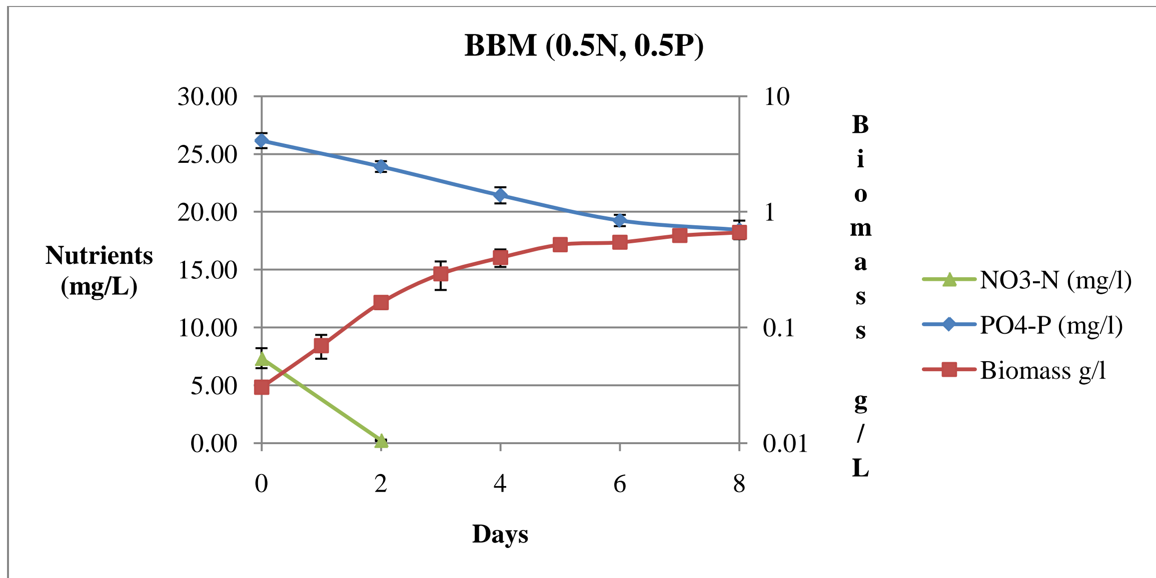

Figure C.13

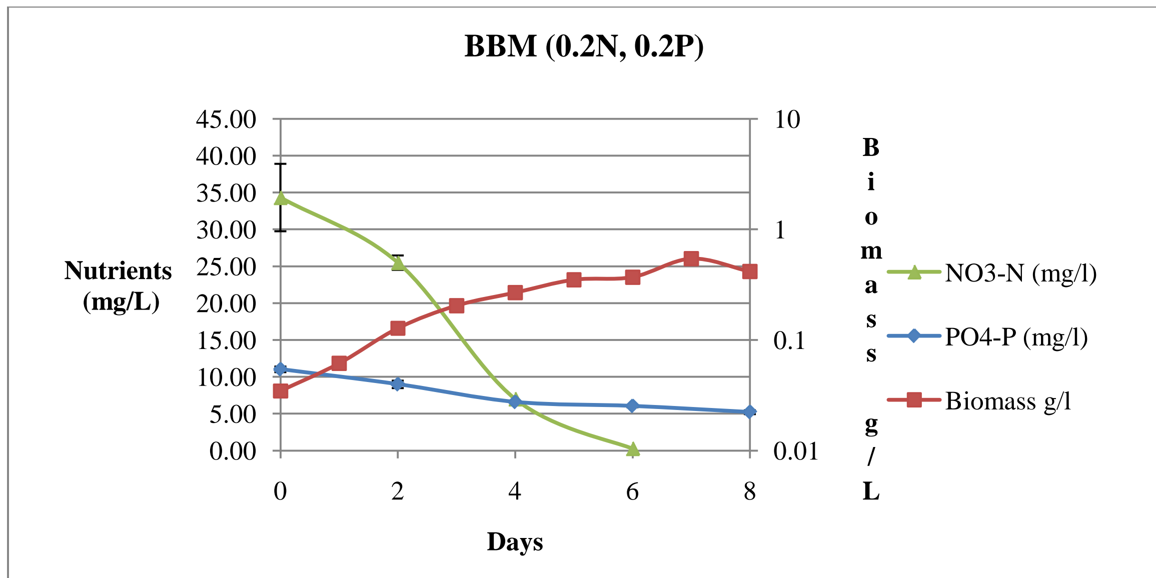

Figure C.14

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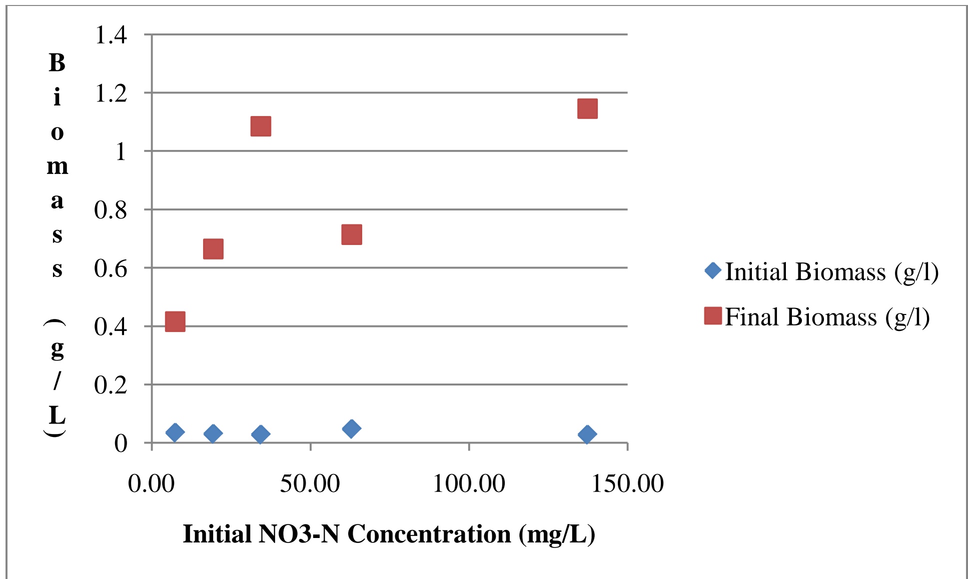

Figure C.15. Variation of biomass (g/L) with initial NO_3-N concentration.

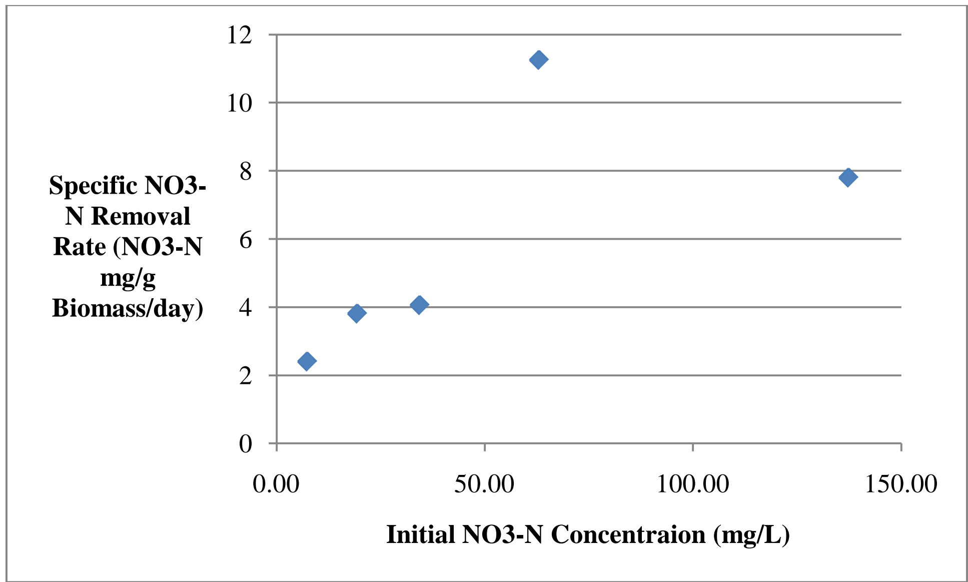

Figure C.16. Effect of initial NO_3-N on specific NO_3-N removal rate.

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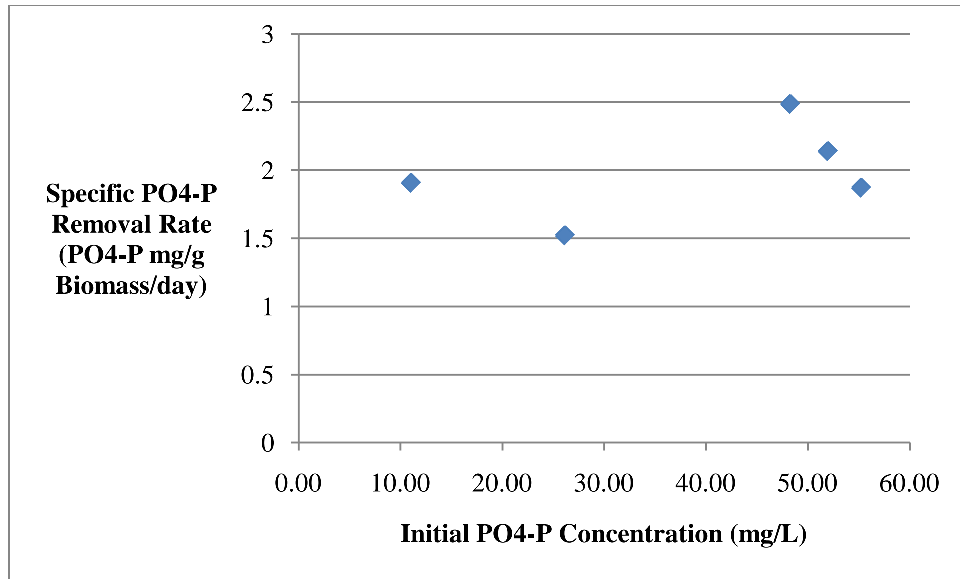

Figure C.17. Effect of initial PO_4-P on specific PO_4-P removal rate.

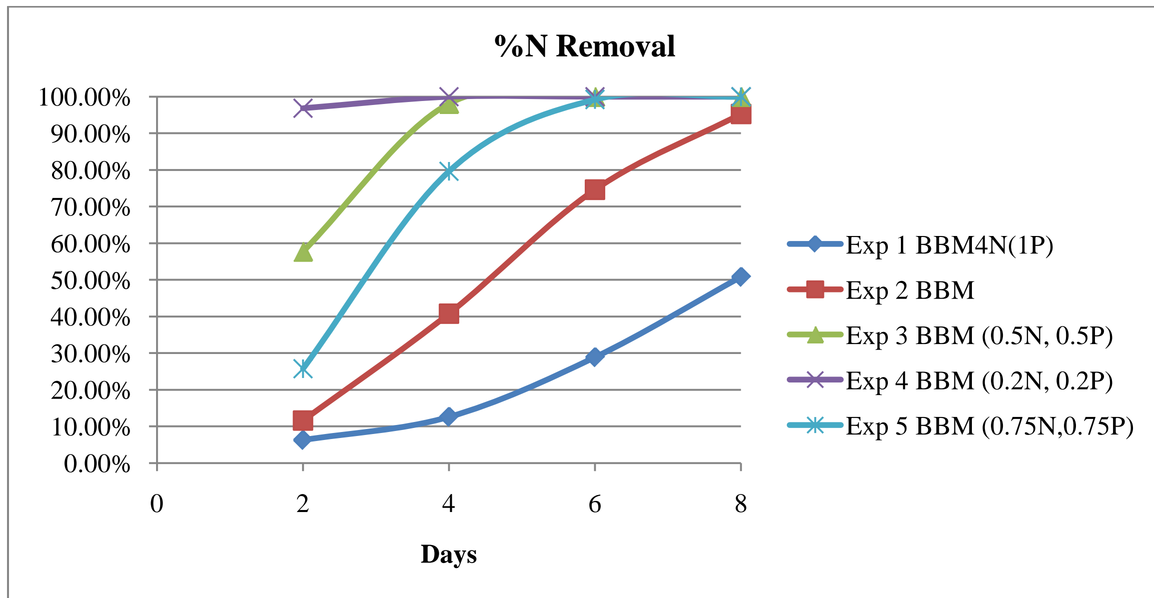

Figure C.18. Effect of media on percent NO_3-N removal.

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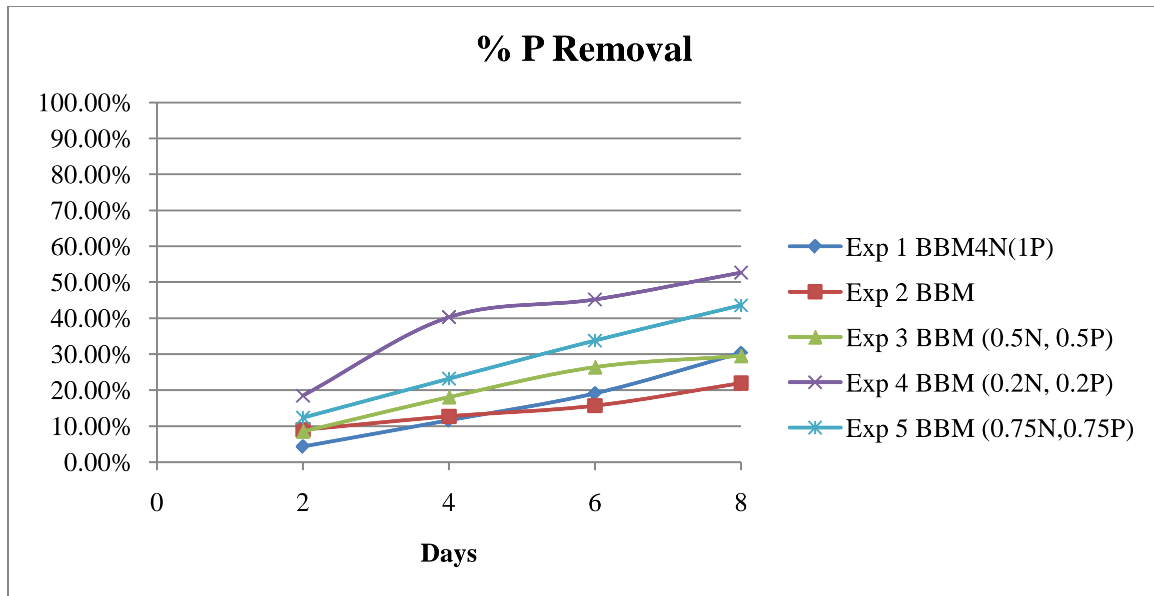

Figure C.19. Effect of media on percent PO_4-P removal.

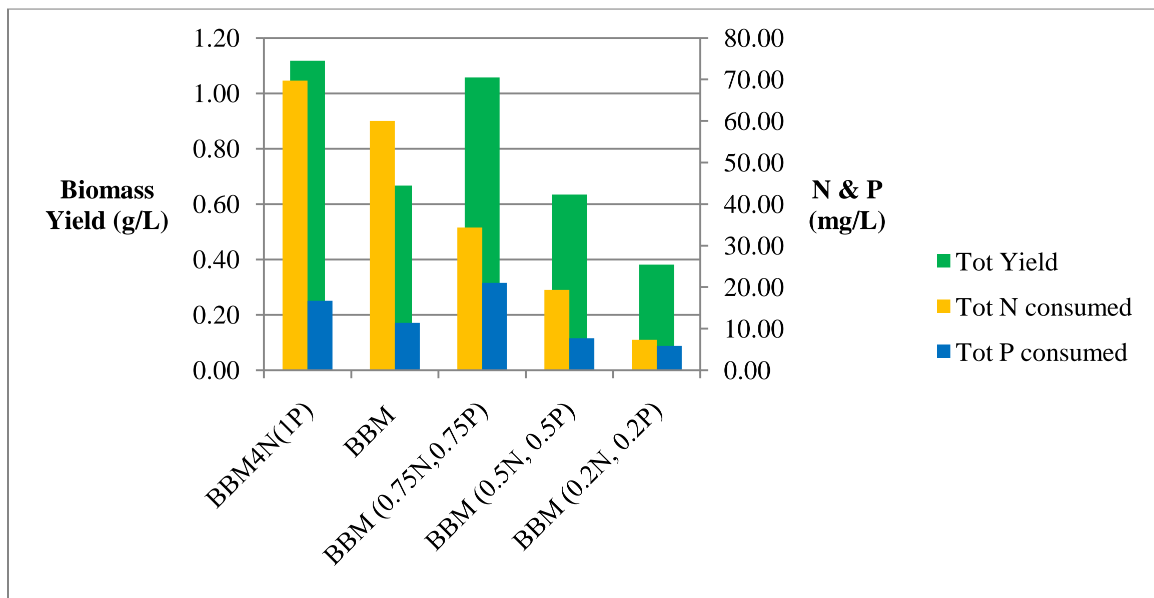

Figure C.20. Biomass produced vs. N and P consumed through day 8.

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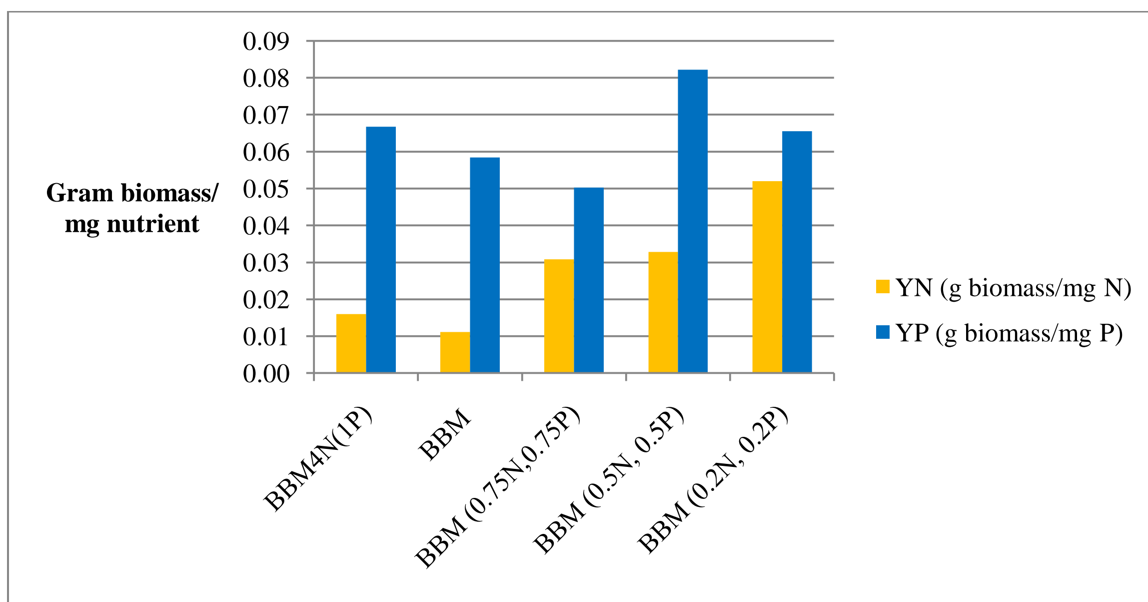

Figure C.21. Gram of biomass produced per mg of nutrient consumed through days 8 for various BBM concentrations.

Appendix D. Experimental Data

Table D.1. Days 0-8 absorbance data (550 nm)

BBM4N

Day	0	1	2	3	4	5	6	7	8
Trial 1	0.181	0.292	0.674	1.420	2.200	4.500	4.610	5.810	6.510
Trial 2	0.169	0.333	0.776	1.850	3.210	5.400	6.160	7.010	7.590
Trial 3	0.165	0.315	0.848	1.990	2.920	5.100	5.840	7.040	7.200
Mean	0.172	0.313	0.766	1.753	2.777	5.000	5.537	6.620	7.100
S.D.	0.008	0.021	0.087	0.297	0.520	0.458	0.818	0.702	0.547
C.I. 95%	0.011	0.027	0.113	0.384	0.673	0.593	1.059	0.908	0.708

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BBM

Trial 1	0.292	0.721	1.260	1.930	2.370	2.720	3.220	3.905	4.490
Trial 2	0.292	0.615	1.505	2.370	2.720	3.840	3.290	3.820	4.300
Trial 3	0.292	0.654	1.193	2.330	2.880	4.210	3.670	4.195	4.485
Mean	0.292	0.663	1.319	2.210	2.657	3.590	3.393	3.973	4.425
S.D.	0.000	0.054	0.164	0.243	0.261	0.776	0.242	0.197	0.108
C.I. 95%	#NUM!	0.069	0.213	0.315	0.338	1.004	0.313	0.254	0.140

BBM (0.5N, 0.5P)

Trial 1	0.189	0.490	0.987	1.410	2.140	2.940	3.420	3.690	4.030
Trial 2	0.189	0.344	0.940	1.820	2.540	3.180	3.230	3.800	4.060
Trial 3	0.189	0.464	1.130	2.180	2.800	3.570	3.500	4.110	4.270
Mean	0.189	0.433	1.019	1.803	2.493	3.230	3.383	3.867	4.120
S.D.	0.000	0.078	0.099	0.385	0.332	0.318	0.139	0.218	0.131
C.I. 95%	0.000	0.101	0.128	0.499	0.430	0.411	0.179	0.282	0.169

BBM (0.2N, 0.2P)

Trial 1	0.206	0.356	0.758	1.260	1.690	2.220	2.270	3.320	2.630
Trial 2	0.217	0.407	0.810	1.260	1.720	2.170	2.340	3.410	2.570
Trial 3	0.220	0.379	0.805	1.280	1.570	2.120	2.270	3.370	2.530
Mean	0.214	0.381	0.791	1.267	1.660	2.170	2.293	3.367	2.577
S.D.	0.007	0.026	0.029	0.012	0.079	0.050	0.040	0.045	0.050
C.I. 95%	0.010	0.033	0.037	0.015	0.103	0.065	0.052	0.058	0.065

BBM (0.75N,0.75P)

Trial 1	0.175	0.339	0.834	1.897	3.340	4.610	5.600	6.390	6.830
Trial 2	0.177	0.370	0.946	2.157	3.850	4.850	5.750	6.250	6.930
Trial 3	0.168	0.350	0.924	1.950	3.480	3.990	4.990	5.835	6.420
Mean	0.173	0.353	0.901	2.001	3.557	4.483	5.447	6.158	6.727
S.D.	0.005	0.016	0.059	0.137	0.264	0.444	0.403	0.289	0.270
C.I. 95%	0.006	0.021	0.077	0.178	0.341	0.574	0.521	0.374	0.350

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Table D.2. Days 0-8 biomass data (g/L)

BBM4N

Biomass g/L									
Day	0	1	2	3	4	5	6	7	8
Trial 1	0.029	0.047	0.109	0.229	0.355	0.726	0.744	0.937	1.050
Trial 2	0.027	0.054	0.125	0.298	0.518	0.871	0.994	1.131	1.224
Trial 3	0.027	0.051	0.137	0.321	0.471	0.823	0.942	1.136	1.161
Mean	0.028	0.051	0.124	0.283	0.448	0.807	0.893	1.068	1.145
S.D.	0.001	0.003	0.014	0.048	0.084	0.074	0.132	0.113	0.088
C.I. 95%	0.002	0.004	0.018	0.062	0.109	0.096	0.171	0.146	0.114

BBM

Biomass g/L									
Trial 1	0.047	0.116	0.203	0.311	0.382	0.439	0.519	0.630	0.724
Trial 2	0.048	0.099	0.243	0.382	0.439	0.619	0.531	0.616	0.694
Trial 3	0.047	0.105	0.192	0.376	0.465	0.679	0.592	0.677	0.723
Mean	0.047	0.107	0.213	0.356	0.429	0.579	0.547	0.641	0.714
S.D.	0.000	0.009	0.026	0.039	0.042	0.125	0.039	0.032	0.017
C.I. 95%	0.000	0.011	0.034	0.051	0.054	0.162	0.051	0.041	0.023

BBM (0.5N, 0.5P)

Biomass g/L									
Trial 1	0.030	0.079	0.159	0.227	0.345	0.474	0.552	0.595	0.650
Trial 2	0.030	0.055	0.152	0.294	0.410	0.513	0.521	0.613	0.655
Trial 3	0.030	0.075	0.182	0.352	0.452	0.576	0.565	0.663	0.689
Mean	0.030	0.070	0.164	0.291	0.402	0.521	0.546	0.624	0.665
S.D.	0.000	0.013	0.016	0.062	0.054	0.051	0.022	0.035	0.021
C.I. 95%	#NUM!	0.016	0.021	0.080	0.069	0.066	0.029	0.045	0.027

BBM (0.2N, 0.2P)

Biomass g/L									
Trial 1	0.033	0.057	0.122	0.203	0.273	0.358	0.366	0.536	0.424

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Trial 2	0.035	0.066	0.131	0.203	0.277	0.350	0.377	0.550	0.415
Trial 3	0.035	0.061	0.130	0.206	0.253	0.342	0.366	0.544	0.408
Mean	0.035	0.061	0.128	0.204	0.268	0.350	0.370	0.543	0.416
S.D.	0.001	0.004	0.005	0.002	0.013	0.008	0.007	0.007	0.008
C.I. 95%	0.002	0.005	0.006	0.002	0.017	0.010	0.008	0.009	0.011

BBM (0.75N,0.75P)

Biomass g/L									
Trial 1	0.028	0.055	0.135	0.306	0.539	0.744	0.903	1.031	1.102
Trial 2	0.029	0.060	0.153	0.348	0.621	0.782	0.927	1.008	1.118
Trial 3	0.027	0.056	0.149	0.315	0.561	0.644	0.805	0.941	1.036
Mean	0.028	0.057	0.145	0.323	0.574	0.723	0.879	0.993	1.085
S.D.	0.001	0.003	0.010	0.022	0.043	0.072	0.065	0.047	0.044
C.I. 95%	0.001	0.003	0.012	0.029	0.055	0.093	0.084	0.060	0.056

Table D.3. NO_3-N removal data (mg/L)

BBM4N

Day	0	2	4	6	8
Trial 1	130.0	137.0	118.0	100.0	75.0
Trial 2	147.0	128.0	117.0	98.0	63.0
Trial 3	135.0	121.0	125.0	95.0	65.0
Ave	137.33	128.67	120.00	97.67	67.67
S.D.	8.74	8.02	4.36	2.52	6.43
CI 95%	11.31	10.38	5.64	3.26	8.32

BBM

Trial 1	63.0	53.0	38.0	17.0	2.0
Trial 2	62.0	54.0	37.0	16.0	2.0
Trial 3	64.0	60.0	37.0	15.0	5.0
Ave	63.00	55.67	37.33	16.00	3.00
S.D.	1.00	3.79	0.58	1.00	1.73
CI 95%	1.29	4.90	0.75	1.29	2.24

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BBM (0.5N, 0.5P)

Trial 1	20.0	9.5	0.3		
Trial 2	19.0	7.0	0.3		
Trial 3	19.0	8.0	0.5		
Ave	19.33	8.17	0.37		
S.D.	0.58	1.26	0.12		
CI 95%	0.75	1.63	0.15		

BBM (0.2N, 0.2P)

Trial 1	6.5	0.2			
Trial 2	8.0	0.2			
Trial 3	7.5	0.3			
Ave	7.33	0.23			
S.D.	0.76	0.06			
CI 95%	0.99	0.07			

BBM (0.75N,0.75P)

Trial 1	30.0	25.0	6.0	0.3	
Trial 2	35.0	25.0	8.0	0.2	
Trial 3	38.0	26.5	7.0	0.3	
Ave	34.33	25.50	7.00	0.27	
S.D.	4.04	0.87	1.00	0.06	
CI 95%	5.23	1.12	1.29	0.07	

Table D.4. PO_4-P removal data (mg/L)

BBM4N

Day	0	2	4	6	8
Trial 1	53.81	52.83	49.89	45.65	40.11
Trial 2	56.42	53.15	46.96	44.02	38.15
Trial 3	55.44	52.50	49.57	44.35	37.18
Ave	55.22	52.83	48.81	44.68	38.48
S.D.	1.32	0.33	1.61	0.86	1.49
CI 95%	1.71	0.42	2.08	1.12	1.93

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BBM

Trial 1	47.61	46.96	46.96	44.68	40.44
Trial 2	56.74	50.55	43.37	43.70	41.42
Trial 3	51.52	44.35	45.65	43.05	39.78
Ave	51.96	47.28	45.33	43.81	40.55
S.D.	4.58	3.11	1.82	0.82	0.82
CI 95%	5.93	4.03	2.35	1.06	1.06

BBM (0.5N, 0.5P)

Trial 1	26.09	23.48	21.85	18.75	19.24
Trial 2	25.60	23.97	20.71	19.57	17.94
Trial 3	26.74	24.29	21.69	19.40	18.10
Ave	26.14	23.91	21.41	19.24	18.42
S.D.	0.57	0.41	0.62	0.43	0.71
CI 95%	0.74	0.53	0.80	0.56	0.92

BBM (0.2N, 0.2P)

Trial 1	10.92	9.29	6.72	5.94	5.28
Trial 2	11.41	9.21	6.52	6.00	4.96
Trial 3	10.76	8.48	6.52	6.20	5.41
Ave	11.03	9.00	6.59	6.04	5.22
S.D.	0.34	0.45	0.11	0.14	0.24
CI 95%	0.44	0.58	0.15	0.18	0.30

BBM
(0.75N,0.75P)

Trial 1	48.26	42.07	37.18	32.94	26.74
Trial 2	51.52	42.72	36.85	28.37	26.90
Trial 3	45.00	42.07	37.18	34.57	28.04
Ave	48.26	42.28	37.07	31.96	27.23
S.D.	3.26	0.38	0.19	3.21	0.71
CI 95%	4.22	0.49	0.24	4.16	0.92

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Appendix E. Summary Statistics

Table E.1. Summary statistics of biomass. ANOVA and Tukey's Test. (*) annotates critical difference (CD) at 0.05 and (**) annotates CD at 0.01.

ANOVA: Single Factor		Day 1			
SUMMARY					
Groups	*Count*	*Sum*	*Average*	*Variance*	
Exp 5 BBM (0.75N,0.75P)	3.00	0.09	0.03	0.00	
Exp 1 BBM4N(1P)	3.00	0.07	0.02	0.00	
Exp 2 BBM	3.00	0.18	0.06	0.00	
Exp 3 BBM (0.5N, 0.5P)	3.00	0.12	0.04	0.00	
Exp 4 BBM (0.2N, 0.2P)	3.00	0.08	0.03	0.00	
ANOVA					
Source of Variation	*SS*	*df*	*MS*	*F*	*F crit*
Between Groups	0.00	4.00	0.00	12.12	3.48
Within Groups	0.00	10.00	0.00		
Total	0.00	14.00			

Day 1

Null Hypoth.	Sign	CD	CD.05	CD.01	
Ho:Exp2=Exp3	*	0.02	0.02	0.02	
Ho:Exp2=Exp4	**	0.03			
Ho:Exp2=Exp5	**	0.03			
Ho:Exp2=Exp1	**	0.04			
Ho:Exp3=Exp4		0.01			
Ho:Exp3=Exp5		0.01			
Ho:Exp3=Exp1		0.02			
Ho:Exp4=Exp5		0.00			
Ho:Exp4=Exp1		0.00			

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Ho:Exp5=Exp1		0.01			

ANOVA: Single Factor		Day 2			
SUMMARY					
Groups	*Count*	*Sum*	*Average*	*Variance*	
Exp 1 BBM4N(1P)	3.00	0.29	0.10	0.00	
Exp 2 BBM	3.00	0.50	0.17	0.00	
Exp 3 BBM (0.5N, 0.5P)	3.00	0.40	0.13	0.00	
Exp 4 BBM (0.2N, 0.2P)	3.00	0.28	0.09	0.00	
Exp 5 BBM (0.75N,0.75P)	3.00	0.35	0.12	0.00	
ANOVA					
Source of Variation	*SS*	*df*	*MS*	*F*	
Between Groups	0.01	4.00	0.00	10.42	*F crit*
Within Groups	0.00	10.00	0.00		3.48
Total	0.01	14.00			

Day 2

Null Hypoth.	Sign	CD	CD.05	CD.01	
Ho:Exp2=Exp3		0.03	0.04	0.05	
Ho:Exp2=Exp4	**	0.07			
Ho:Exp2=Exp5	*	0.05			
Ho:Exp2=Exp1	**	0.07			
Ho:Exp3=Exp4	*	0.04			
Ho:Exp3=Exp5		0.02			
Ho:Exp3=Exp1		0.04			
Ho:Exp4=Exp5		0.02			
Ho:Exp4=Exp1		0.00			
Ho:Exp5=Exp1		0.02			

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ANOVA: Single Factor		Day 3			
SUMMARY					
Groups	*Count*	*Sum*	*Average*	*Variance*	
Exp 1 BBM4N(1P)	3.00	0.77	0.26	0.00	
Exp 2 BBM	3.00	0.93	0.31	0.00	
Exp 3 BBM (0.5N, 0.5P)	3.00	0.78	0.26	0.00	
Exp 4 BBM (0.2N, 0.2P)	3.00	0.51	0.17	0.00	
Exp 5 BBM (0.75N,0.75P)	3.00	0.88	0.29	0.00	
ANOVA					
Source of Variation	*SS*	*df*	*MS*	*F*	
Between Groups	0.04	4.00	0.01	5.33	
Within Groups	0.02	10.00	0.00		*F crit*
					3.48
Total	0.05	14.00			

Day 3

Null Hypoth.	Sign	CD	CD.05	CD.01	
Ho:Exp2=Exp3		0.05	0.10	0.14	
Ho:Exp2=Exp4	**	0.14			
Ho:Exp2=Exp5		0.01			
Ho:Exp2=Exp1		0.05			
Ho:Exp3=Exp4		0.09			
Ho:Exp3=Exp5		0.03			
Ho:Exp3=Exp1		0.01			
Ho:Exp4=Exp5	*	0.13			
Ho:Exp4=Exp1		0.09			
Ho:Exp5=Exp1		0.04			

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ANOVA: Single Factor		Day 4			
SUMMARY					
Groups	*Count*	*Sum*	*Average*	*Variance*	
Exp 1 BBM4N(1P)	3.00	1.26	0.42	0.01	
Exp 2 BBM	3.00	1.14	0.38	0.00	
Exp 3 BBM (0.5N, 0.5P)	3.00	1.12	0.37	0.00	
Exp 4 BBM (0.2N, 0.2P)	3.00	0.70	0.23	0.00	
Exp 5 BBM (0.75N,0.75P)	3.00	1.64	0.55	0.00	
ANOVA					
Source of Variation	*SS*	*df*	*MS*	*F*	
Between Groups	0.15	4.00	0.04	13.60	
Within Groups	0.03	10.00	0.00		*F crit*
					3.48
Total	0.18	14.00			

Day 4

Null Hypoth.	Sign	CD	CD.05	CD.01	
Ho:Exp2=Exp3		0.01	0.13	0.18	
Ho:Exp2=Exp4	*	0.15			
Ho:Exp2=Exp5	*	0.16			
Ho:Exp2=Exp1		0.04			
Ho:Exp3=Exp4	*	0.14			
Ho:Exp3=Exp5	*	0.17			
Ho:Exp3=Exp1		0.05			
Ho:Exp4=Exp5	**	0.31			
Ho:Exp4=Exp1	**	0.19			
Ho:Exp5=Exp1		0.13			
ANOVA: Single Factor		Day 5			
SUMMARY					

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Groups	*Count*	*Sum*	*Average*	*Variance*	
Exp 1 BBM4N(1P)	3.00	2.34	0.78	0.01	
Exp 2 BBM	3.00	1.60	0.53	0.02	
Exp 3 BBM (0.5N, 0.5P)	3.00	1.47	0.49	0.00	
Exp 4 BBM (0.2N, 0.2P)	3.00	0.95	0.32	0.00	
Exp 5 BBM (0.75N,0.75P)	3.00	2.09	0.70	0.01	
ANOVA					
Source of Variation	*SS*	*df*	*MS*	*F*	
Between Groups	0.39	4.00	0.10	17.01	
Within Groups	0.06	10.00	0.01		*F crit*
					3.48
Total	0.45	14.00			

Day 5

Null Hypoth.	Sign	CD	CD.05	CD.01	
Ho:Exp2=Exp3		0.04	0.19	0.25	
Ho:Exp2=Exp4	*	0.22			
Ho:Exp2=Exp5		0.16			
Ho:Exp2=Exp1	*	0.25			
Ho:Exp3=Exp4		0.18			
Ho:Exp3=Exp5		0.20			
Ho:Exp3=Exp1	**	0.29			
Ho:Exp4=Exp5	**	0.38			
Ho:Exp4=Exp1	**	0.46			
Ho:Exp5=Exp1		0.08			

ANOVA: Single Factor		Day 6			
SUMMARY					
Groups	*Count*	*Sum*	*Average*	*Variance*	
Exp 1 BBM4N(1P)	3.00	2.60	0.87	0.02	

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Exp 2 BBM	3.00	1.50	0.50	0.00	
Exp 3 BBM (0.5N, 0.5P)	3.00	1.55	0.52	0.00	
Exp 4 BBM (0.2N, 0.2P)	3.00	1.01	0.34	0.00	
Exp 5 BBM (0.75N,0.75P)	3.00	2.55	0.85	0.00	

ANOVA					
Source of Variation	*SS*	*df*	*MS*	*F*	
Between Groups	0.66	4.00	0.16	34.40	
Within Groups	0.05	10.00	0.00		*F crit*
					3.48
Total	0.71	14.00			

Day 6

Null Hypoth.	Sign	CD	CD.05	CD.01	
Ho:Exp2=Exp3		0.02	0.17	0.23	
Ho:Exp2=Exp4		0.16			
Ho:Exp2=Exp5	**	0.35			
Ho:Exp2=Exp1	**	0.37			
Ho:Exp3=Exp4		0.18			
Ho:Exp3=Exp5	**	0.34			
Ho:Exp3=Exp1	**	0.35			
Ho:Exp4=Exp5	**	0.52			
Ho:Exp4=Exp1	**	0.53			
Ho:Exp5=Exp1		0.01			

ANOVA: Single Factor		Day 7			
SUMMARY					
Groups	*Count*	*Sum*	*Average*	*Variance*	
Exp 1 BBM4N(1P)	3.00	3.12	1.04	0.01	
Exp 2 BBM	3.00	1.78	0.59	0.00	
Exp 3 BBM (0.5N, 0.5P)	3.00	1.78	0.59	0.00	
Exp 4 BBM (0.2N, 0.2P)	3.00	1.53	0.51	0.00	
Exp 5 BBM (0.75N,0.75P)	3.00	2.90	0.97	0.00	

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ANOVA					
Source of Variation	*SS*	*df*	*MS*	*F*	
Between Groups	0.71	4.00	0.18	50.87	
Within Groups	0.04	10.00	0.00		*F crit*
					3.48
Total	0.75	14.00			

Day 7

Null Hypoth.	Sign	CD	CD.05	CD.01	
Ho:Exp2=Exp3		0.00	0.15	0.20	
Ho:Exp2=Exp4		0.09			
Ho:Exp2=Exp5	**	0.37			
Ho:Exp2=Exp1	**	0.45			
Ho:Exp3=Exp4		0.08			
Ho:Exp3=Exp5	**	0.37			
Ho:Exp3=Exp1	**	0.45			
Ho:Exp4=Exp5	**	0.46			
Ho:Exp4=Exp1	**	0.53			
Ho:Exp5=Exp1		0.07			

ANOVA: Single Factor		Day 8			
SUMMARY					
Groups	*Count*	*Sum*	*Average*	*Variance*	
Exp 1 BBM4N(1P)	3.00	3.35	1.12	0.01	
Exp 2 BBM	3.00	2.00	0.67	0.00	
Exp 3 BBM (0.5N, 0.5P)	3.00	1.90	0.63	0.00	
Exp 4 BBM (0.2N, 0.2P)	3.00	1.14	0.38	0.00	
Exp 5 BBM (0.75N,0.75P)	3.00	3.17	1.06	0.00	

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ANOVA					
Source of Variation	*SS*	*df*	*MS*	*F*	
Between Groups	1.15	4.00	0.29	135.00	
Within Groups	0.02	10.00	0.00		*F crit*
					3.48
Total	1.17	14.00			

Day 8

Null Hypoth.	Sign	CD	CD.05	CD.01	
Ho:Exp2=Exp3		0.03	0.12	0.15	
Ho:Exp2=Exp4	**	0.29			
Ho:Exp2=Exp5	**	0.39			
Ho:Exp2=Exp1	**	0.45			
Ho:Exp3=Exp4	**	0.25			
Ho:Exp3=Exp5	**	0.42			
Ho:Exp3=Exp1	**	0.48			
Ho:Exp4=Exp5	**	0.68			
Ho:Exp4=Exp1	**	0.74			
Ho:Exp5=Exp1		0.06			

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14. ABSTRACT

Chlorella vulgaris was cultured in microbioreactors using Bold's Basal medium at varying nitrogen and phosphorus concentrations to define nitrogen and phosphorus utilization standards. Nutrient concentration was varied between 137 mg/L to 7.33 mg/L NO3-N and between 55.2 mg/L to11.0 mg/L PO_4-P in five test scenarios. All were grown under a constant photoperiod at 22±2 °C and a mixture of 4 to 10% carbon dioxide/air. Maximum yield and growth rate occurred with the highest initial nitrogen and phosphorus concentrations. A statistically significant difference in biomass was found among all test levels at the end of the eight day growth period. Applying both Liebig's Law of the Minimum and the Blackman Limitation, it was determined that nitrogen was the limiting factor over the range of concentrations tested. Michaelis-Menten biokinetic coefficients (k), the reaction rate constant; the half saturation constant (K_m); and Y_x, the yield coefficients were also determined. To maximize *C. vulgaris* growth initial N concentration values should be 137 mg/L and should not be allowed to fall below 69 mg/L. No equivalent recommendation for P was determined. Yield coefficient calculations suggested that the N:P ratio should be at least 3:1. This study was conducted as a part of the ongoing advanced jet fuel project at the University of Dayton Research Institute and a part of the military objective to reduce the carbon footprint of jet fuel production.

15. SUBJECT TERMS

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