**2016 Project Abstract**
For the Period Ending June 30, 2019

**PROJECT TITLE: Utilization of Dairy Farm Wastewater for Sustainable Production**
**PROJECT MANAGER: Bradley Heins**
**AFFILIATION: University of Minnesota**
**MAILING ADDRESS: 46352 State Hwy 329**
**CITY/STATE/ZIP: Morris, MN 56267**
**PHONE: 320-589-1711**
**E-MAIL: hein0106@umn.edu**
**WEBSITE:** http://wcroc.cfans.umn.edu/
**FUNDING SOURCE:** Environment and Natural Resources Trust Fund
**LEGAL CITATION:** M.L. 2016, Chp. 186, Sec. 2, Subd. 07d

**APPROPRIATION AMOUNT: $475,000**
**AMOUNT SPENT: $451,386**
**AMOUNT REMAINING: $23,614**

**Sound bite of Project Outcomes and Results**
The project benefited lakes and streams through the development of novel methods to clean affected waterways through microalgae production and testing. We developed technology that recycled nutrients and added value to nutrients in wastewater from dairy farms in Minnesota that will reduce environmental impact.

**Overall Project Outcome and Results**
The work was a collaboration between the West Central Research and Outreach Center in Morris and in the Department of Bioproducts and Biosystems Engineering and Food Science and Nutrition at the University of Minnesota. The goal of our project was to use dairy cattle wastewater to produce green energy, foods, and feed for livestock. The project utilized algae cultivation to remove nutrients from dairy wastewater and produce algae biomass for dairy calf feed, as well as hydroponic vegetables. Chlorella species of algae was the most predominant algae studies in the project, and the fatty acid profile of Chlorella indicated it would be superior of livestock feed and energy. Our project found that algae can successfully remove nitrogen, phosphorous, and dissolved solids in dairy wastewater, thus improving the environmental effects of wastewater from livestock farms. Results also suggest that feeding algae grown from dairy wastewater provided acceptable nutritional requirements for dairy cattle and mice. No adverse growth of calves or feed intake was observed when adding algae to dairy calf rations. This project suggests that algae can clean Minnesota waterways through reduced nitrogen and phosphorous from agricultural runoff.

**Project Results Use and Dissemination**
We have provided tours of the algae biomass system at the WCROC to legislators, farmers, and industry representatives. We have also hosted dairy field days and the Midwest Farm Energy Conference at the WCROC that have shown the results and bioreactors to the public as well. Over 2,000 people have viewed the system and have responded with favorable interest in the system. Our graduate student on the project presented an abstract at the Algal Biomass Conference in Denver, CO on biomass production for livestock. So far, 18 peer reviewed papers have been published with more to follow. The website will be updated with the final results of the project and infographics for promotion of the project. An abstract on calf feeding with be presented at the American Dairy Science Association meeting in 2020. This applied algae livestock feeding component is the Master's thesis of Siane Luzzi in the Department of Bioproducts and Biosystems Engineering at the University of Minnesota and she will defend her thesis in 2019.

# Environment and Natural Resources Trust Fund (ENRTF)
# M.L. 2016 Work Plan Final Report

**Date of Report:** August 16, 2019

**Final Report**

**Date of Work Plan Approval:** June 7, 2016

**Project Completion Date:** June 30, 2019

**PROJECT TITLE: Utilization of Dairy Farm Wastewater for Sustainable Production**

**Project Manager:** Bradley Heins

**Organization:** University of Minnesota

**Mailing Address:** 46352 State Hwy 329

**City/State/Zip Code:** Morris, MN 56267

**Telephone Number:** (320) 589-1711

**Email Address:** hein0106@umn.edu

**Web Address:** http://wcroc.cfans.umn.edu/research-programs/dairy

**Location:** Statewide

| **Total ENRTF Project Budget:** | | |
|---|---|---|
| | **ENRTF Appropriation:** | **$475,000** |
| | **Amount Spent:** | **$451,386** |
| | **Balance:** | **$23,614** |

**Legal Citation:** M.L. 2016, Chp. 186, Sec. 2, Subd. 07d

**Appropriation Language:**

$475,000 the second year is from the trust fund to the Board of Regents of the University of Minnesota for the West Central Research and Outreach Center in Morris to develop and evaluate an integrated system that recycles and uses nutrients in dairy wastewater from feedlots and milk processing, thereby reducing nutrients from agricultural runoff, and to provide outreach on adoption of new technologies. This appropriation is subject to Minnesota Statutes, section 116P.10. This appropriation is available until June 30, 2019, by which time the project must be completed and final products delivered.

**I. PROJECT TITLE: Utilization of Dairy Farm Wastewater for Sustainable Production**

**II. PROJECT STATEMENT:**

The dairy industry in Minnesota generates over $3.2 billion dollars in economic activity. However, the 450,000+ dairy cows on over 4,000 dairy farms in Minnesota generate a significant amount of waste. Dairy producers from across the state have manure lagoons on their farms to store all of the waste generated. Nutrient removal, in particular nitrogen and phosphorus, from wastewater is a growing regulatory need and the use of algae may create a unique amalgamation between dairy wastewater treatment and livestock feed production. This project will benefit all size dairy operations in Minnesota ranging from 50 to 500 cows. We will clean the dairy waste stream through algae production before it moves to farm fields and streams instead of applying the dairy waste directly to the land. This will reduce the environmental impact of dairy waste from entering streams and watersheds. Dairy producers will learn about the remediation of dairy farm wastewater through research, demonstration, and outreach experiences.

This project will develop and demonstrate an integrated facility to utilize and recycle nutrients from dairy farm wastewater, as well as carbon dioxide emissions on-site to simultaneously produce "green" energy, clean water, food, and livestock feed. Nutrient laden wastes are a direct result of the dairy industry in Minnesota. Dairy wastewater is comparatively poor in organic matter but typically rich in nitrogen and phosphorus. This wastewater is used to irrigate agricultural cropland; however, runoff of excess nitrogen and phosphorus leads to anthropogenic eutrophication of Minnesota watersheds and rivers. Reduction of the nitrogen and phosphorus in dairy wastewater through engineered alga and hydroponics systems will allow for more control of the nutrient content in cropland irrigation water while supply feed for livestock. Other systems partly fix the problem by removing some nutrients, such as organic matter or sulfur. Overall, an integrated approach is needed and the proposed system represents a more intelligent nutrient recycling strategy that mitigates adverse environmental consequences such as eutrophication and pollution of Minnesota watersheds.

Specifically, we will develop and evaluate a novel, integrated facility consisting of a microalgae photobioreactor, and hydroponic system, which will be operated next to an existing underground dairy wastewater lagoon. This combination of systems will be utilized to interrupt waste streams, by cleaning dairy wastewater before it flows to fields and streams from land application. Briefly, wastewater discharged from the lagoon contains substantial amounts of nutrients that are well suited to serve as a water and nutrient source for the integrated system, yielding growth of microalgae. Excess clean water after from the systems may be utilized for other applications (e.g. washing the dairy barn or irrigation). The outcomes of the proposed system will be clean water and air, and microalgae as a livestock feed. This project would be scalable to dairy farms of all sizes in Minnesota.

In addition, we will utilize the microalgae biomass produced from the system to conduct demonstrations directed at the potential use of microalgae as livestock feed for cattle and swine. Livestock feed is an opportunity for additional source of income for farmers adopting a green technology that doesn't add cost of livestock production, but generates income. This technology will enable dairy producers to meet greenhouse gas emission reductions and other current and future environmental regulatory requirements. The West Central Research and Outreach Center in Morris, is uniquely positioned as an excellent resource to use for conducting this research because of its national prominence in research and outreach involving renewable energy, environmental sustainability, and alternative livestock production systems.

**III. OVERALL PROJECT STATUS UPDATES:**

**Project Status as of *January 1, 2017*:**
A thermophilic AD process with light vacuum was developed to treat animal wastewater for algae and vegetable cultivation. The process was implemented in a lab scale system and tested. Main process variables including initial pH, vacuum, reaction volume and time were studied in terms of methane production and nutrient removal. The initial results show that the process can produce methane steadily and effectively remove ammonia. A number of algal systems and hydroponics parts were purchased/constructed and tested to grow algae from the dairy wastewater. Their ability to clean the wastewater to the extent that the treated water could be used for fish cultivation was evaluated. We have also developed prototype algae bag reactors for use in the scaled up version at the WCROC.

**Project Status as of *January 1, 2018*:**
The algae component has many positive effects that have been achieved from the wastewater cultivation system. In daily operations, algae can help balance pH value, add oxygen, and control ammonia in the wastewater system. Although algae have lower productivity comparable to vegetables and maybe economically unfavorable to a grower, algae can remove nitrogen more efficiently than vegetables due to higher nitrogen content in algae. In terms of water treatment, algae have a unique role in the system and could be placed at the final stage of the system for further ammonia removal. The results also showed the pretreated manure and corn stover mixture can significantly improve the specific methane yield under higher pH. Chlorella vulgaris grew well on minimally diluted pretreated manure. The algae could be used as biodiesel feedstock and the algal biomass could be used as an animal feed if cultivated in wastewater production system. The algae bag reactor system at the WCROC has been scaled and tested with different concentrations of dairy wastewater to optimize algae growth.

**Project Status as of *July 1, 2018*:**

A cultivation system with daily recycling of post-harvest culture broth was set up. The system was firstly used for microalgae cultivation on pretreated swine manure. Chlorella vulgaris grow very well in this system with moderate recycling ratios. Chemical compositions in this alga varied with the change of recycling ratios. The algal biomass grown in this system could be used as a biodiesel feedstock. We have begun experiments with the algae bioreactor system at the WCROC. The experiments have utilized different concentrations of wastewater to maximize algae production. We have also began to centrifuge and test the algae for properties to maximize feed quality to feed to livestock. We will begin feeding trials this fall with the algae harvested during the summer of 2018.

**Project Status as of *January 1, 2019*:**

An algae biomass production system using dairy wastewater was utilized at the West Central Research Center during the summer and fall of 2018. Our objective was 2 fold: 1)to identify which wastewater dilution produces the greatest amount of algae biomass in the reactor, and 2) to produce algal biomass for feeding to dairy livestock. We used a dilution rate of 1:10, 1:20, 1:30, and 1:40 of wastewater and water in the bioreactor. We collected data on algae cell growth and algae biomass yield. We also evaluated the average consumption of ammonium and nitrate of the algae growing in the biomass system. Wastewater dilution of 1:10 had greater algae biomass production than the 1:30 dilution. This dilution may produce enough algae for feeding livestock using wastewater. Biofilm formation was observed, which caused variation in biomass measurements. Furthermore, the nutrient removal rates show that algae are capable of reducing the amount of nutrients in dairy wastewater. The final feeding phase of the project will be completed in the early months of 2019 and final analysis of algae data will be completed as well. Planning has begun for our Midwest Farm Energy Conference in

July 2019 at the West Central Research and Outreach Center in Morris, where we will discuss the algae production systems as a renewable and sustainable energy source.

**Overall Project Outcomes and Results:**

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*Thermophilic AD work*

The untreated liquid manure is a poor-digestible substrate for the methane (CH4) production through anaerobic digestion because of the high ammonia content (3000 mg/L – 5000 mg/L) and low carbon-to-nitrogen ratio (C: N) (~3). Even the thermophilic anaerobic digestion has the benefits of short hydraulic retention time and high solid degradation rate, the expected methane production of untreated manure is low because of the potential inhibitions by high ammonia and low C: N ratio.

The Thermal-Vacuum Stripping treatment applied in this project can easily remove the 98% of total ammonia content at 55 0C and pH = 10 within an hour treatment. Adding the grounded Corn Stover as the supplement of carbon also showed over 4 times CH4 productivity (CH4-mL/ g-VSadded) improvement through balanced the C: N ratio and support high quality carbon source. When we did both Thermal-Vacuum Stripping treatment and Corn Stover supplement at the same time, the C: N ratio of treated LSM would raise from 3 to 19 and 80% - 90% of total ammonia content was recovered as ammonium sulfate. Therefore, the CH4 yield of the thermophilic anaerobic digestion of treated manure was significantly enhanced.

In this project, the combination of Thermal-Vacuum Stripping and Corn Stover supplement pointed out an efficient way to convert the available carbon source to renewable energy (CH4) from poor-digestible animal wastewater. The abundant ammonia nitrogen source in the LSM can be fixed as ammonium sulfate and therefore utilized as the nitrogen fertilizers of the crops. Then the low ammonia inhibition solution should boost the productivity of the algae cultivation of the thermophilic anaerobic digestion effluent.

During the study of this project, we also found that the thermal insulation and the salinity are the critical concern of the thermophilic anaerobic digestion. Without the careful design and operation, the serious depression of CH4 yield would be observed with the low C: N ratio and high ammonia content of the manure.

*Microalgae cultivation work*

The effluent from the AD with much of the ammonia stripped was used for microalgae cultivation. The algae strain Chlorella vulgaris was grown on minimally diluted (2×, 3× and 4×) autoclaved and non-autoclaved pretreated anaerobic digestion manure in a batch-culture system for 7 days. Results showed that C. vulgaris grew best on 3× AD effluent, and effectively removed NH4+-N, TN, TP and COD by 98.48-99.82%, 49.21-55.41%, 19.95-23.09%, 31.22-33.95% and 99.84-99.92%, 67.35-70.83%, 49.34-54.36%, 73.56-78.66% in differently diluted autoclaved and non-autoclaved AD effluent, respectively. The results of the chemical compositions in C. vulgaris indicated that carbohydrate may be converted to lipids with increasing dilution of pADSM. The fatty acid profiles of C. vulgaris suggested that this alga could be used as animal feed if cultivated in autoclaved the AD effluent and as good-quality biodiesel feedstock if cultivated in non-autoclaved AD effluent.

*Hydroponic cultivation work*

Three vegetables, namely arugala, purple kohlrabi, lettuce, were hydroponically grown on artificial and AD manure effluent with and without added algae to the culture media. The effect of microalgae on production and nutrient removal was assessed for three vegetable crops (arugula, purple kohlrabi and lettuce) grown entirely using synthetic wastewater in greenhouse hydroponics. Results showed the effect of algae on the productivity of

different vegetable species varied. Algae have a negative effect on arugula growth, but in purple kohlrabi or lettuce groups, there were no negative effects or even an increase in vegetable production. After 35 days, the mean harvested biomass of purple kohlrabi in natural state was 31.6 g/plant (2.16 kg/m2), which was significantly greater than that of arugula, lettuce or purple kohlrabi in all other conditions. The dissolved oxygen and algae biomass concentration in the Chlorella addition group is relatively higher. The existence of algae, especially Chlorella addition, can significantly improve the removal rate of total dissolved solids, total nitrogen, total phosphorus, but not COD. These results from experiments using synthetic wastewater provide clear pictures how microalgae and vegetables interacted. Similar results were obtained from experiments using AD manure effluent as the culture media.

*Waste water algae feeding work*

Young male mice (n= 8/group) were fed the chows containing 0, 5% , and 10% green algae (Chlorella Vulgaris) grown in waste water from slaughterhouse and dairy processing facilities, respectively, for 21 days. The metabolic effects of waste water algae were investigated by growth performance, blood chemistry, and liquid chromatography-mass spectrometry (LC-MS)-based metabolomics. The results showed that growth performance and blood chemistry, including glucose, triacylglycerol, cholesterol, and blood urea nitrogen, were not significantly affected by either slaughterhouse or dairy algae. Metabolomic analysis of liver, cecum, feces, and urine samples identified the metabolic changes shared by both sources of algae and the source-specific metabolic changes. The levels of B vitamins in urine were significantly increased by both waste water algae treatment. Short-chain fatty acids in feces were only increased by 10% slaughterhouse algae. Muricholic acid and deoxycholic acid in feces were decreased by both 10% slaughterhouse and 10% dairy algae, while lithocholic acid was reduced only by 10% slaughterhouse algae. Liver metabolites including oxidized glutathione, niacinamide and adenosine were only reduced by 5% slaughterhouse algae. Overall, the results suggested that feeding waste water algae in an appropriate include rate is an acceptable practice for animal nutrition.

*Applied dairy waste water algae*

Chlorella sp. was cultured in mixotrophic conditions using different ratios of raw dairy wastewater in water, in bench-scale (1.25L) and pilot-scale (70L). The influence of extra CO2, pH, temperature, solar radiation, photosynthetic active radiation, were tested for the cell growth, biomass productivity and nutrients ($NH_4^+$, $PO_4^{3-}$, $NO_3^-$) removal. The aim of this study was to find the best ratio (1:10, 1:20, 1:30 or 1:40) of dairy wastewater in water, where Chlorella sp. biomass could be produced, removing the highest amounts of nutrients possible, and then test the significance of adding extra CO2 into the system. In the first experiment, lab and pilot-scale had the same biomass growth behavior, where control had the highest productivity and was followed by 1:10 (1.315 ± 0.240 g/L and 1.575 ± 0.599 g/L, respectively for lab-scale, and 0.752 ± 0.397 g/L and 0.434 ± 0.355 g/L, respectively for pilot-scale), both of them being harvested after seven days of experiment. Ratio 1:30 showed to be an alternative for reactions of four days long because 1:30 was not significantly different 1:10 when the goal was to remove nutrients. However, it was significant different for biomass production. In pilot-scale, none of the treatments (control, controlN, 1:10, 1:10N, 1:30 and 1:30N) were significantly different from each other, considering the nutrients ($NH_4^+$, $PO_4^{3-}$, $NO_3^-$) removal rates and biomass, when adding or not, extra CO2.

*Waste water algae calf feeding*

Agricultural environmental problems may be caused by the excessive addition of nutrients due to livestock production. The objective of this study was to evaluate the taste preference of calves fed Chlorella sp. produced from dairy lagoon wastewater. Chlorella sp. biomass was produced outdoors using bioreactors. The biomass was sterilized and kept frozen at -4°C until the feeding trial. Holstein and crossbred dairy heifer calves were fed 0, 30, and 60 g of Chlorella sp. daily in a sequential elimination study. No difference were found for dry matter intake of calves fed 0, 30, or 60 g of Chlorella sp., indicating that microalgae may be added to the rations of calves without any adverse effects.

**IV. PROJECT ACTIVITIES AND OUTCOMES:**

**ACTIVITY 1: Development of an integrated system to recycle and more effectively utilize nutrients in dairy wastewater to reduce agricultural runoff.**
**Description:**

We will develop an integrated system to utilize and treat dairy waste water and simultaneously produce green energy and feeds. Specifically, the system will consist of a bioreactor to recover energy and ammonia through thermophilic anaerobic digestion (AD) of dairy wastewater with consistent methane production and a minor vacuum to recover ammonia and remove odor, a photo-bioreactor (PBR) for algae cultivation, and a hydroponic bioreactor. The system is designed to facilitate several processes intended to fully utilize dairy wastewater and at the same time produce clean water. Due to the scaling down of the project, a smaller scale of the complete system will be developed and tested on the St. Paul campus to demonstrate the feasibility of the proposed concept and respective processes, as well as testing of algal strains to clean dairy wastewater. However, a reasonably large PBR and algal cultivation system for cleaning dairy wastewater will be developed and demonstrated on the West Central Research and Outreach Center dairy site.

Dairy wastewater contains a large amount of nitrogen which is turned into ammonia during fermentation. Ammonia is an inhibitor in AD process as well as algae growth. Therefore, it is beneficial to remove ammonia during the AD process and prior to algae cultivation. Furthermore, if ammonia could be recovered and used as fertilizer, substantial value is captured and air pollution is reduced. There are several methods to remove ammonia from dairy manure, including crystallization/precipitation, acidic solution-sprayed scrubbers and bio-filters, and chemicals such as acidified clays and sodium hydrogen sulfate, gas-permeable membrane extraction, etc. Some of these methods are promising, but have limited use due to high cost, lack of ammonia recovery for beneficial uses, and the complexity of operation.

We propose to implement a thermophilic process in combination with vacuum volatilization and acid absorption to not only remove ammonia from the digestate but also capture ammonia in the form of ammonium sulfate. Recently, control over the point sources of N and P shifted from removal to recovery, with a particular emphasis on improving the sustainability of agricultural activities. Global demand for the nitrogenous fertilizer has been increasing steadily. Therefore, the current attempts are not only to clean and reuse the water resources, but also to extract the maximum amounts of N from dairy manure.

Thermophilic AD, which has been well documented in the literature, is usually operated at about 55°C (131°F). At these elevated temperatures, the reaction rates in thermophilic AD are significantly higher than those in normal AD, and ammonia is produced at higher rates as well. To help ammonia escape from the liquid manure, we propose to apply low vacuum to draw ammonia out of the liquid and the AD bioreactor and inject the air stream into dilute sulfuric acid solution where ammonium sulfate will be produced. The solution containing ammonium sulfate can be used directly as liquid fertilizer or made into ammonium sulfate granules for convenient transport and storage. The value from production of ammonium sulfate is expected to at least offset the cost associated with raising temperature for thermophilic AD process. In this project, the costs of the proposed system and process will be evaluated and compared with normal AD process.

Figure 1 is a schematic illustration of the thermophilic AD bioreactor combined with vacuum volatilization and acid absorption. We have prior experience in designing and operating an AD reactor. For this project, we will construct a 100 gallon bioreactor with a heating and temperature control apparatus to maintain the necessary thermophilic conditions for AD. A 20 gallon absorption column will be constructed and placed next to the AD bioreactor. A vacuum pump will connect the AD bioreactor and the absorption column. The key process

parameters to be studied and optimized will include AD temperature, pH, low vacuum, feed depth, cycle length. The yield and composition of biogas (methane), yield of ammonium sulfate, energy consumption, and the overall productivity will be evaluated.

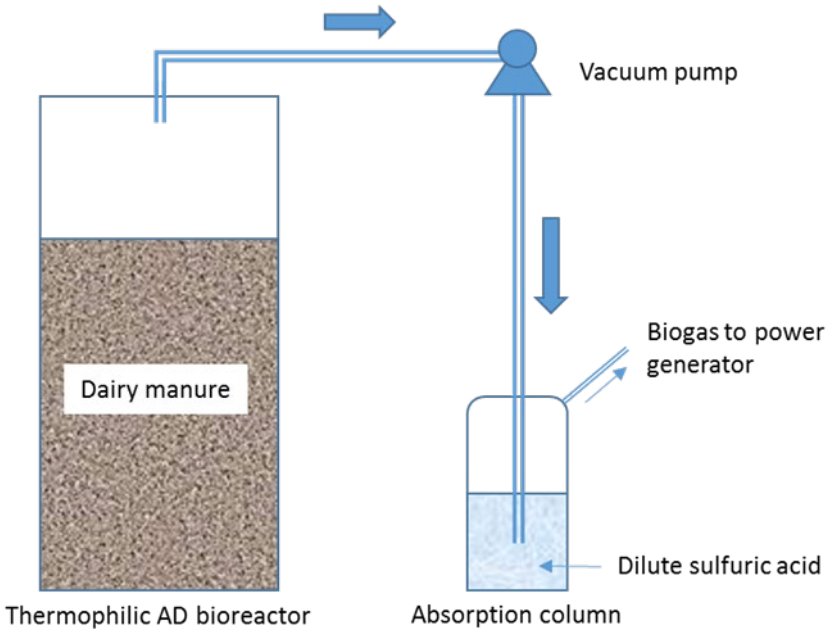



Figure 1. Thermophilic AD bioreactor with vacuum volatilization and acid absorption for ammonia removal and recovery.

Additionally, our previous research found that while algae utilize the carbon source in animal wastewaters quite effectively, one issue with these wastewaters is that there is insufficient carbon source to support complete utilization and removal of N and P, meaning that there will be 25-40% residual N and P in the culture broth after algae are removed. In order to fully utilize the resources in dairy wastewater, we proposed to incorporate a hydroponic bioreactor into the facility to grow vegetables and further utilize the residual N and P and clean the water for dairy housing wash, irrigation or discharged. We will source components and construct a hydroponic bioreactor in-house (Figure 2). We will evaluate this bioreactor's capacity of cleaning water through hydroponic production. The nutrient uptake will be monitored daily. The experience we obtained from another project involving an aquaponics system will guide us to maintain healthy growth of vegetables.

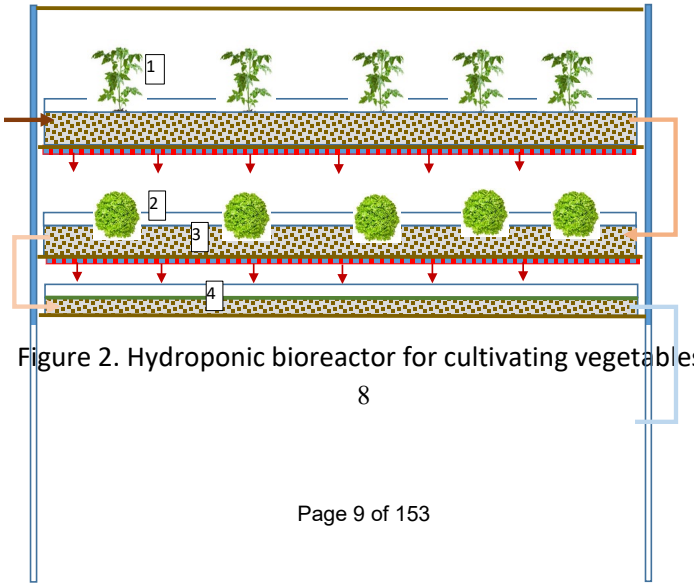



Figure 2. Hydroponic bioreactor for cultivating vegetables.

1. Tall plants, with 2-ft growth space.
2. Short plants with 1-ft growth space.
3. Gravel, fix the plant's root, bacteria, and filtration.
4. Sand filtration with algae attaching on the surface, 4-6 inch.

**Summary Budget Information for Activity 1:**

**ENRTF Budget: $ 196,610**
**Amount Spent: $ 196,610**
**Balance: $ 0**

| Outcome | Completion Date |
|---|---|
| 1. Develop and optimize parameters and production of algae for the integrated facility to remove 25 to 40% of the residual nitrogen and phosphorus in dairy wastewater. | 7/1/2017 |
| 2. Develop and test the microalgae photobioreactor and hydroponic systems to characterize the WCROC wastewater in terms of nitrogen, phosphorus, inorganic and organic carbon concentrations, and a complete micronutrient analysis. | 7/1/2017 |
| 3. Integrate and test the facility to determine the efficacy and efficiency of the systems for screening of over 25 existing algal culture collections . | 7/1/2017 |
| 4. Optimize nutrient removal rate of algae production system with dairy wastewater to clean 100% of dairy wastewater as it move from the algae production through the hydroponic system. | 7/1/2017 |

**Activity Status as of *January 1, 2017*:**

**Develop thermophilic AD process with light vacuum to treat animal wastewater**
In the first period of this project, we have developed a thermophilic AD process and implemented in a lab scale reactor as shown in Figure 1. The temperature was maintained around 55 °C with or without vacuum (18 KPa). The effects of key process variables such as initial pH, vacuum, reaction volume and time on methane production and nutrient removal were studied.

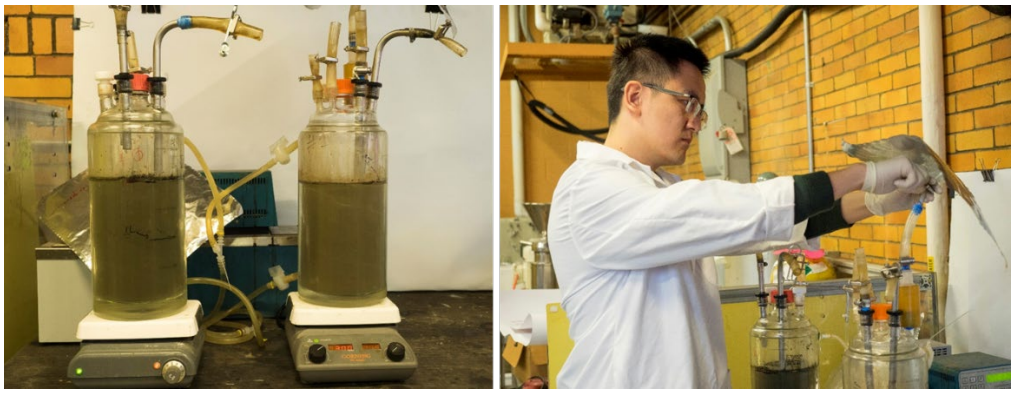


Figure 1. The experimental apparatus for thermophilic AD with light vacuum to treat animal wastewater.

*Initial pH*
Fig 2 shows that higher ammonia removal efficiency and lower final ammonia concentration were obtained at pH=10 at the beginning of the vacuum process. Since the total nitrogen includes organic nitrogen, nitrite, nitrate and ammonia, the results of nitrite and nitrate indicate that most of the reduction in total nitrogen was due to

ammonia removal (Table 1). Higher solid degradation rate was found at pH=8 condition and no significant variation occurred in COD, TP, and VS (Table 1). However, the lower $NH_3$/Total nitrogen rates at pH=9 and pH=10 indicate the organic nitrogen in the liquid swine manure could be converted to ammonia and be removed when ammonia concentration stop decrease. The relationship between pH and ammonia concentration suggests that higher pH could lead to lower organic nitrogen. Volatile free acids (VFAs) showed highest level at pH=10 (Table 1). The percentage of acetic acid increased when the percentage of butyric acid decreased at pH=10 condition, and propionic acid is most stable acid during thermal-vacuum process (Fig. 3).

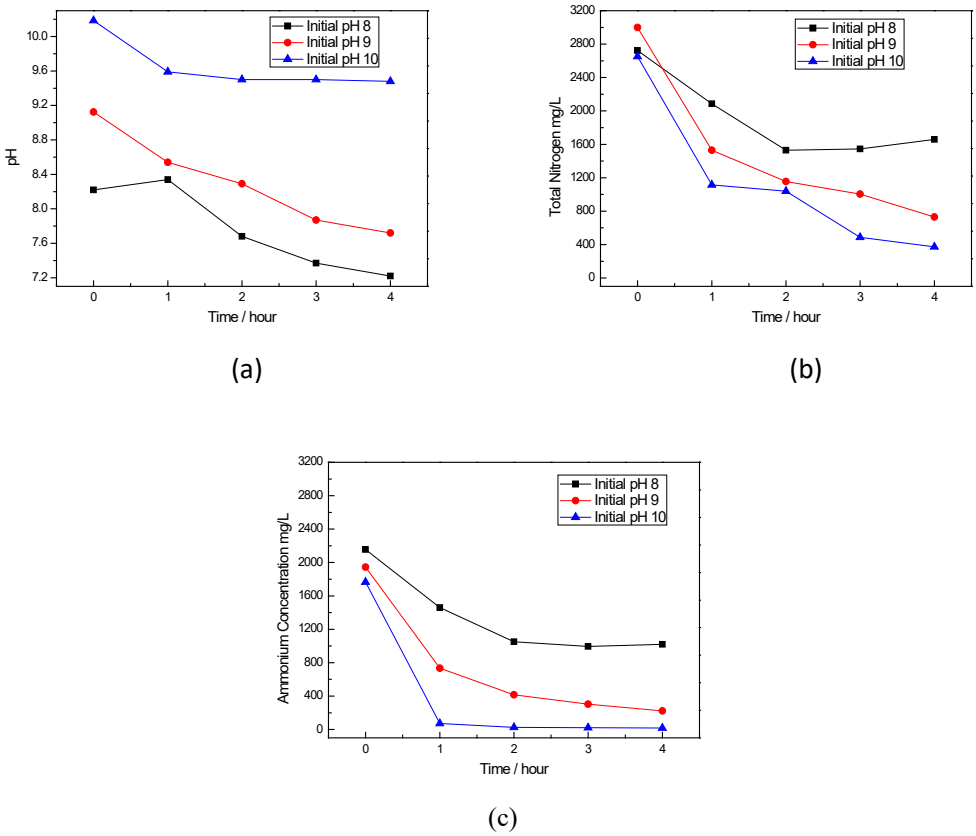



*Figure 2. Effect of initial pH on ammonia removal with Thermal-Vacuum process. (a): pH, (b): TN, (c): ammonia*

*Table 1 Variance of swine manure characters by Thermal-Vacuum process.*

| 4 hours treatment | PH=8 | | PH=9 | | PH=10 | |
|---|---|---|---|---|---|---|
| | innital | end | innital | end | innital | end |
| $NO_3^-$-N mg/L | 10.0 | 7.9 | 10.6 | 9.0 | 11.8 | 10.4 |
| $NO_2^-$-N mg/L | 0.6 | 0.4 | 0.7 | 0.5 | 0.8 | 0.6 |
| COD mg/L | 20225 | 19100 | 21125 | 19250 | 19700 | 18550 |
| TP mg/L | 580.0 | 590.0 | 273.3 | 247.5 | 345.5 | 406.5 |
| TS g/kg | 24.6 | 22.4 | 20.1 | 20.5 | 27.5 | 27.6 |
| VS g/kg | 14.6 | 13.1 | 11.2 | 11.5 | 13.2 | 13.2 |
| VFAs mg/L | 4836.6 | 5527.0 | 5398.3 | 5339.6 | 3401.3 | 6547.8 |

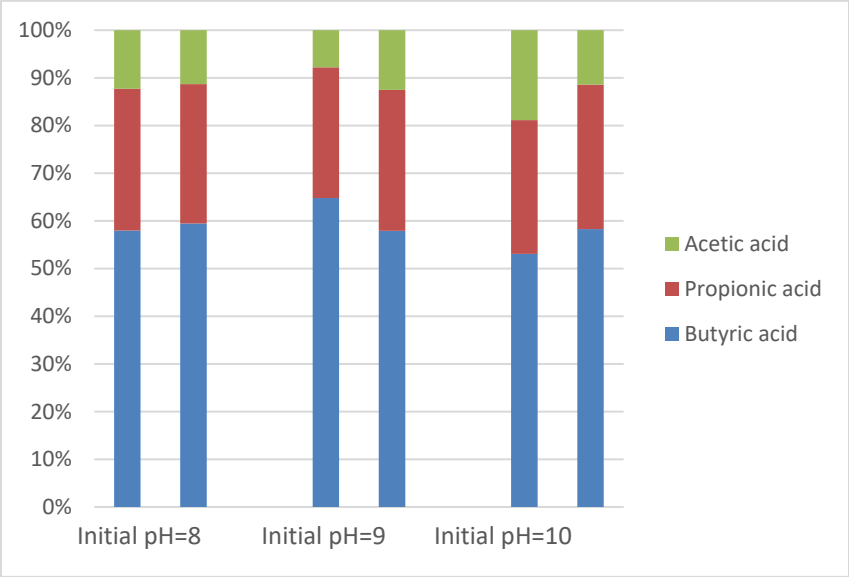



*Figure 3. Influence of initial pH on VFAs distribution*

*Reaction volume*

Fig. 4 shows that the complete ammonia removal time increased with increasing reaction volume. The data shows that when the reaction volume was only 0.1 L, over 95 % ammonia removed from the liquid swine manure within 1 hour. However, when the reaction volume increased to 15 times of original volume, the reaction time for same final concentration increased to 4 hours. The total ammonia nitrogen (TAN) removal efficiency data in Fig. 5 provided further evidence of reaction volume effect on Thermal-Vacuum process. The ammonia removal efficiency in first hour was highest in all volume conditions. Only 60 % ammonia can be removed from samples when the reaction volume is 1.5 L, which was around 40% decreased compared to 0.1 L condition.

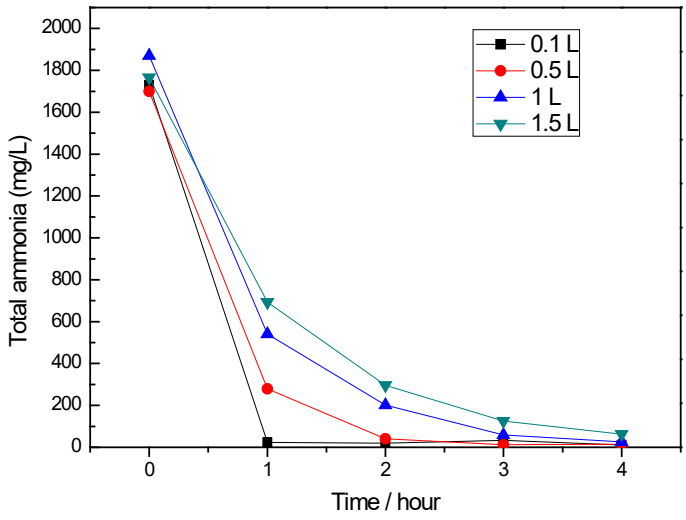



*Figure 4. The influence of reaction volume of Thermal-Vacuum process on total ammonia concentration.*

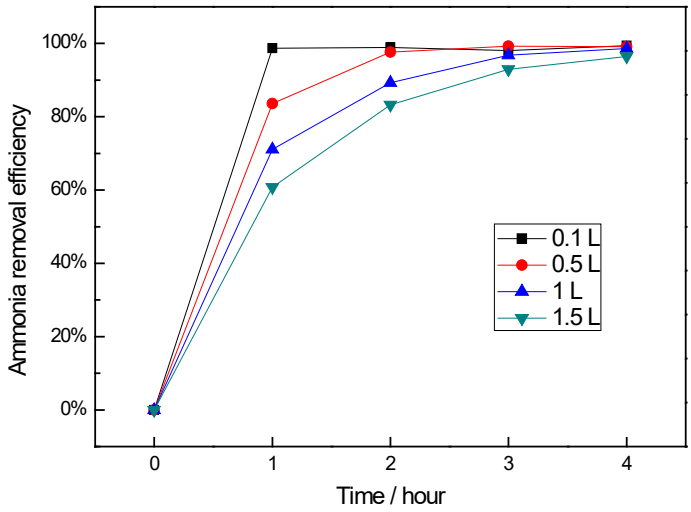



*Figure 5. The influence of reaction volume of Thermal-Vacuum process on total ammonia removal.*

However, the decrease in ammonia removal efficiency was not equal to the mass of ammonia decreased. The data is Fig. 6 indicated that larger reaction volume could produce more ammonia salts in the same time in theory. The data in Fig. 5 does not show the upper limit of the ammonia removal when the reaction volume increased by 15 times, which provided the potential industrialized ability of the Thermal-Vacuum process.

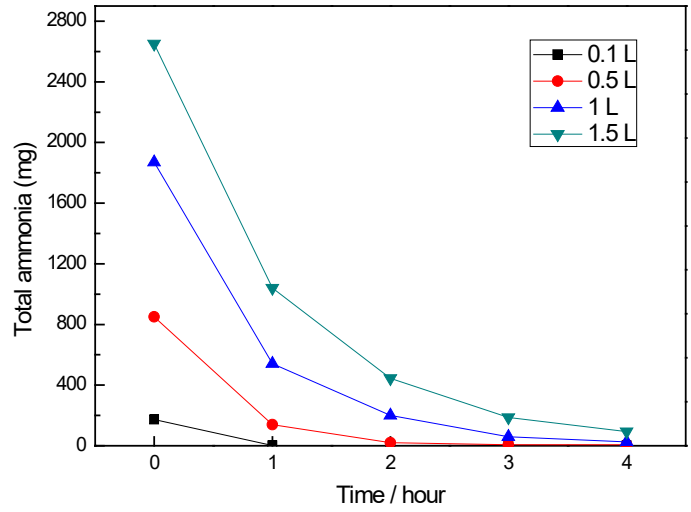



*Figure 6. TAN removal amount trends*

When further scale-up to 4 L reaction volume, the results in Fig. 7 reveals that the final ammonia concentration depends on the pH value. When the pH is lower than 8.8, no more ammonia can be stripped from the liquid. Since the free ammonia percentage is lower than 50 % in pH 8.8, we suspect that the interval of free ammonia percentage in liquid phase and gas phase is the key for Thermal-Vacuum process. The data in Fig. 8 also show that when we regulated the pH to 10 again, the final ammonia concentration restarted to drop from 200 mg/L to 20mg/L. Additionally, the pretreated liquid swine manure has pH control benefit for anaerobic digestion

process in Fig. 9. When we set the thermal-vacuum unit before thermophilic anaerobic digestion, the system shows higher pH-buffer ability than normal thermophilic anaerobic digestion.

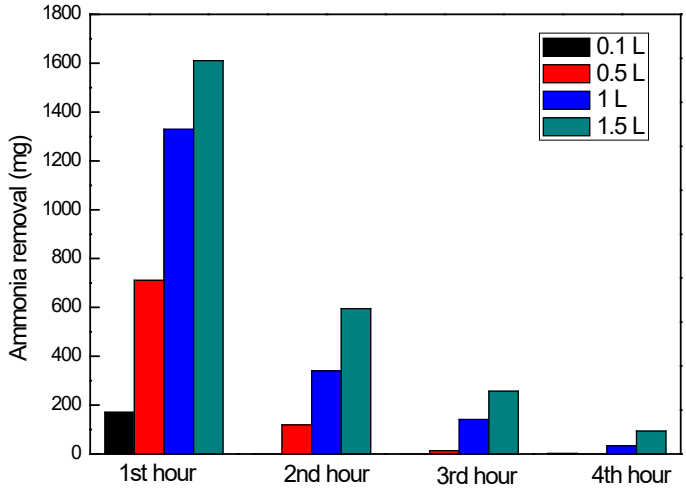



*Figure 7. TAN removal efficiency of mass rate*

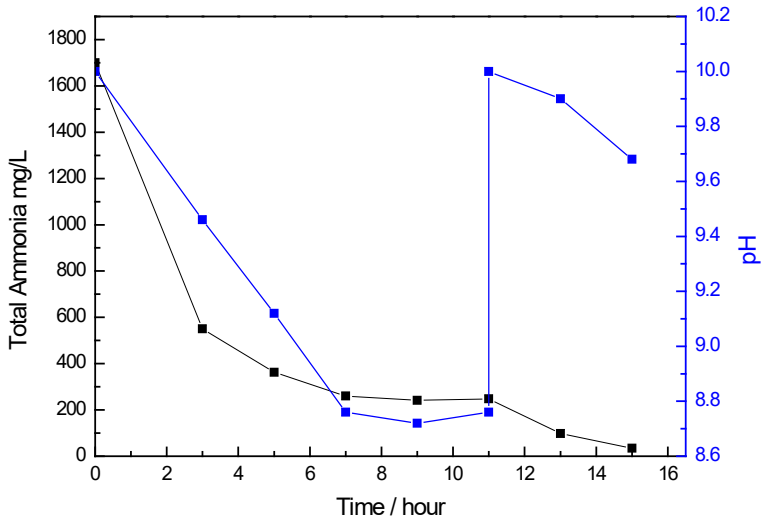



*Figure 8. TAN removal under 4L reaction volume*

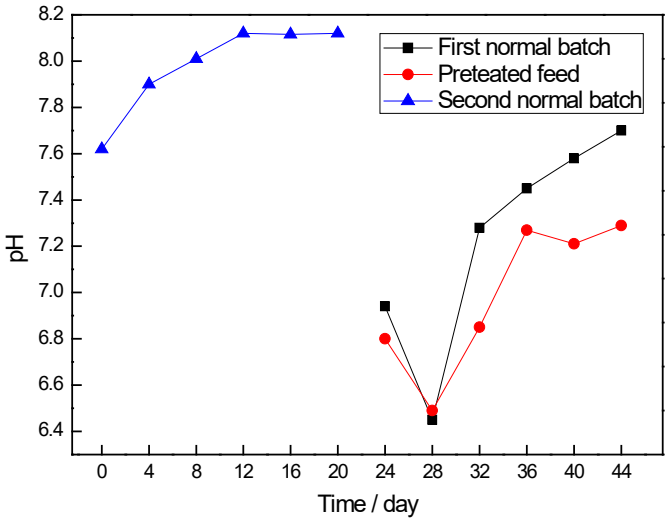



*Figure 9. Change in pH during Thermal-Vacuum pretreatment*

Fig. 10 shows the total carbon utilization rate that is measured as chemical oxygen demand (COD). TCOD degradation rate reached above 1300 mg/L/day, which is equal to 2%/day. The main contribution of thermophilic anaerobic digestion is to degrade large particle (observed as TCOD) to VFAs that can be further converted to methane. The data in Fig. 10 reveal that our thermophilic anaerobic digestion has reached a stable status. The data in Fig. 11 show our thermophilic anaerobic digestion can produce methane stably.

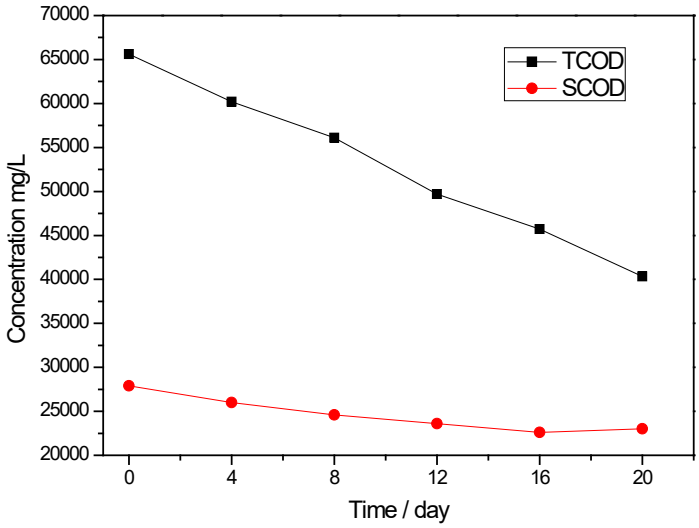



*Figure 10. Carbon utilization through Chemical oxygen demand trend*

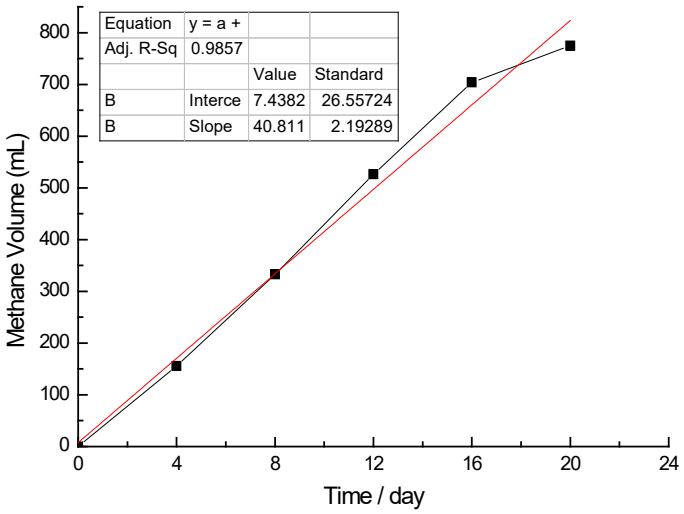



*Figure 11. Methane accumulation in thermophilic anaerobic digestion.*

**Develop aquaponics process**

In the past six months, four aquaponics /hydroponics systems have been purchased/constructed. They are two Nelson and Pade (F5) aquaponics systems that were purchased and used for parallel comparison test, one Will Allen system and one media filled system were constructed. Figure 1-3 showed the four systems. Two aquaponics systems (F-5 Fantastically Fun Fresh Food Factory) were purchased from Nelson and Pade Inc. and set up in a greenhouse on Saint Paul campus at the University of Minnesota. One vegetable tray was used for each system. The water discharged from the fish tank enters the clarification tank where the fish waste can be separated out. The central baffle will force the water to flow downwards and then upwards so that the solid will precipitate to the bottom of the tank. It is suggested by the manufacturer to change the water and wash out the solid every two days. The mineralization tank is filled with plastic orchard netting that is used for bacteria to grow on and filtering the solids. In mineralization tank, undigested solid will be converted to ammonia and other micronutrients needed by the plant. Due to anaerobic condition developed in the mineralization tank, nitrogen gas resulted from denitrification process and other harmful gasses such as hydrogen sulfide and methane can be formed. Therefore in the degassing tank, aeration is used to release these gasses into the air. In each vegetable tray, 45 plants can be planted.

Figure 1. Nelson and Pade F5 system.

The Will Allen system was constructed using wood structure with liner material. Two layers of vegtable trays provide 160 sqft vegtable area. The fish tank can holds up to 500 gallon of water. This system is also using float raft for plant support. Upper level is good for tall vegtable that require high sunlight, bottom level is good for small plant with low light/temperature requirement.

Figure 2. Will Allen system.

Differ from above two systems, the third system used the expansion clay as the filled media for root zone support. An algae component was added in this system for pH control, oxygen addition, and ammonia reduction. The first vegetable tray has constant water level, and the second vegetable tray has the bell and drain system where the water level goes up and down so that the oxygen can enter the root zoon for oxygenation. This system was placed in an indoor condition therefore light is needed 24 h/day for illumination.

Figure 3. Media filled system.

All systems can be set up as either aquaponics or hydroponics system. After the systems were stabilized with vegetable and fish, the plan for next six month is to introduce digested manure into the selected systems and monitoring water quality and fish condition.

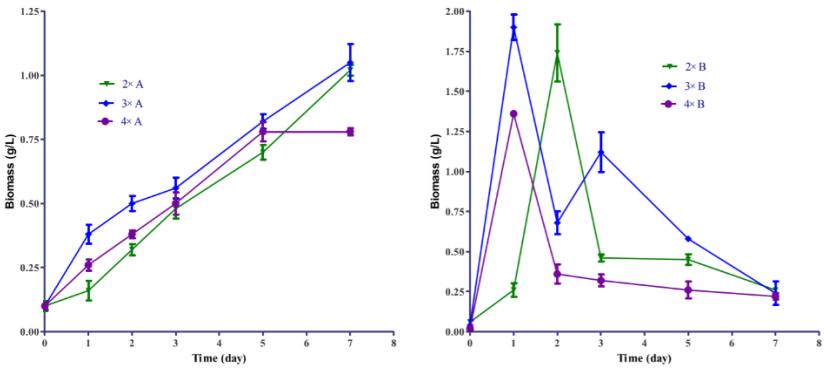
**Activity Status as of *January 1, 2018*:**

In this project period, liquid swine manure was treated using thermophilic anaerobic digestion at 55°C for 21 day, during which ammonia was stripped by vacuum at the 55°C and 100 mmHg (More details about the process is provided in later part of the report). Once the ammonia level reached about 200 ppm, the digestate was centrifuged to remove some of the solids in the liquid. The resultant solution was used for algae cultivation. Since the ammonia level and the turbidity of the digestate was reduced, lower dilution rates (2×, 3× and 4×) were used to grow *Chlorella vulgaris* (UTEX 2714) in a batch-culture system for 7 days.

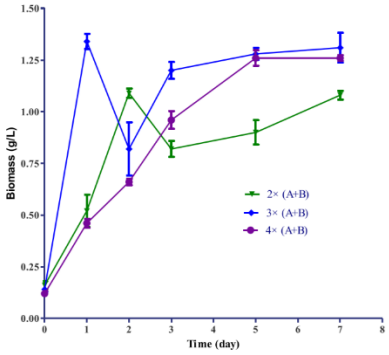
The experiments set up: The pretreated anaerobic digestion swine manure (PADSM) was diluted with distilled water (v/v) to three different dilution multiples of 2, 3 and 4 before inoculation, which were labeled as 2×, 3× and 4×, respectively. Then, the 2×, 3× and 4× PADSM were divided into two equal aliquots. One aliquot was autoclaved at 121 C for 20 min in order to prevent interference from other microorganisms (mainly bacteria), and the other non-autoclaved aliquot was directly used for experiments where bacterial growth was expected. When C. vulgaris (UTEX 2714) grew to the exponential growth phase with a biomass of 0.8 g/L during pre-cultivation on TAP media, a total of 25 mL of the pre-culture was centrifuged (6000g, 4 C, 10 min) to collect the microalgal cells. The collected cells were washed twice with sterile distilled water, and then inoculated into the 2, 3 and 4 autoclaved and non-autoclaved PADSM with an initial biomass of about 0.1 g/L, which were labeled as 2 A, 3 A, 4 A and 2 (A + B), 3 (A + B), 4 (A + B), respectively. The other flasks filled with 2, 3 and 4 autoclaved and non-autoclaved PADSM without algae inoculation were labeled as 2 C, 3 C, 4 C and 2 B, 3 B, 4 B. In this work, batch experiments were performed in 500 mL Erlenmeyer flasks (A-algae, B-Bacteria, C-autoclaved wastewater). Table 1 indicated the characteristic of the manure for the pretreated AD swine manure before and after autoclave.

**Table 1**
Physicochemical characteristics of pretreated anaerobic digestion swine manure (PADSM) before and after autoclave. All measurements were performed in triplicate, and results are expressed as mean value ± standard deviation (SD).

| Parameter | PADSM | Autoclaved PADSM |
|---|---|---|
| TVSS (g/L) | 0.3 ± 0.03 | 0.4 ± 0.06 |
| Chemical oxygen demand (COD) (g/L) | 18.6 ± 0.2 | 19.7 ± 0.4 |
| Ammonium ($NH_4^+$-N) (mg/L) | 255.4 ± 4.9 | 169.4 ± 2.8 |
| Total nitrogen (TN) (mg/L) | 463.0 ± 6.7 | 400.8 ± 13.0 |
| Total phosphorus (TP) (mg/L) | 113.3 ± 7.0 | 116.6 ± 2.5 |
| Total volatile fatty acids (g/L) | 7.9 ± 0.3 | 7.5 ± 0.3 |
| Acetic acid (g/L) | 3.8 ± 0.1 | 3.9 ± 0.1 |
| Propionic acid (g/L) | 1.4 ± 0.07 | 1.3 ± 0.02 |
| Butyric acid (g/L) | 0.3 ± 0.08 | 0.3 ± 0.06 |
| Salinity (%) | 2.3 ± 0.03 | 2.4 ± 0.02 |
| pH | 7.0 ± 0.03 | 7.0 ± 0.01 |




(a) (b)




(c)

Fig. 1. Growth profiles of Chlorella vulgaris (UTEX 2714) grown on minimally diluted (2, 3 and 4) autoclaved (a) and non-autoclaved (c) pretreated anaerobic digestion swine manure (PADSM) and biomass changes of bacteria in non-autoclaved PADSM (b).

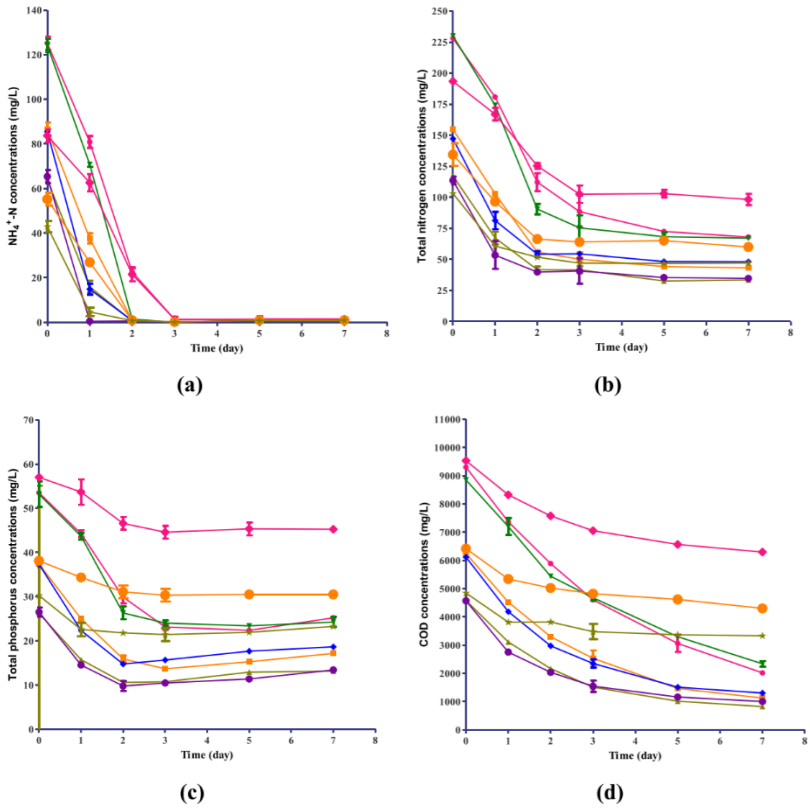



Fig. 2. Nutrient removals and pH change in minimally diluted (2, 3 and 4) autoclaved and non-autoclaved pretreated anaerobic digestion swine manure (PADSM) by Chlorella vulgaris (UTEX 2714) within 7 days. (a) NH4 + -N removal; (b) TN removal; (c) TP removal; (d) COD removal.

Table 2 Chemical compositions of Chlorella vulgaris (UTEX 2714) cultivated in pretreated anaerobic digestion swine manure (PADSM). 2 (A + B), 3 (A + B), 4 (A + B) and 2 A, 3 A, 4 A represent the treatments of this alga grown on 2, 3 and 4 non-autoclaved and autoclaved PADSM, respectively.

| Chemical compositions | Content (%) | | | | | |
|---|---|---|---|---|---|---|
| | 2 × (A + B) | 3 × (A + B) | 4 × (A + B) | 2 × A | 3 × A | 4 × A |
| *(a) Chemical composition of Chlorella vulgaris based on a dry matter basis* | | | | | | |
| Chl *a* | 1.1 ± 0.05 | 1.3 ± 0.07 | 1.1 ± 0.08 | 2.0 ± 0.11 | 2.2 ± 0.09 | 2.0 ± 0.08 |
| Chl *b* | 0.5 ± 0.03 | 0.5 ± 0.02 | 0.4 ± 0.03 | 0.4 ± 0.01 | 0.6 ± 0.04 | 0.5 ± 0.02 |
| Carotenoid | 0.7 ± 0.02 | 0.8 ± 0.03 | 0.7 ± 0.04 | 0.7 ± 0.05 | 0.7 ± 0.06 | 0.7 ± 0.03 |
| Pigment | 2.3 ± 0.11 | 2.5 ± 0.09 | 2.3 ± 0.10 | 3.1 ± 0.13 | 3.4 ± 0.15 | 3.2 ± 0.12 |
| Carbohydrate | 16.6 ± 1.53 | 10.8 ± 0.92 | 10.4 ± 1.02 | 12.2 ± 1.48 | 9.1 ± 0.86 | 8.8 ± 0.75 |
| Protein | 47.1 ± 3.26 | 54.0 ± 1.87 | 51.4 ± 2.61 | 51.0 ± 4.37 | 58.8 ± 2.19 | 48.7 ± 4.29 |
| Lipids | 16.9 ± 1.02 | 17.7 ± 1.21 | 21.6 ± 1.32 | 22.0 ± 1.74 | 17.4 ± 1.89 | 33.7 ± 2.01 |
| *(b) Fatty acid profile of Chlorella vulgaris* | | | | | | |
| Palmitic acid (C16:0) | 11.8 ± 0.22 | 18.8 ± 0.12 | 20.3 ± 0.02 | 19.0 ± 0.24 | 20.6 ± 0.08 | 20.1 ± 0.03 |
| Stearic acid (C18:0) | 1.7 ± 0.00 | 1.9 ± 0.01 | 1.8 ± 0.00 | 1.3 ± 0.01 | 0.5 ± 0.00 | 0.5 ± 0.00 |
| Saturated fatty acids (% of total fatty acids) | 13.5 ± 0.19 | 20.7 ± 0.11 | 22.1 ± 0.02 | 20.3 ± 0.22 | 21.2 ± 0.07 | 20.6 ± 0.03 |
| Palmitoleic acid (C16:1) | 10.9 ± 0.03 | 7.5 ± 0.00 | 7.0 ± 0.01 | 11.3 ± 0.03 | 13.0 ± 0.00 | 12.7 ± 0.03 |
| Oleic acid (C18:1) | 49.2 ± 0.19 | 43.0 ± 0.17 | 40.4 ± 0.08 | 22.3 ± 0.12 | 13.2 ± 0.00 | 9.9 ± 0.06 |
| Monounsaturated fatty acids (% total fatty acids) | 60.1 ± 0.12 | 50.6 ± 0.12 | 47.4 ± 0.07 | 33.6 ± 0.11 | 26.2 ± 0.01 | 22.6 ± 0.07 |
| Hexadecadienoic (C16:2) | 5.5 ± 0.02 | 5.2 ± 0.01 | 5.3 ± 0.00 | 6.6 ± 0.02 | 8.1 ± 0.00 | 9.5 ± 0.01 |
| Linoleic acid (C18:2) | 20.3 ± 0.10 | 23.2 ± 0.06 | 24.9 ± 0.07 | 18.6 ± 0.03 | 21.3 ± 0.00 | 24.4 ± 0.04 |
| Linolenic acid (C18:3) | 0.6 ± 0.01 | 0.4 ± 0.01 | 0.4 ± 0.01 | 20.8 ± 0.04 | 23.2 ± 0.08 | 22.9 ± 0.00 |
| Polyunsaturated fatty acids (% total fatty acids) | 26.4 ± 0.09 | 28.8 ± 0.05 | 30.5 ± 0.04 | 46.1 ± 0.06 | 52.7 ± 0.06 | 56.8 ± 0.06 |

Figure 1 shows that *C. vulgaris* (UTEX 2714) can grow and grow best on 3× diluted pre-treated swine manure, and $NH_4^+$-N, TN, TP and COD can be effectively removed by 99.8-99.9%, 67.4-70.8%, 49.3-54.4%, 73.6-78.7%, respectively. Based on these research a paper was published by Deng et al. (2017). It was concluded that (1) C. vulgaris (UTEX 2714) grew best in 3 PADSM; (2) C. vulgaris (UTEX 2714) was capable of completely depleting NH4 + -N, TN, TP and COD from PADSM, particularly when it was cultivated with bacteria; and (3) C. vulgaris (UTEX 2714) grown on autoclaved PADSM could be used as animal feed, while oil from this strain cultivated in non-autoclaved PADSM could be used as a good-quality biodiesel resource. Thus, an integrated process of LSM pretreatment and microalgae cultivation could be employed as an effective way to utilize nutrients in the LSM to produce efficient valuable microalgal products.

**Integrating algae into aquaponics/hydroponics for wastewater treatment and biomass production**

In this study, algae and aquaponics/hydroponics integrated systems were used to evaluate the algae effect on the aquaponic/hydroponic system and to determine the nutrient removal rate and algae production rate. Figures 1 and 2 show the experiment setup where 1/3 of the area was replaced by algae.

The experiments were conducted in two different stages. The first study was conducted in a newly started system and run for several weeks. After the first study the system was maintained with regular effort including changing water, feeding fish, harvesting vegetables, adjusting pH if needed. It was expected that the nitrate level would be higher in a later stage that algae might have higher productivity, therefore the second study was in a more matured stage (Addy et al., 2017).

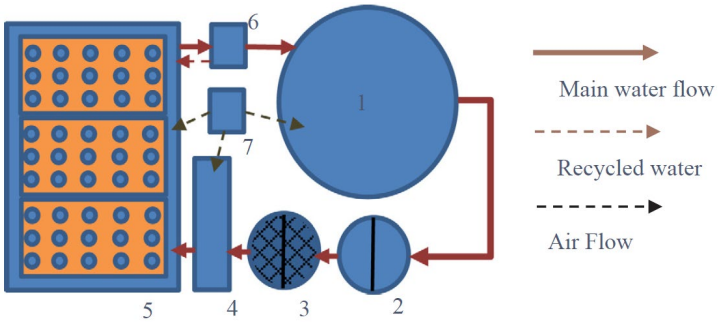



**Figure. 1. The aquaponic system (80″× 100″) from Nelson and Pade Inc. (1) fish tank (110 gallon), (2) clarification tank (30 gallon) with central baffle, (3) mineralization tank (15 gallon) with central baffle and net, (4) degassing tank (5 gallon), (5) vegetable tray (42″×67″), (6) water pump, (7) air pumps**.

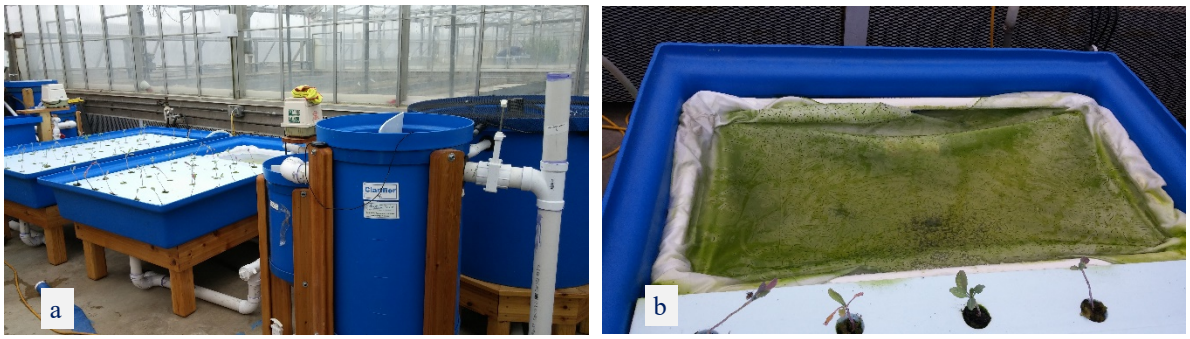



**Figure 2. Nelson and Pade system. a) Initial setup, b) algae attached on a cloth.**

To evaluate the algae effect in the aquaponic/hydroponic system, several aspects were considered. Algae as a microorganism are able to utilize both ammonia and nitrate, but their utilization preference is species dependent. When algae were placed in the system, both forms of nitrogen present. Many studies indicated that C. vulgaris preferred ammonia when ammonia nitrate was used, and higher algal yields could be obtained when nitrate was substituted by ammonia. Another research also indicated that ammonia present in the solution stopped nitrate assimilation completely. Once ammonia was used up, the nitrate assimilation would restart again. These findings suggest that nitrogen assimilated by algae would be mostly from the ammonia since there was often low levels of ammonia detected in the system that inhibited algae using nitrate (Figure 3). From this point of view, the algae were unlikely to compete with vegetable for nitrate utilization but reduce excess ammonia from the system. Total nitrogen content in leafy vegetable was about 4% on dry basis (Fontes et al., 1997). The vegetable was tested to have 96% water content. For a productivity of 155 g/m2·day vegetable would remove 0.25 g (155 × 0.04 x 0.04) nitrogen, comparing with 4.15 g/ m2·day algae production on dry basis, which was removing 0.29 g/day (4.15 × 0.07) of nitrogen (Figure 5). Although more productive, the vegetable is not necessarily more efficient than algae for nitrogen removal. When considering the economic profile, growing vegetable will still be more profitable than growing algae.

When proper amount of vegetable to fish ratio was used, the pH and ammonia in an aquaponic system can be less an issue, but it still relies on high level of management. Extensive knowledge, monitoring and control system are generally required to make quick response to change that only large scale system has this level of management. Small systems are often based on experience and manually control and therefore pH fluctuation and excessive ammonia were the potential threats to the system stability. Algae can certainly act as a buffer zone and add more diversity and resilience to the system. For the second question about the competing relationship of algae and vegetable, the situation was more complicated. Algae are unlikely competing for nitrate since there is often low level of ammonia present in the water that algae would use ammonia first, but the algae would compete for space and the total nitrogen source. For better management, the algae section should be arranged as the last section in the water cycling system before entering the fish tank. The nitrifying bacteria would be offered the first opportunity to convert the ammonia to nitrate for vegetable usage, and the ammonia not used up could be assimilated by algae. Therefore algae have less chance to compete for ammonia but to further treat the wastewater and make sure the recycled water has the lowest ammonia content. The algae cultivation system has more potential to further increase the productivity by using increased attaching area that is vertically inserted in water, and at the same time, better lighting could also improve algae biomass productivity.

It then concluded that the algae component has many proven positive effects in the system. In daily operations, algae can help balance pH value, add oxygen, and control ammonia in the system. Figure 3 shows that the system with the algae section had a lower ammonia level. Although algae have lower productivity comparable to vegetable and economically unfavorable to grower, but algae can remove nitrogen more efficiently than vegetable due to higher nitrogen content in algae. Moreover algae are unlikely to compete with vegetable for nitrate nitrogen but compete for total nitrogen resource and growth space. In term of water treatment, algae have a unique role in the system and could be placed at the final stage of the system for further ammonia removal when situation allows.

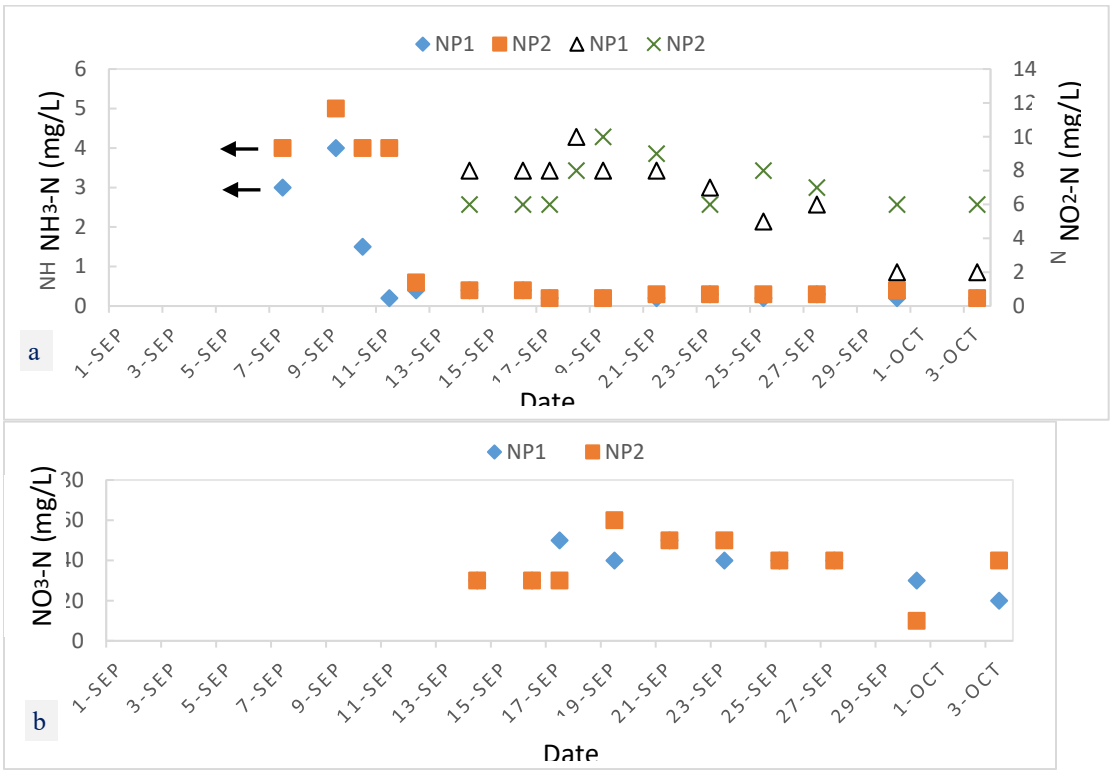
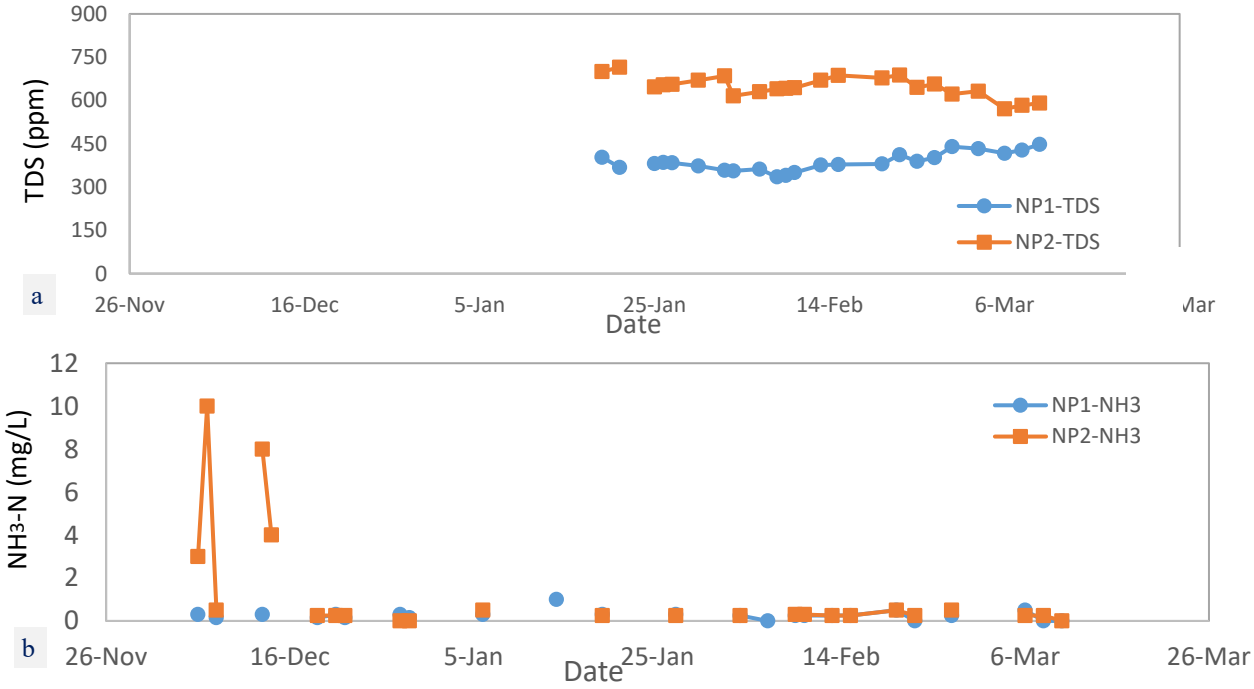



Figure 3. The (a) $NH_3$-N and $NO_2$-N, and (b) $NO_3$-N of the NP1 and NP2 systems in study 1.



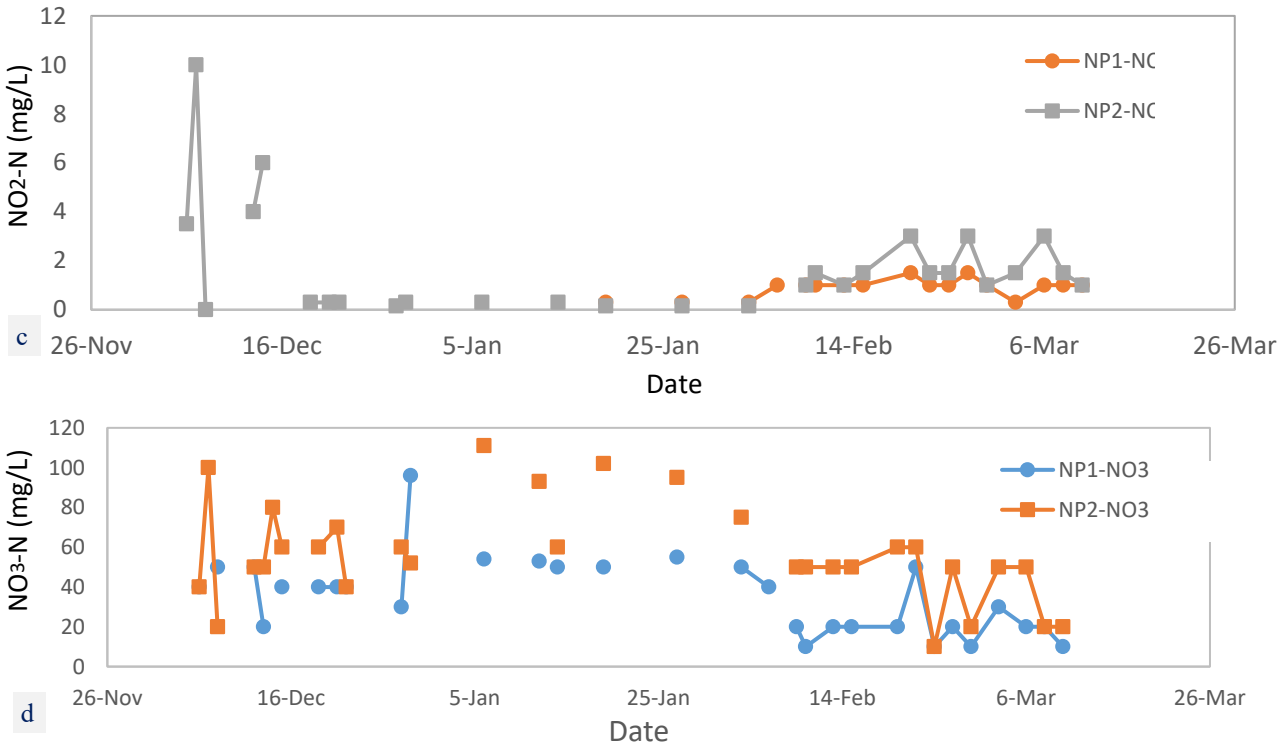



Figure 4. The (a) TDS, (b) $NH_3$-N, (c) $NO_2$-N, and (d) $NO_3$-N of the NP1 and NP2 systems in study 2.

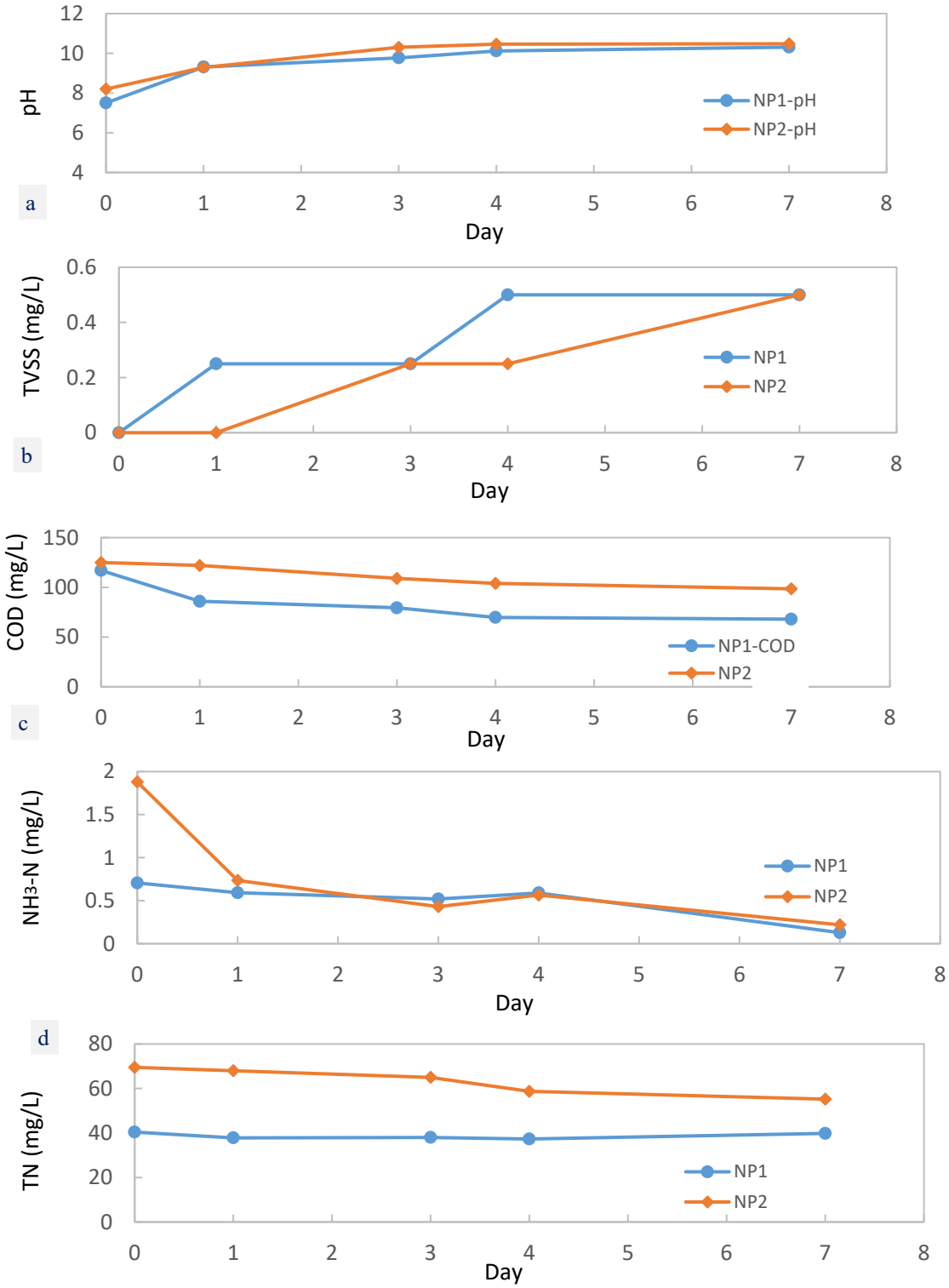



Figure 5. Algae cultivated in the lab condition. (a) pH, (b) TVSS, (c) COD, (d) TN, and (e) $NH_3$-N.

**An innovative intermittent-vacuum assisted thermophilic anaerobic digestion process for effective animal manure utilization and treatment**

The objective of this study was to prove the possibility of intermittent-vacuum assisted thermophilic anaerobic digestion (IVS-TAD) system through investigating the synergistic effect of temperature, pH and pressure on total ammonia nitrogen (TAN) removal efficiency in a short-term thermal-vacuum stripping (TVS) treatment to

simulate intermittent stripping process in IVS-TAD. Then, total organic nitrogen (ONt) degradation and nitrogen forms inter-conversion in TVS were monitored to evaluate the long-term TAN accumulation risk of IVS-TAD and the nitrogen source adaptability for algae cultivation. Furthermore, to investigate the hydrolysis and methanogenesis improvement of TAD though the short-term TVS, biogas composition analysis and nutrients variance between partial liquid swine manure (PLSM) and liquid swine manure (LSM) were inspected through biomethane potential testing under thermophilic condition (55 $^0$C) (Table 1).

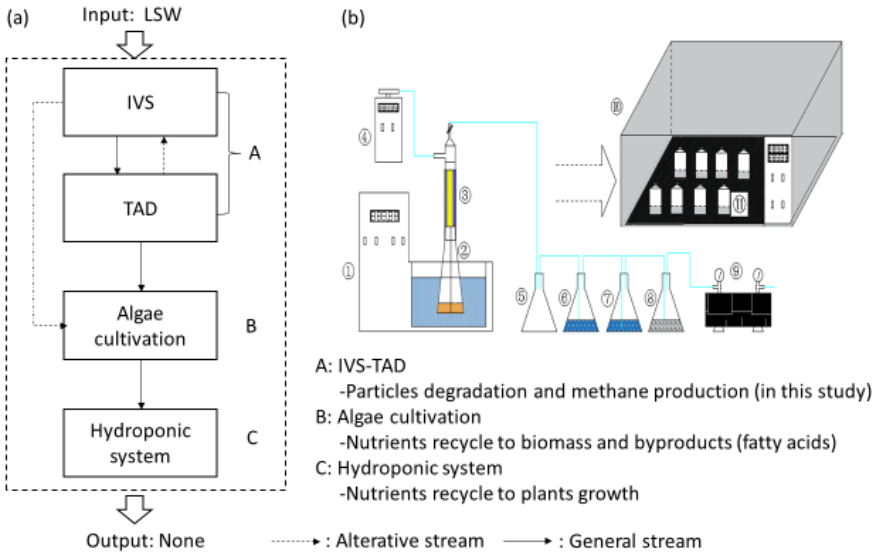



**Fig. 1 (**a) Overview of complete nutrients utilization system for LSM; (b) IVS-TAD system: 1- waterbath, 2- substrates container, 3- condenser with water, 4- Manometer, 5- Liquid trapping bottle, 6- sulfuric acid solution, 7- sodium hydroxide, 8 – dehydrated agent, 9 – vacuum pump, 10 – temperature controlled shaker, 11 – thermophilic digesters (serum bottles).

Intermittent -vacuum stripping (IVS) was developed as a pretreatment for thermophilic anaerobic digestion (TAD) to improve methanogenesis and hydrolysis activity through preventing free ammonia and hydrogen sulfide ($H_2S$) inhibition from liquid swine manure (LSM). Over 98% of ammonia and 38% organic nitrogen were removed in 60 minutes from 55 $^0$C to 85 $^0$C with vacuum pressure (from 100.63 ± 3.79 mmHg to 360.91 ± 7.39 mmHg) at initial pH 10.0 by IVS (figure 2). Thermophilic methanogenesis and hydrolysis activity of pretreated LSM increased 52.25% (from 11.56 ± 1.75% to 17.60 ± 0.49%) in 25 days and 40% (from 10 days to 6 days) in bio-methane potential assay (figure 4). Over 80% $H_2S$ and total nitrogen were removed by IVS assistance, while around 70% nitrogen was recycled as ammonium sulfate (figure 3)(Zhang et al., 2017). Therefore, IVS-TAD combination could be an effective strategy to improve TAD efficiency, whose elution is more easily utilized in algae cultivation and/or hydroponic system.

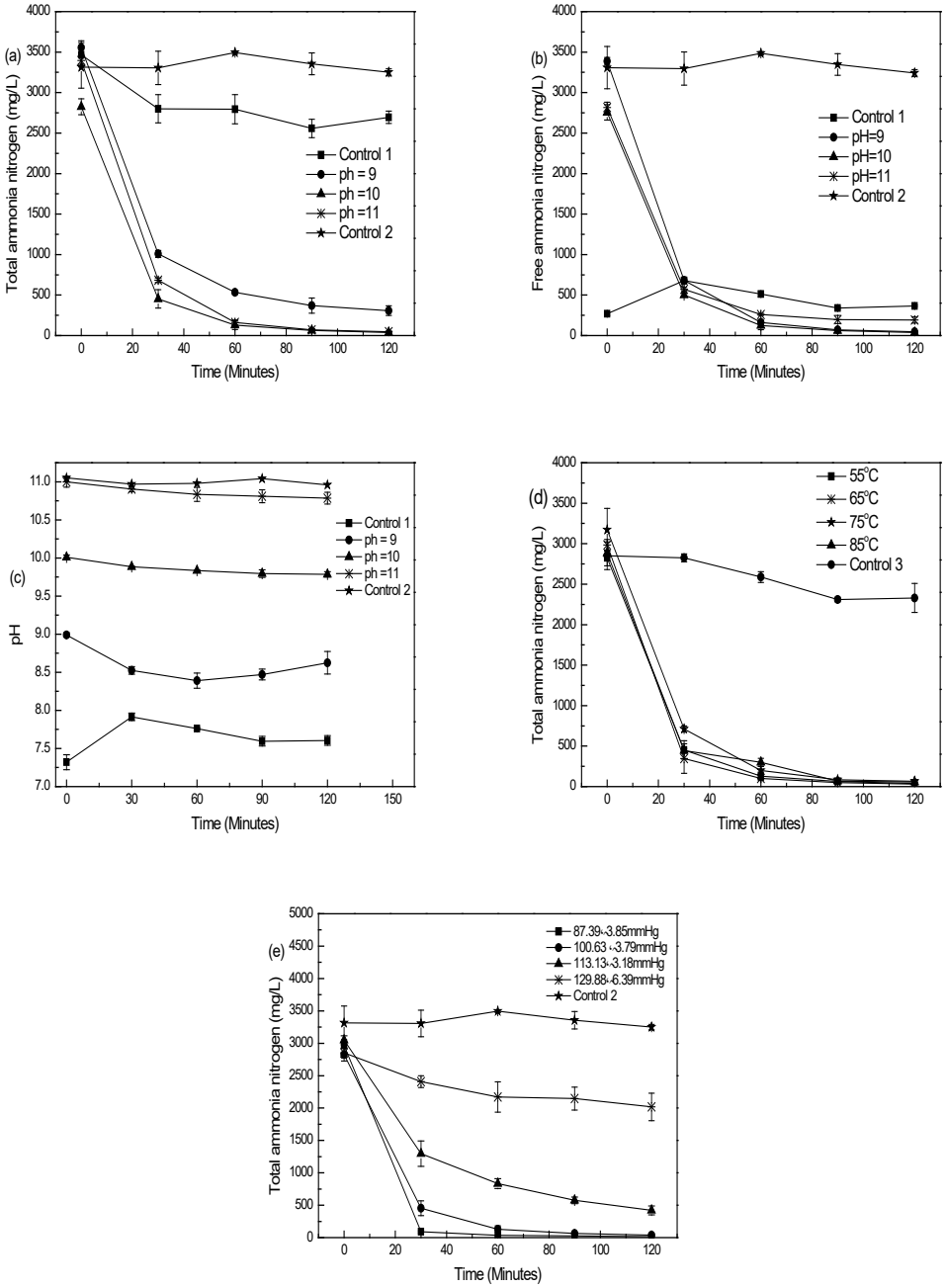



**Fig. 2** Effects of temperature, pH and pressure on TAN removal efficiency in TVS: (a-c) Effect of initial pH conditions on TAN, FAN removal efficiency and pH trends at 55 $^0$C and 100 mmHg with TVS; (d) Effect of different temperature on TAN removal efficiency of IVS at 85% relative pure water boiling pressure and initial pH 10; (e) Effect of pressure on TAN removal efficiency of IVS at 55 $^0$C and initial pH 10. Control 1: Natural LSM at 55 $^0$C and 100 mmHg; Control 2: Initial pH 11 at 55 $^0$C and atmosphere; Control 3: pH 10 LSM at 85 $^0$C and atmosphere.

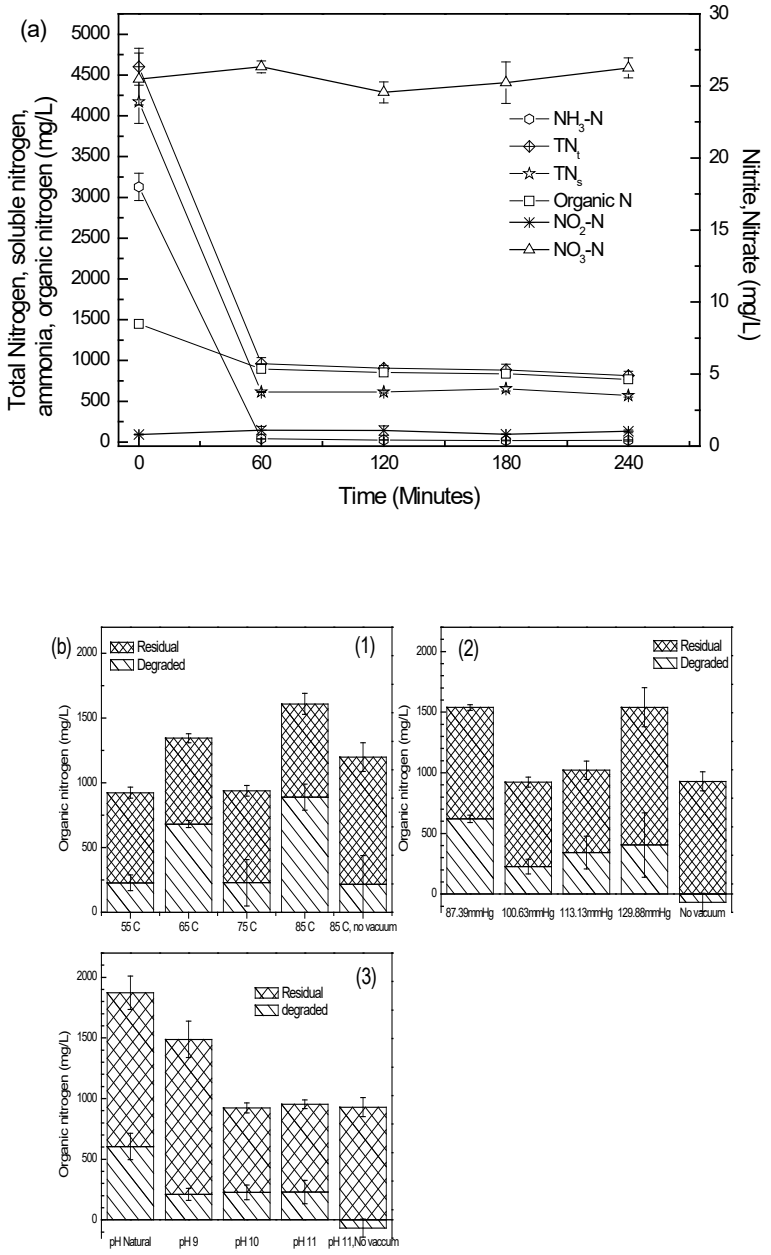



**Fig. 3** Nitrogen conversion and degradation in IVS: (a) Nitrogen conversion variance in IVS; (b) Effect of pH, pressure and temperature on ONt degradation in IVS.

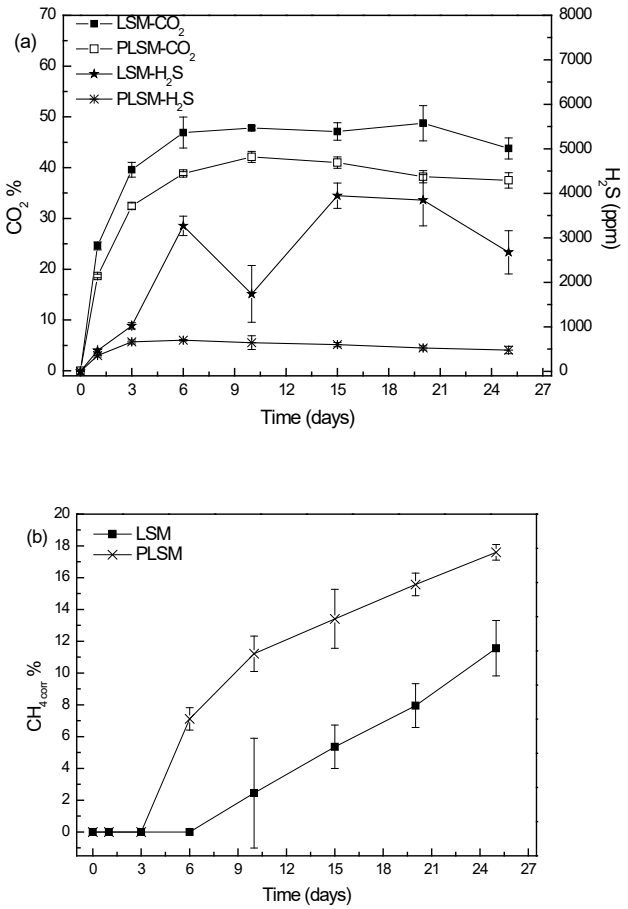



**Fig. 4** Biogas compositions comparison of LSM and PLSM by BMP test: (a) Carbon dioxide and hydrogen sulfide variances in biogas; (b)Correction methane yield variance in biogas.

Table 2. Characteristics of materials used in IVS and TAD.

| Particular | Units | LSM | PLSM |
|---|---|---|---|
| CODt | g/L | 72.48 ± 0.92 | 66.30 ± 0. 69 |
| CODs | g/L | 43.07 ± 0.15 | 53.03 ± 0.15 |
| TNt | g/L | 5.12 ± 0.26 | 1.01 ± 0.03 |
| TNs | g/L | 4.23 ± 0.12 | 0.73 ± 0.01 |
| TPs ($PO_4^-$-P) | mg/L | 198.35 ± 1.71 | 244.50 ± 3.42 |
| TAN ($NH_4^+$-N) | mg/L | 3357.50 ± 12.58 | 43.30 ± 0.62 |
| pH* | | 7.80 ± 0.16 | 10.29 ± 0.08 |
| Nitrate ($NO_3^-$-N) | mg/L | 25.70 ± 0.49 | 28.65 ± 5.04 |
| Nitrite ($NO_2^-$-N) | mg/L | 0.78 ± 0.05 | 1.10 ± 0.09 |
| TS | g/kg | 24.91 ± 1.76 | 42.48 ± 1.53 |
| VS | g/kg | 13.11 ± 0.98 | 15.59 ± 1.62 |

"±" means standard deviation and all values presented are the means of independent quadruplicate (n = 4).
* means the pH value was measured under room temperature.

**Develop 40 L thermophilic AD process with light vacuum to treat animal wastewater**

In the second period of this project, we have scaled-up thermophilic AD process with a stripping side in a pilot scale (40L) as shown in Figure 1. Corn Stover was supplied as the nutrient balance component for thermophilic anaerobic co-digestion with animal wastewater. The biomethane potential (BMP) yield of different pH and salinity conditions were analyzed under thermophilic condition (55 °C). The performance of biogas composition, methane yield, nutrient utilization and solid degradation were studied under 40 L scale reactor.

Figure 1. The experimental apparatus for thermophilic AD with light vacuum side to treat animal wastewater.

Corn stover was mixed with liquid animal waste water as carbon source supplement since the C:N ratio of single animal waste water is not high enough to achieve a high nutrient utilization efficiency. The previous results also showed the corn stover addition can truly improve the animal waste water digestion ability.

To investigate the effect of thermal vacuum stripping on corn ctover, the BMP tests were used under thermophilic condition. The results showed the Thermal vacuum striping (TVS) may reduce the corn stover benefits (Fig. 2a), if animal waste water was treated without corn stover. However, an obviously improvement from pretreated animal waste water and Corn Stover together condition indicated that the TVS mainly affect the corn stover part. The potential reason for the weakness in pretreated animal waste water group may due to the VFAs losing during TVS process, which may also reduce the hydrolysis efficiency in thermophilic anaerobic co-digestion. However, the alkali (pH =10) and high temperature (55 C) condition may help cellulose and hemicellulose degradation, which can be considered as significant benefits from TVS, since the most thermophilic anaerobic co-digestion was sensitive to high pH condition.

The results also showed the pretreated manure and corn stover mixture can significant improve the specific methane yield under higher pH condition (Fig. 2b), which may due to the lower ammonia inhibition and abundant VFAs in pretreated substrate.

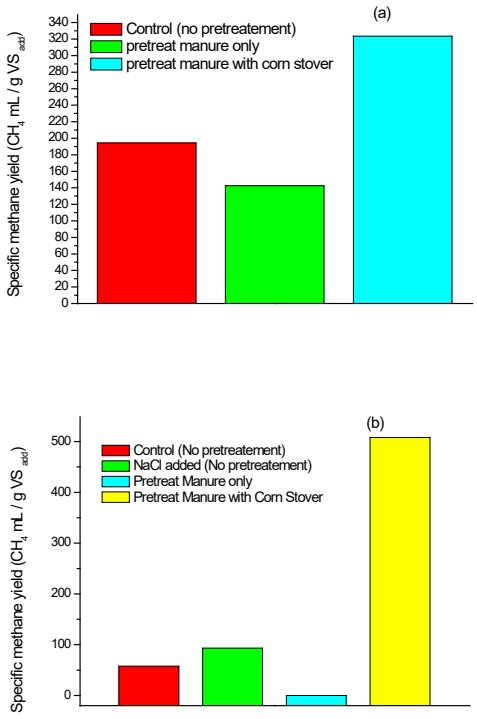



Figure 2. (a) BMP test under Initial pH 7.5; (b) BMP test under initial pH 8.5.

Table 3. Substrates profile for thermophilic co-digestion.

| Substrate | LSM | PLSM |
|---|---|---|
| $COD_t$ | 77800 ± 2000 | 74550 ± 1850 |
| $COD_s$ | 38950 ± 3750 | 39050 ± 450 |
| TAN | 5280 ± 331 | 1625 ± 15 |
| $TN_t$ | 6925 ± 375 | 3523 ± 178 |
| $TN_s$ | 5775 ± 25 | 2468 ± 3 |
| $TP_t$ ($P\text{-}PO_4^-$) | 1128 ± 18 | 1053 ± 28 |
| $TP_s$ ($P\text{-}PO_4^-$) | 290 ± 3 | 323 ± 1 |
| $NO_3^-$ | 40.33 ± 2.18 | 61.75 ± 2.25 |
| $NO_2^-$ | 0.69 ± 0.57 | 2.47 ± 0.12 |
| VS % | 6.32 ± 0.24 | 4.25 ± 0.87 |
| TS % | 7.99 ± 0.24 | 9.15 ± 0.08 |
| C:N | 3.74 | 7.05 |

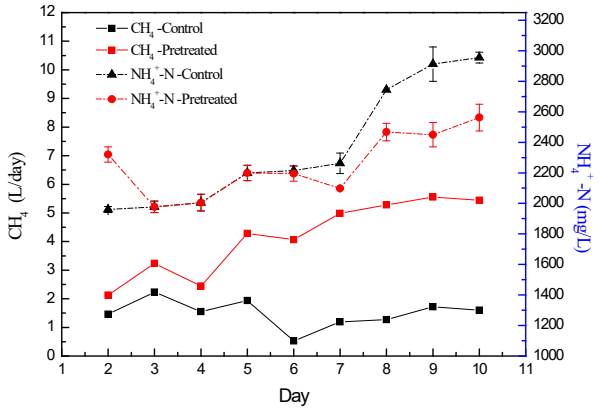



Figure 3. The relationship between methane yield and TAD ammonia concentration by pretreated and untreated substrates.

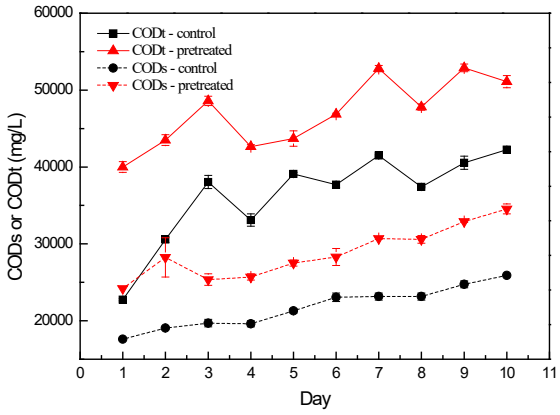



Figure 4. Degradation variance from total COD to soluble COD by pretreated and untreated substrates.

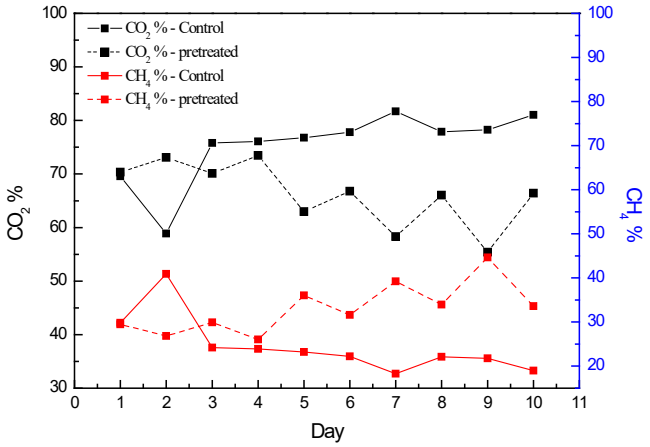



Figure 5. Degradation variance from total COD to soluble COD by pretreated and untreated substrates.

Methane average yield was improved by TVS from 1.62 L/day to 4.17 L/day with a 2.57 time increment in first 10 days (as shown in Figure 3), when ammonia inhibition variance was not significantly due to less accumulation

in the starting stage. The total COD consumption rate of pretreated group was higher than untreated group (as shown in Figure 4). However, the concentration of soluble COD in TAD showed similar trends in in first 10 days, which means concentration of CODs was not affected by thermo-vacuum treatment (as shown in Figure 4). Methane concentration of biogas was increased from 20% to 40% by thermo-vacuum pretreatment. The methane percentage of pretreated group dropped after every two days feeding and raised later, which shows that the daily loading rate can also be increased after thermo-vacuum pretreatment compared with untreated group. (as shown in Figure 5). After TVS pretreatment the C:N ratio of mixture substrate was almost double than before, which may also contribute to the improved fermentation activity of TAD (Table 2).

The lower removal TAN efficiency of scale-up TVS equipment may be the reason for over 1000 mg/L TAN left in PLSM substrate. And longer stripping time was applied due to the lower efficiency, which may also make the VFAs lose during TVS process.

A majority of the activities and outcomes for activity 1 have been completed or tested. We have developed and optimized parameters for testing algae in the integrated wastewater system. In daily operations, algae was found to balance pH value, add oxygen, and control ammonia in the wastewater system. Chlorella vulgaris grew well on minimally diluted pretreated manure. The algae could be used as biodiesel feedstock and the algal biomass could be used as an animal feed if cultivated in wastewater production system. In the benchtop system, we have optimized nutrient removal rate of algae production system with wastewater to clean swine and dairy wastewater.

**Activity Status as of *July 1, 2018*:**

A majority of the work has been completed for activity 1. Publications for the work will be published in 2018 and 2019. In summary of our recent study, a cultivation system with daily recycling of the post-harvest culture broth was set up and performed in order to reuse the water and nutrients in pretreated anaerobically digested swine manure, which was used as media to cultivate Chlorella vulgaris (UTEX 2714) at different recycling ratios. Results showed that the alga grew well in the system with an accumulative algal biomass and productivity of 1.68–3.47 g/L and 234.1–532.2 mg/L/d, respectively, at the end of the cultivation. Additionally, chemical compositions in this alga varied with the change of recycling ratios, and the highest productivities of carbohydrate, protein and lipids (76.4, 257.2 and 183.7 mg/L/d, respectively) were obtained in the system with a recycling ratio of 1/4 or 1/6. Fatty acid profiles indicated that this alga could be used as a good-quality feedstock with a productivity of 9.65–40.1 mg/L/d.

**Activity Status as of *January 1, 2019*:**

Publications for the work were published in 2018 and more will be in 2019. Briefly, High salinity was a more serious inhibition than high concentration ammonia in Thermophilic Anerobic Digestion (TAD). Half the CH4 production was loss in TAD, while the salinity increased from 2% to 4%. Methanogenesis was completely inhibited at 8% salinity in TAD. Butyrate was a indicator of the non-salinity inhibited methanogenesis in TAD. In another study that evaluated an integrated process for anaerobically digested swine manure, flocculation and struvite precipitation were successfully used as pretreatment. Co-cultivation of algae and bacteria provided excellent biological treatment. Activated carbon adsorption ensured the final complete digestate treatment. Higher biomass and nutrients removal were achieved with the integrated process.

**Final Report Summary:**

*Microalgae cultivation work*

The effluent from the AD with much of the ammonia stripped was used for microalgae cultivation. After stripping the ammonia, we could largely reduce the dilution rate e.g. from 20 times dilution to 5 times dilution.

The algae strain *Chlorella vulgaris* was grown on minimally diluted (2×, 3× and 4×) autoclaved and non-autoclaved pretreated anaerobic digestion manure in a batch-culture system for 7 days. Results

showed that *C. vulgaris* grew best on 3× AD effluent, and effectively removed $NH_4^+$-N, TN, TP and COD by 98.48-99.82%, 49.21-55.41%, 19.95-23.09%, 31.22-33.95% and 99.84-99.92%, 67.35-70.83%, 49.34-54.36%, 73.56-78.66% in differently diluted autoclaved and non-autoclaved AD effluent, respectively. The results of the chemical compositions in *C. vulgaris* indicated that carbohydrate may be converted to lipids with increasing dilution of the AD effluent. The fatty acid profiles of *C. vulgaris* suggested that this alga could be used as animal feed if cultivated in autoclaved the AD effluent and as good-quality biodiesel feedstock if cultivated in non-autoclaved AD effluent (Deng, X. et al. 2018).

Since the size of the microalgae is too small to be harvested, a filamentous algae Tribonema sp. was used in this project. We expected that the filamentous algae can act as a harvesting tool for catch the individual Chlorella. It aims to reduce the cost of wastewater treatment and improves the economics of algae harvesting. It was found that the co-cultivation may induce auto flocculation of the microalgae due to filamentous excrete cationic polymer, which bind non-filamentous microalgae cells and increase the harvesting efficiency. At the same time, the co-cultured system can reduce the ammonia nitrogen, total nitrogen, total phosphorus in the wastewater for about 95% and COD for 50%(Salim et al., 2011; Gonçalves et al., 2016).

In Test 1, the optimization of inoculation ratio for the two strains in co-culture was explored. Figure 3 shows the shape of two strains. The cells size ranged from 2 to 15 μm in diameter. Chlorella zofingiensis as a potential producer of astaxanthin, with the 0.3%-0.8% of the dry biomass.

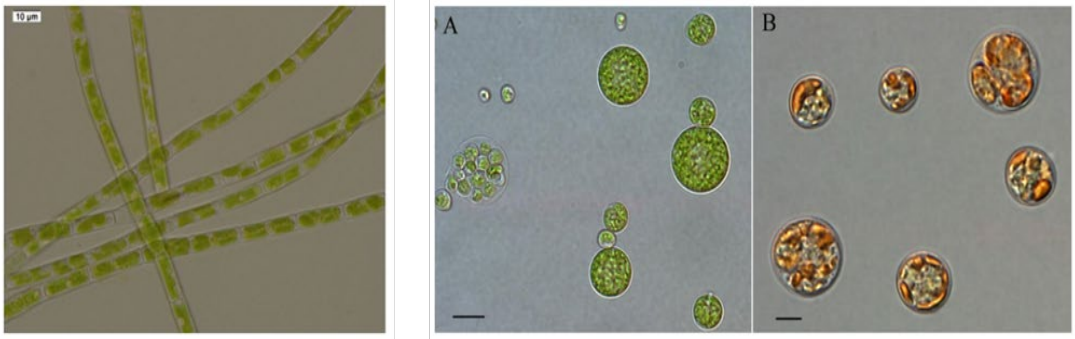



*Figure 6 The picture of the two algae species used in this project, left picture is Tribonema sp, Right picture is the chlorella zofingiensis*

In order to reduce freshwater usage, the aquaculture wastewater was used to dilute the swine manure wastewater and the nutrient profile is in table 4.

Table 4 Aquaculture wastewater sampled from a fishery (Liu et al., 2019)

| Parameters (mg. L-1) | COD | NO3-N | NO2-N | NH 4-N | TP |
|---|---|---|---|---|---|
| | 32.4 | 0.35 | 24.7 | 6.25 | 1.83 |

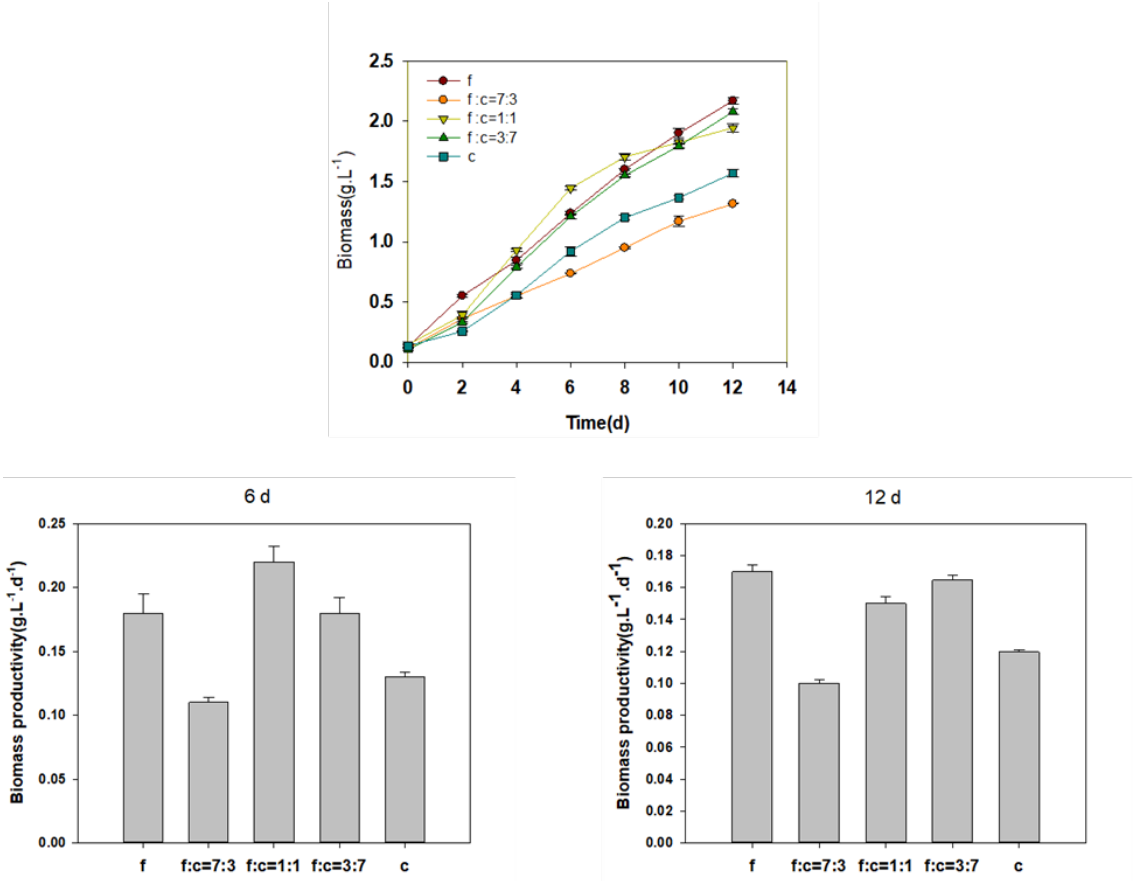



Figure 7 The growth of co-culture of the two strains in the BG-11.

Note: Air conditions, No CO2; Light intensity: 55 μ mol m-2s-1; the initial biomass of Filamentous sp. is 0.15 gL-1, the initial biomass of Chlorella zofingiensis is 0.14 gL-1.

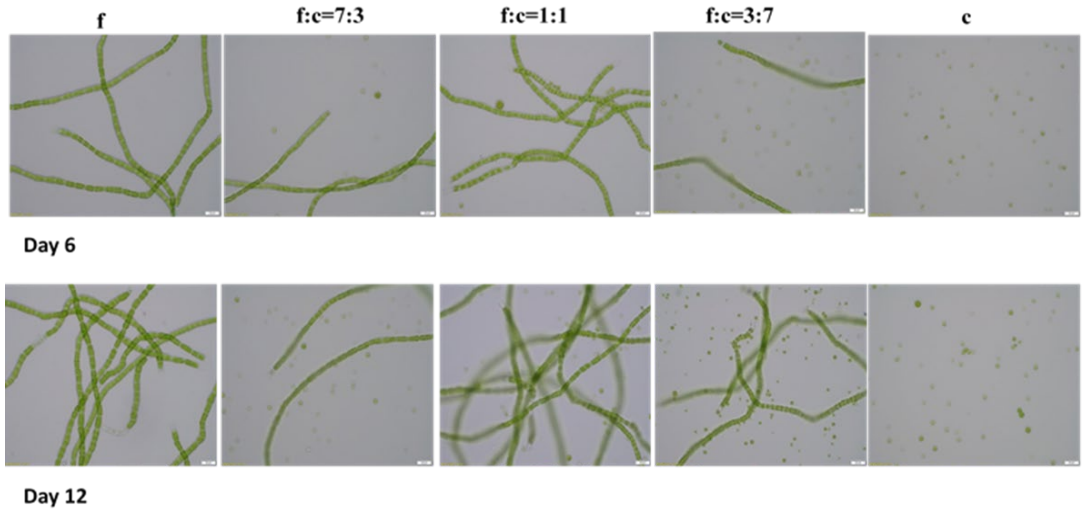



Figure 8 The growth of the two strains in co-culture in BG-11.

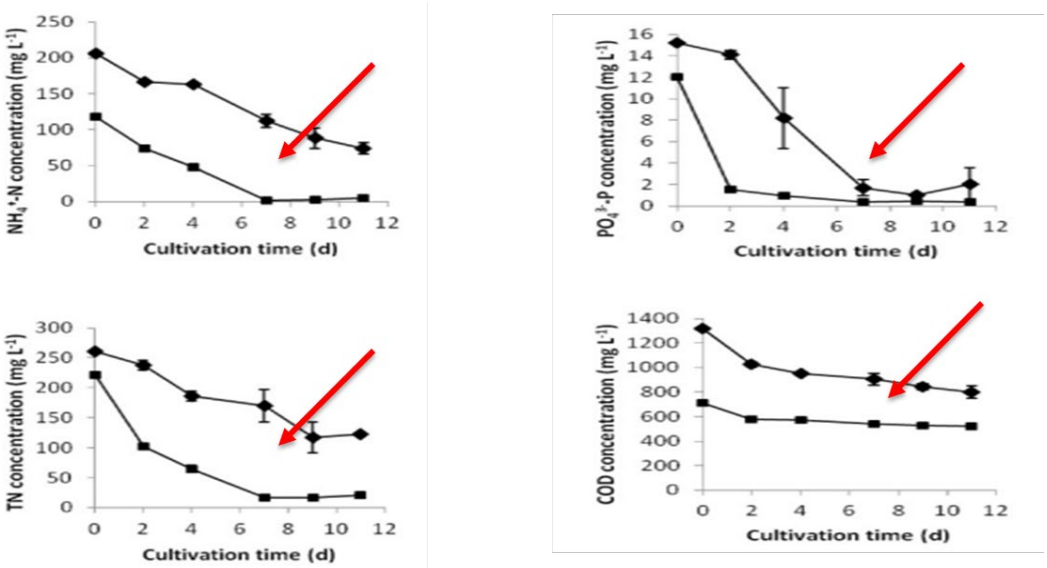



*Figure 9 Nutrient removal of the growth of co-culture in swine wastewater*

It was presented in Fig. 6, when treat swine wastewater with microalgae, the pollutants in wastewater drop the fastest in the first few days, and the change tended towards stable at about 4-6 days. (Fig. 6)

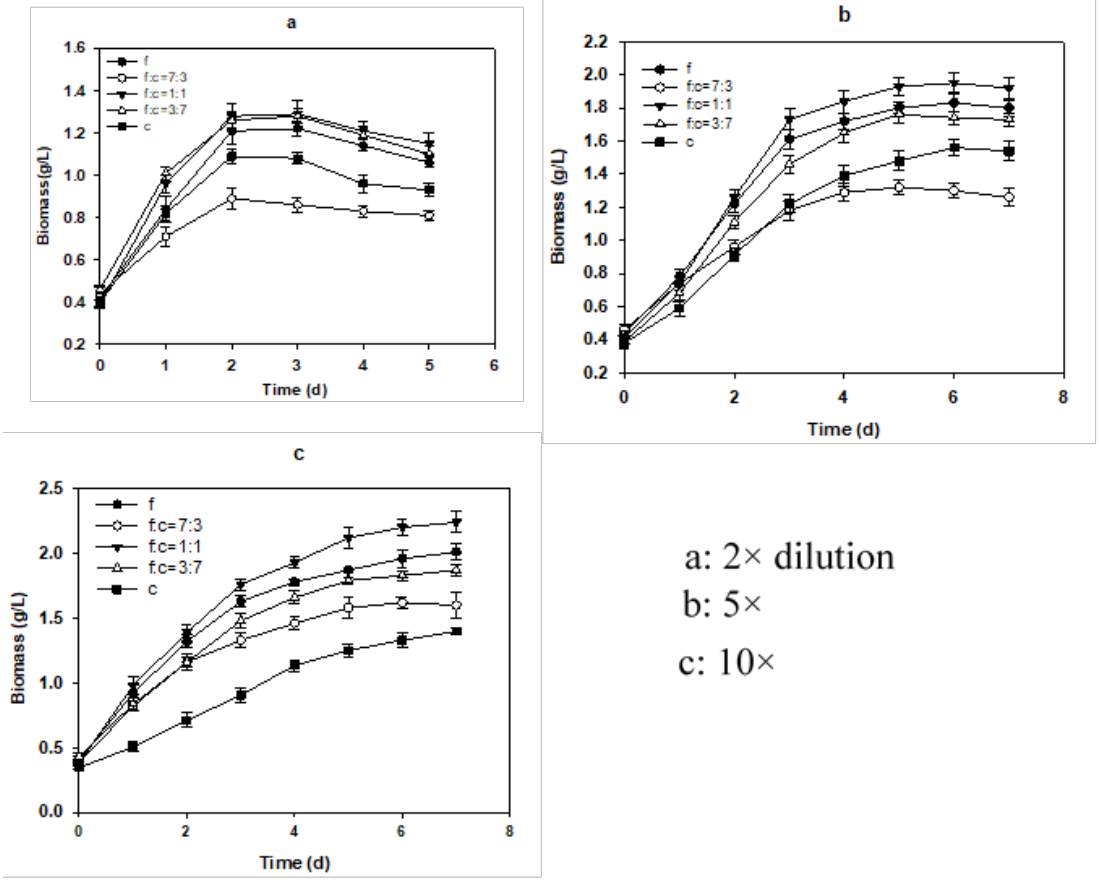



Figure 10 Co-culture of the two strain in wastewater diluted with aquaculture wastewater with different dilution rate.

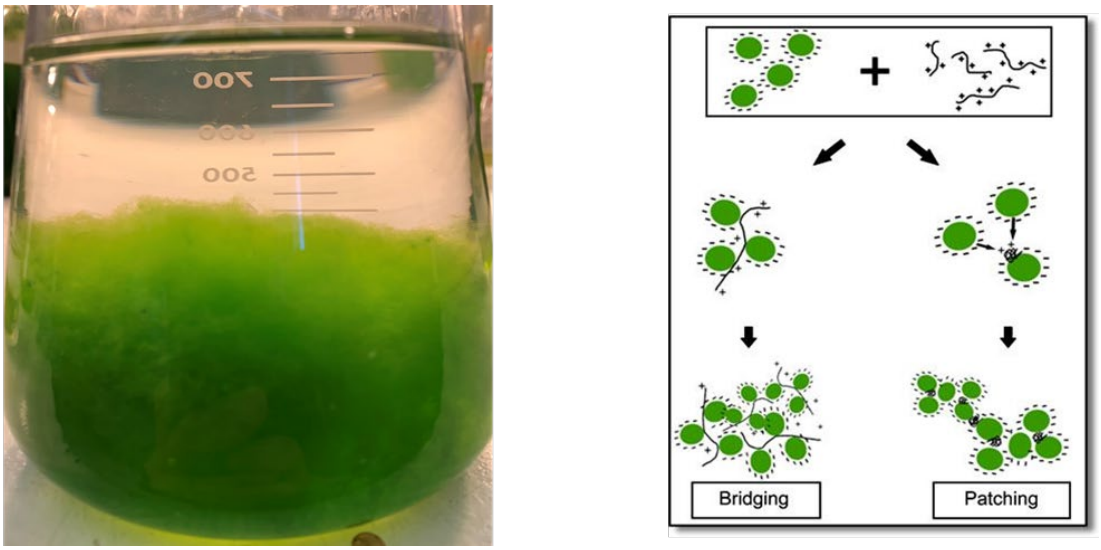



Figure 11 Auto flocculating Filamentous may be able to induce sedimentation of non-flocculating microalgae Chlorella zofingiensis and may increase the harvesting efficiency.

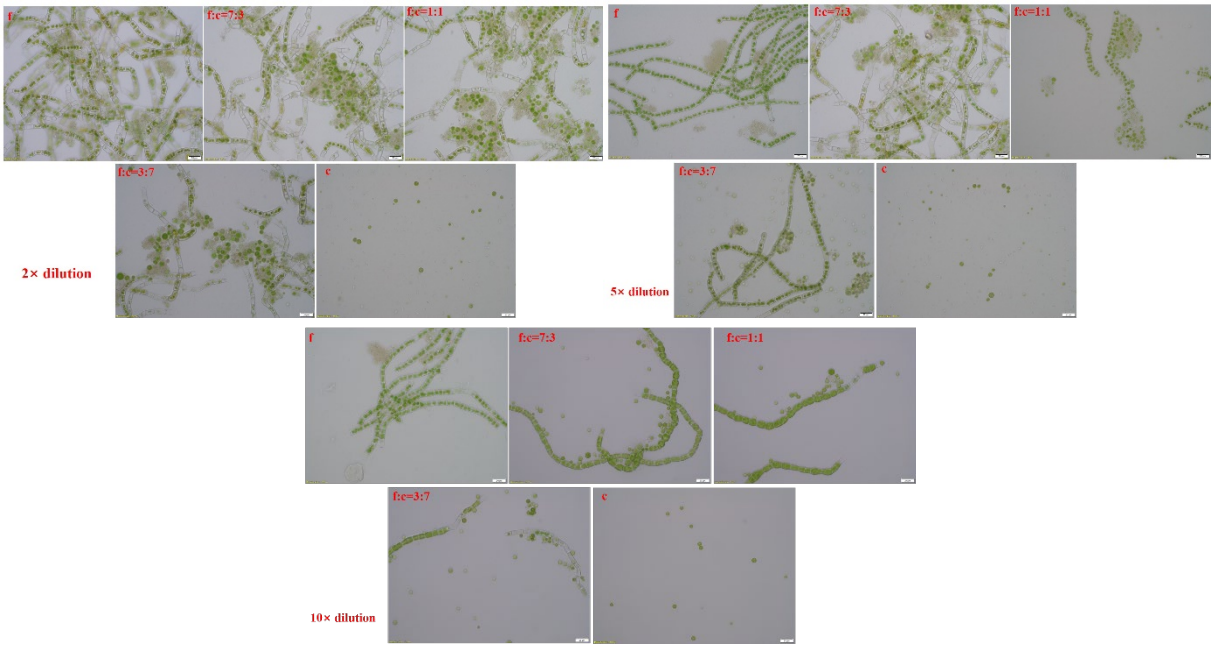



Fig. 9 Comparison of algal cells microscope observation on co-culture of different initial inoculum ratios

As Figure 7 and Figure 9 shown, the co-culture effect of wastewater with different dilution concentration on two algae was different, and the better ratio is 1:1. It may be that the two algae complement each other in their need for nutrients in the wastewater to promote growth. In poor inoculation ratio of f:c=7:3, there were more flocculating microalgae filamentous, and more chlorella was wrapped, which may reduce the light intensity of the two algae. Using one flocculating Filamentous sp. for harvesting of another high-value microalga Chlorella zofingiensis can be applied as the controlled and reliable pre-concentration step in harvesting of the high-value microalgae. Filamentous algae may promote the flocculation of Chlorella

by secreting extracellular substances. (Fig.7 and Fig. 9)

*Hydroponic cultivation work*

Three vegetables, namely arugala, purple kohlrabi, lettuce, were hydroponically grown on artificial and AD manure effluent with and without added algae to the culture media. The effect of microalgae on production and nutrient removal was assessed for three vegetable crops (arugula, purple kohlrabi and lettuce) grown entirely using synthetic wastewater in greenhouse hydroponics. Results showed the effect of algae on the productivity of different vegetable species varied. Algae have a negative effect on arugula growth, but in purple kohlrabi or lettuce groups, there were no negative effects or even an increase in vegetable production. After 35 days, the mean harvested biomass of purple kohlrabi in natural state was 31.6 g/plant (2.16 kg/m2), which was significantly greater than that of arugula, lettuce or purple kohlrabi in all other conditions. The dissolved oxygen and algae biomass concentration in the Chlorella addition group is relatively higher. The existence of algae, especially Chlorella addition, can significantly improve the removal rate of total dissolved solids, total nitrogen, total phosphorus, but not COD. These results from experiments using synthetic wastewater provide clear pictures how microalgae and vegetables interacted. Similar results were obtained from experiments using AD manure effluent as the culture media.

Hydroponic systems are efficient and industrialized green vegetable production systems. The growth rate of a hydroponic plant is 30-50% faster than a soil plant. Hydroponics can realize the automatic control of irrigation and fertilization, ensure a clean planting environment, and save space for multilayer vertical production (Wada, 2019). However, algae often thrive in hydroponic systems with a recirculating nutrient solution. It appears that algae have some negative effects on vegetable yield in hydroponic systems. It is generally accepted that algae will affect the water quality parameters of pH, DO, and consume the nutrients in the water and compete with the target vegetables. Algae can even act as a buffer, preventing effective chemical sanitization even with bleach treatment. Thus, it is important that algal levels are kept to a minimum in hydroponic systems (Tesoriero et al., 2010).

In this project, experiments were carried out to monitor the effect of microalgae (naturally grown algae or *Chlorella* addition) on the growth of three typical vegetables (arugula, purple kohlrabi and lettuce) and nutrient removal in greenhouse hydroponics with synthetic wastewater. Also, as an important feed additive, health food and biofuel raw material (Khan et al., 2018; Huo et al., 2018), algae biomass accumulation together with vegetable production in hydroponic systems could be strongly recommended as a new strategy for a better, cheaper and ecofriendly co-cultivation. The concentrations of the main components are shown in Table 5.

Table 5 Characteristics of synthetic wastewater

| Parameters | Concentration (mg/L) |
|---|---|
| Chemical Oxygen Demand | 120 |
| Total dissolved solids,TDS | 455 |
| Ammonia | 18.4 |
| Nitrates | 62.5 |
| Total nitrogen | 80.9 |
| Total phosphorus | 13.8 |
| Potassium | 69.8 |
| Calcium | 19.8 |
| Magnesium | 9.9 |
| iron | 1.0 |
| pH | 6.9 |

Under different culture conditions, algae have different effects on the growth of the three kinds of vegetables. As Figure 9 shows, after 35 days, the arugula crop has the highest biomass of 13.9 g/plant under shading state condition. Under the conditions of *Chlorella* addition or natural state, the existence of algae is not conducive to arugula growth and is the worst in the natural state. This result may be caused by algae competitive nutrition. Also, algae on the containers and expanded clay pellets can act as a food source for fungus gnats. Fungus gnats can directly reduce plant growth by larval feeding of roots, but they can also spread spores of plant pathogens (Tesoriero et al., 2010). In the purple kohlrabi group, under natural state conditions, the biomass of purple kohlrabi can reach up to 31.6 g/plant. Algae does not seem to pose a threat to the growth of purple kohlrabi. Under natural state conditions, vegetables, algae and bacteria can build a harmonious system. However, the biomass accumulation of vegetables in the *Chlorella* addition group is very poor. The oxygen produced by algal photosynthesis prevents anaerobiosis in the root system of the crop and removes $CO_2$ fertilization from crop root respiration (Jansson et al., 2010). Furthermore, Barone et al. (2019) found that growth-promoting biostimulant extracts (amino acids, enzymes or hormones) from *Chlorella* vulgaris and S.quadricauda in the early stages of plant growth in sugar beet. Those substance are beneficial for plant culture and can possibly increase nutrient uptake and stress response (Shaaban et al, 2010; Michalak et al, 2016). In the lettuce group, the biomass of lettuce with *Chlorella* addition before the fourth week is the highest at 12.0 g/plant, and the biomass is the highest under natural state conditions at 13.9 g/plant in the fifth week. The putative bio-stimulation mechanism induced by microalgae is a very complex mechanism and not completely understood.

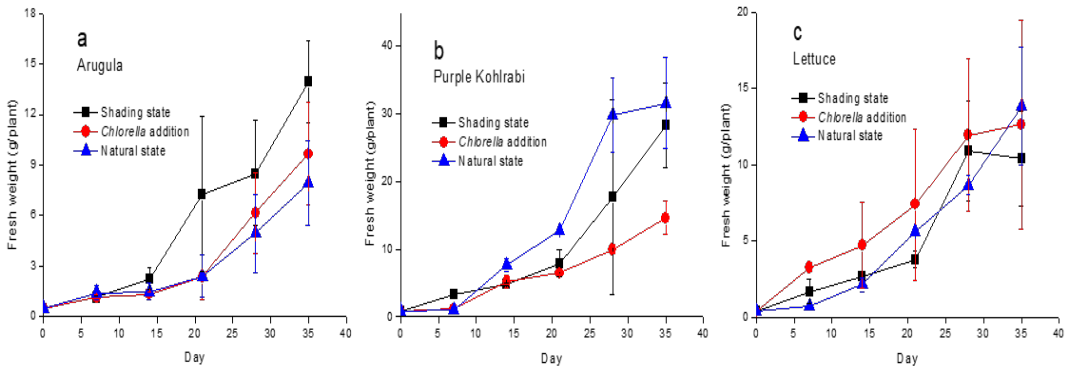



Figure 12 Biomass accumulation of vegetable plant in the hydroponics

From Table 2, it can be seen that the algae biomass in *Chlorella* addition group is relatively higher than that in natural state condition, especially in the purple kohlrabi and lettuce groups. The algae biomass in the purple kohlrabi and lettuce groups increased by 80% and 75%, respectively, compared with that in the natural state condition. *Chlorella* grows fast and has strong adaptability to the environment. But it is easily preyed on by protozoans such as rotifers (Huo et al., 2017). Algae can increase the dissolved oxygen concentration in the hydroponic system (Fang et al., 2017), and the dissolved oxygen in *Chlorella* addition group is relatively higher than that in natural state condition.

The growth rate of vegetable root length is faster than stem length, and the growth rate of vegetable root length is positively related to vegetable productivity. Normally, the root system and epigeous part of the plant grow at the same rate. The root and shoot ratio (both on the basis of fresh and dry weight) can be heavily affected by stress conditions (Erice et al. 2010). In arugula and purple kohlrabi groups, algae significantly affected the root-shoot ratio, no matter whether the condition was *Chlorella* addition or Natural state. As Table 6 shows, vegetable crops were harvested at 35 days, and the mean harvest biomass of purple kohlrabi in natural state was 2.16 kg/$m^{2,}$ which is significantly greater than that of arugula, lettuce, or purple kohlrabi in the other conditions. Horiuchi et al. (2019) found in comparisons among rice, perilla, and lettuce that lettuce showed the largest growth rate, the fresh weight was 5.8g/plant at 28 days. In this experiment, the minimum dry weight exceeded 8.7g/plant (the natural state) and the 12.0 g/pant (the *Chlorella* addition) at 28 days. All species used in this study were grown at much higher densities than is recommended under standard growing practices in soil-based systems. Recommended densities for most of the species ranged from 11 to 33 plants/$m^2$ (Buzby et al., 2017), while the densities used were 68.4 plants/$m^2$.

Table 6 Vegetable growth and microalgae biomass accumulation in the hydroponic system

| Group | | Microalgal productivity ($g \cdot m^{-2} \cdot d^{-1}$) | Microalgal harvest biomass ($g \cdot L^{-1}$) | Dissolved oxygen ($g \cdot mL^{-1}$) | Vegetable stem length ($cm \cdot d^{-1}$) | Vegetable root length ($cm \cdot d^{-1}$) | Vegetable productivity ($g \cdot m^{-2} \cdot d^{-1}$)[a] | Vegetable harvest biomass ($kg \cdot m^{-2}$) |
|---|---|---|---|---|---|---|---|---|
| Arugala | Shading state | - | - | 7.73±0.77 | 0.69±0.13 | 1.25±0.60 | 2.44±0.95 | 0.95±0.17 |
| | *Chlorella* addition | 1.47±0.23 | 0.56±0.08 | 7.95±0.73 | 0.61±0.14 | 0.64±0.12 | 1.73±0.73 | 0.66±0.21 |
| | Natural state | 1.37±0.04 | 0.51±0.01 | 7.89±0.78 | 0.43±0.19 | 0.43±0.21 | 1.36±0.71 | 0.54±0.17 |
| Purple Kohlrabi | Shading state | - | - | 7.89±0.91 | 0.70±0.15 | 1.00±0.24 | 5.15±4.38 | 1.94±0.42 |
| | *Chlorella* addition | 1.41±0.23 | 0.54±0.08 | 8.23±0.98 | 0.39±0.01 | 0.44±0.14 | 2.77±0.11 | 1.00±0.17 |
| | Natural state | 0.78±0.19 | 0.29±0.07 | 8.09±0.89 | 0.80±0.09 | 1.85±0.33 | 8.84±1.66 | 2.16±0.46<br>0.46 |
| Lettuce | Shading state | - | - | 7.96±0.91 | 0.41±0.08 | 0.84±0.12 | 3.21±0.99 | 0.71±0.21<br>0.21<br>3.10483 |
| | *Chlorella* addition | 1.86±0.19 | 0.71±0.07 | 8.17±1.0 | 0.43±0.06 | 0.86±0.13 | 3.53±1.53 | 0.86±0.47 |
| | Natural state | 1.06±0.06 | 0.40±0.02 | 8.13±0.99 | 0.35±0.05 | 0.65±0.08 | 2.52±0.19 | 0.95±0.27 |

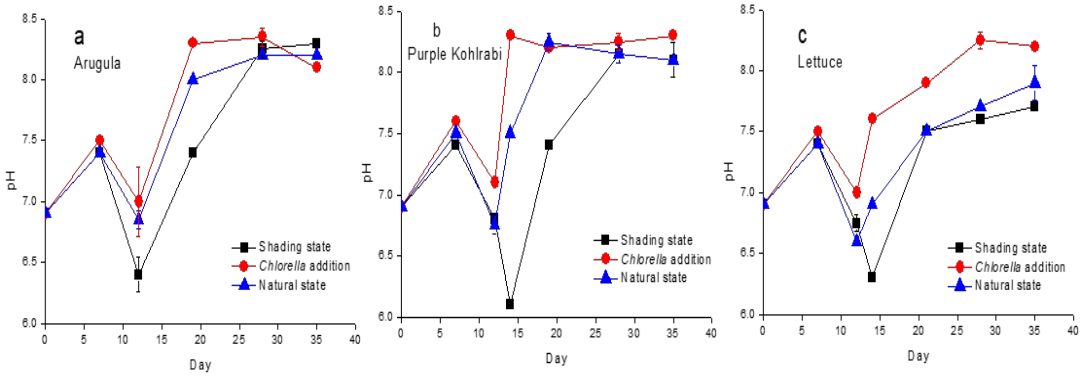



Figure 13 pH changes in the hydroponics

The pH changes for the hydroponic systems showed greater variation with the competition between the algal, vegetable and even bacterial populations, since N and P removal may alter the pH (Lee et al., 2018). As shown in Figure 10 the pH increases may be due to microalgal photosynthesis, resulting in consumption of inorganic carbon such as $HCO3^-$ and accumulation of $OH^-$ in the solution. Plants intake nitrogen in the form of nitrate, while the algae absorb more nitrogen as ammonia (Hellebust et al., 1989). Due to the fast consumption of ammonia nitrogen in the system, the pH drops. The consumption rate of ammonia nitrogen in the shade groups of purple kohlrabi and lettuce is slower (Figure 10), the pH reaches the lowest in 14 days, and the pH in the *Chlorella* addition group is significantly higher than that in the other two groups. When ammonia nitrogen is consumed, nitrate nitrogen consumption can increase pH. In general, the tolerance range for most plants is 5.5–7.5 (Somerville et al., 2014). Most plants prefer mildly acidic conditions. If the pH goes outside of this range, although the nutrients are present in the water the plants are unable to use them. However, there could be biological interactions occurring between the plant roots, bacteria and fungi that may allow nutrient uptake even at higher pH levels than those shown (Somerville et al., 2014).

As Figure 11 shows, the presence of algae, especially under *Chlorella* addition, can significantly improve the removal rate of TDS, and the removal rate of TDS in purple kohlrabi group can reach 56.7%. In the arugula group, compared with *Chlorella* addition and natural state groups, the shading group vegetables growth contributed more to TDS removal at the later stage of culture. Similar to the results of Malla et al. (2017), algae can significantly remove TDS due to the absorption of salts and solids present in the wastewater, *Chlorella minutissima* removed about 90-98%.

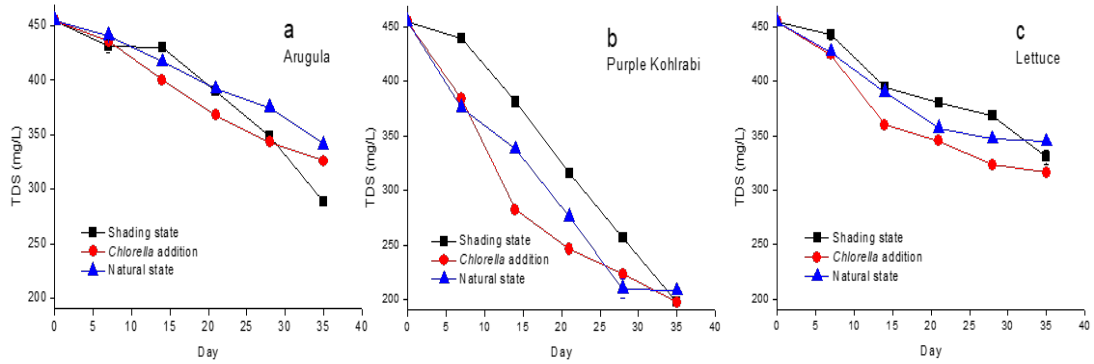



Figure 14 Total dissolved solids (TDS) removal in the hydroponics

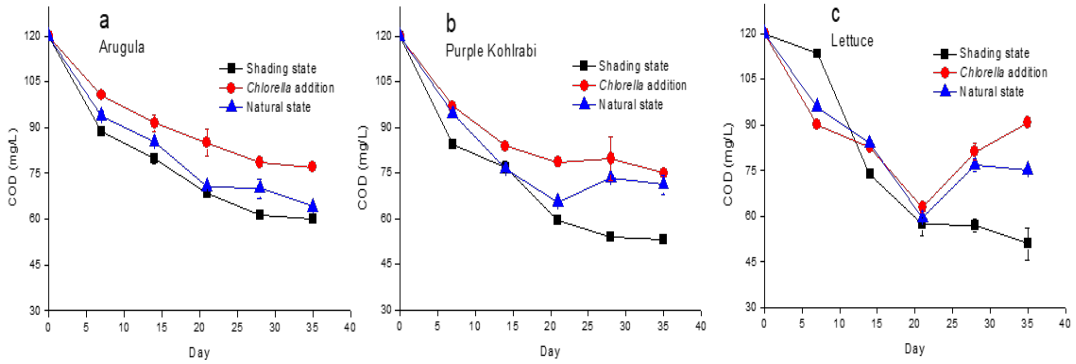



Figure 15 chemical oxygen demand (COD) removal in the hydroponics

As figure 12 shows, algae cannot improve COD removal rate in the hydroponic system. COD removal in purple kohlrabi shading group can reach a remarkable 55.8%. This was inconsistent with previous results. Deng et al. (2018) found *Chlorella vulgaris* (UTEX 2714) grew well on the diluted digested swine manure wastewaters, COD removal rate was 0.94-2.14 g/L/d . It is possible that

*Chlorella* is replaced by environmental microalgae during its growth. COD removal mainly depends on the synergistic effect of vegetables and bacteria. Plant roots play an important role in the absorption and adsorption of organic pollutants, and also provide suitable conditions for root microorganisms to form a unique water-plant roots-microbial ecosystem. COD degradation was accelerated by the synergistic removal of plants and microorganisms. In lettuce groups, COD increased after three weeks under *Chorella* addition and natural state conditions. It may be that algae release some organic matter. These results are similar to the results of Xi et al. (2015) who found that hydroponic vegetables can effectively remove organic pollutants from duck farms. The COD average removal rate of lettuce, water spinach and amaranth reached 83.68-89.95%, and water celery 66.05%.

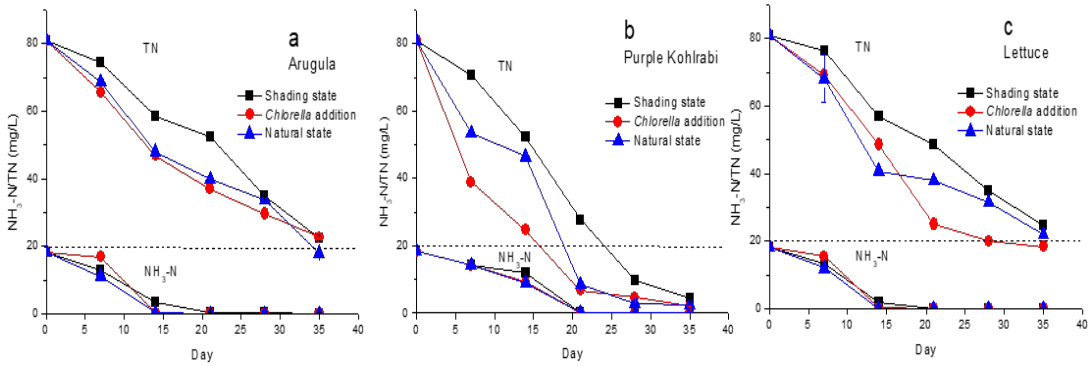



Figure 16 Total nitrogen (TN) and ammonia nitrogen (NH3-N) removal in the hydroponics

Many algae favor ammonium uptake over nitrate (Hellebust et al., 1989; Addy et al., 2017). Vegetables can remove NH3-N from wastewater either by plant absorption or by nitrification/denitrification. The oxygen produced by photosynthesis is transferred to the root and diffused into the water environment through the aerenchyma. The rhizosphere can form an aerobic/anaerobic zone, which promotes nitrification/denitrification bacteria to form a biofilm on the root surface, on which nitrification or denitrification can take place (Xi et al., 2015). As shown in Figure 13, under natural state or *Chlorella* addition, ammonia nitrogen is basically completely removed within two weeks. Arugula requires three weeks to remove ammonia nitrogen completely under Shading state condition. For the total nitrogen (TN) removal, removal rate of TN in purple kohlrabi group can reach 94.6-97.6%. In the presence of algae, especially the condition of *Chlorella* addition, the removal rate of total nitrogen can be significantly increased to 97.6%. High levels Nitrate (> 250 mg/L) or ammonia nitrogen (> 30 mg/L) have a negative impact on plant growth (Somerville et al., 2014). At the beginning of this experiment, $NO_3$-N 62.5 mg/L and NH3-N 18.4 mg/L did not inhibit the growth of vegetables.

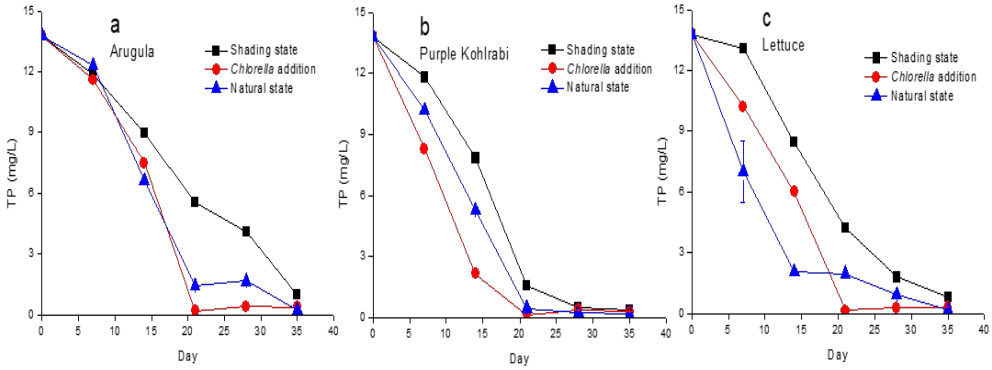



Figure 17 Total phosphorus (TP) removal in the hydroponics

Phosphorus removal ability is related to plant species (phosphorus absorption efficiency), phosphorus precipitation and microbial immobilization (Xi et al., 2015). As Figure 14 shows, the

removal rate of TP in purple kohlrabi group can reach 97.4-98.8%. Microalgae can significantly improve the removal of nitrogen and phosphorus. *Chlorella* addition can significantly improve the removal of nitrogen and phosphorus in the purple kohlrabi group, while in the lettuce group, the removal of nitrogen and phosphorus is fastest in natural state, which slows down after two weeks. The removal of nitrogen and phosphorus by the *Chlorella* addition group is still effective.

For different kinds of vegetables, algae can have different effects on their growth. Under natural algae conditions, purple kohlrabi grows fastest, and the biomass can reach 31.6 g/plant. But in the Arugula group, algae may have certain negative effects on plant production. The algal biomass and dissolved oxygen concentration in the *Chlorella* addition group was relatively higher than that of natural state. Algae existence, especially *Chlorella* addition, can significantly improve removal of TDS, nitrogen and phosphorus content, but not COD. The correlation between algae and the changes of plant and even system microbial communities needs more in-depth analysis.

**ACTIVITY 2: Evaluate the technical and environment impact of an integrated wastewater management facility at the research and outreach center in Morris and conduct algal feeding demonstrations with livestock. Description:**

A microalgae production system will be installed at the West Central Research and Outreach Center's dairy facility for the production of various microalgae strains for use in livestock feeds. Wastewater from the dairy will be utilized for the microalgae production system to produce quantities needed to conduct feeding trails and demonstrations of feeding diets containing microalgae to dairy and swine.

We will characterize the WCROC wastewater in terms of nitrogen, phosphorus, inorganic and organic carbon concentrations, and a complete micronutrient analysis. Additionally, we will isolate microalgae from WCROC wastewater capable of heterotrophic, mixotrophic, autotrophic growth using the nitrogen and phosphorus available from the wastewater. Care will be taken to monitor any strain for cyanotoxin production which could potential poison calves, pigs, and other livestock. Not any one strain is expected to perform all characteristics and community culturing or isolated culturing may be required; additionally, WCROC wastewater is different from other wastewater and strains will need to be optimized for production on it.

Screening of existing algal culture collections for known organisms capable of the properties discussed above will be conducted. We will scale growth of the top candidates to produce enough biomass to begin feeding studies. Outdoor cultivation is essential to ensure the feeding studies will represent algal strains that will actually be used in production. Optimization of the temporal and environmental influence on productive strains of algae will be implemented. We will implement a high productive system to optimize the selected strains for outdoor cultivation and treatment of WCROC wastewater. Selection of most suitable algae species will include their capacity for growth, biomass yield, and nutritional composition. Algae species will be sent for analysis of dry matter, crude protein, ether extract, starch, and neutral detergent fiber as well as calcium, phosphorus and heavy metals (i.e., Zn, Cu, Pb, Ni, Cd, As, Cr).

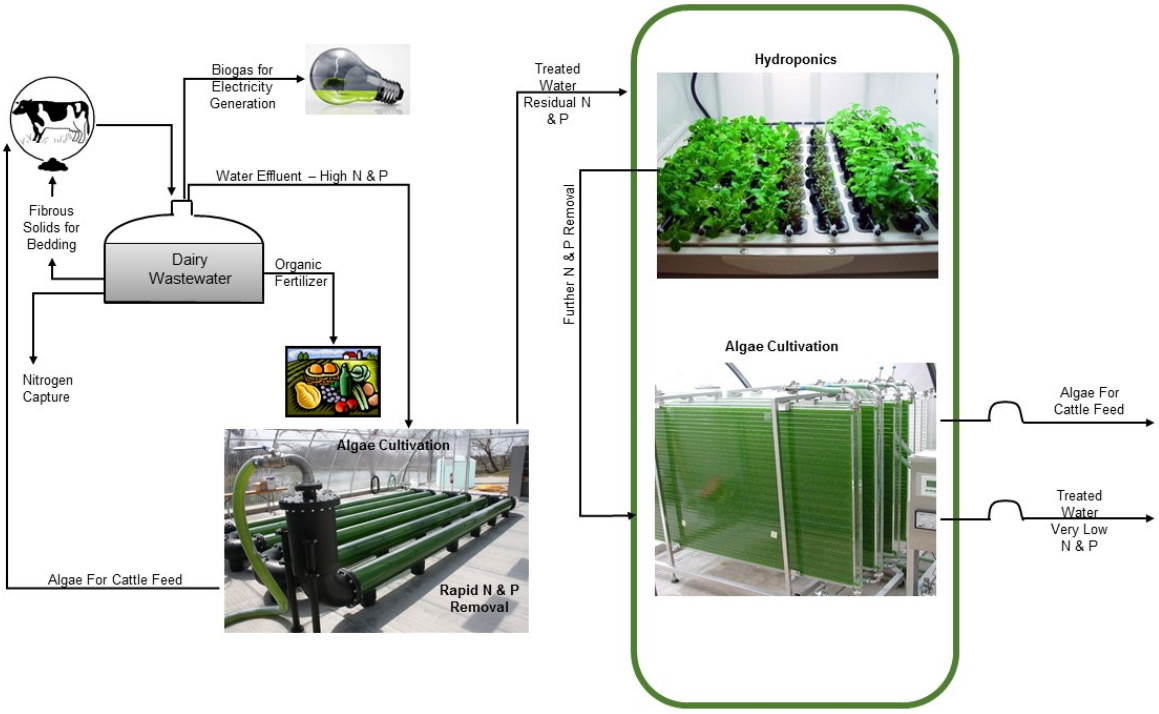



Figure 3. Fully scalable system to clean dairy wastewater, to recycle nutrients, and produce biomass algae for livestock feed.

Replicated feeding studies will be demonstrated and results verified using production strains from the outdoor WCROC wastewater system which has been optimized for high algal production and nitrogen/phosphorus removal. We will continue to demonstrate continual wastewater treatment and algal feed productivity.

For the algal feeding studies, the objective of this experiments will be to determine if calves prefer the taste of algae added to starter grain using the sequential elimination procedure. The algae will be added to calf starter grain and milk and mixed. The experiment will be conducted 1 calf at a time. A calf feeder will be used to determine the taste preference studies. The diets will be offered for 7 days, with the first 2 days used for adaptation to surroundings and day 3 to the end of day 7 used for data collection. After the third day of collection (day 5), the treatment with the overall greatest consumption will be removed and replaced by an empty container. During the last 2 day (day 6 and 7), the remaining treatments will be used to determine second preference.

**Summary Budget Information for Activity 2:**

**ENRTF Budget: $ 265,890**
**Amount Spent: $ 253,988**
**Balance: $ 11,902**

| Outcome | Completion Date |
|---|---|
| 1. Install an algal production system to clean 20% of the dairy wastewater at the research center and produce biomass to feed at least 40 calves. | 7/1/2018 |
| 2. Graduate students will conduct three feeding trials with dairy calves on algae potential as a livestock feed at the research dairy. | 7/1/2018 |

| | |
|---|---|
| 3. Evaluate the potential of feeding algae from the algal production system through an economic analysis to determine if feed costs can be reduced by 10% with algae. | 7/1/2018 |
| 4. Evaluate the environmental impact of the dairy wastewater remediation system through measurement of the amount of dairy wastewater cleaned. | 7/1/2018 |

**Activity Status as of *January 1, 2017*:**

Alternative algae photobioreactors were designed and constructed at the University of Minnesota West Central Research and Outreach center for use in testing multiple algae species. That works is currently ongoing.

**Activity Status as of *January 1, 2018*:**

We have scaled up the alage bioreactor demonstration and have begun testing algae concentrations. Data analysis is in preparation.

At our dairy we have fed whole milk for many years, so we needed a system that could work with our whole milk feeding. We installed a Holm and Laue HL 100 automated calf feeder, with 2 calf Hygiene Stations for feeding. One of the Hygiene Stations has a forefoot calf weigh scale attached to record daily growth of calves. For feeding whole milk, we added a 200-gallon Milk Jug cooling tank from Calf-Star in New Franken, WI. We also have a

Holm and Laue Milk Taxi that can pasteurize milk and transport the milk to the Milk Jug and calf feeder. We installed our automated feed in a 60 ft by 60 ft barn that we retrofitted to fit the feeder. We have an unconventional system, because I wanted our calves to have access to the outdoors even in the winter time. We will save that discussion for another day why I think calves should be outside. We have 2 pens in our barn with 15 calves each that will provide for 49 square feet per calf. We also bed with a lot of dry straw to allow calves to burrow into the straw and keep warm in the winter. Our calves are being fed 8 L per day in 2 L increments of whole milk. We start them on the feeder at 5 days of age and allow them to drink 6.8 L per day. They are ramped up to 8 L within 7 days. We also will start gradually weaning the calves off milk starting at 49 days and will wean by 56 days. The system provides us valuable data. The algae calf study will begin in March 2018.

**Activity Status as of *July 1, 2018*:**

Many strains of microalgae grow in the WCROC dairy manure lagoon. We are growing strains flat-panel bioreactors outside the WCROC dairy barn. The 2 reactors are made of clear, recyclable, 22-gallon plastic bags hanging on south-facing frames. It takes about a week for the reactors to fill with microalgae. The harvested algae are freeze-dried. Current algae yields are about 5 grams per liter of wastewater.
We have begun an experiment that is testing different dairy wastewater concentrations and water to maximize algae growth. We are using a control, 1:10, 1:20, 1:30, and 1:40 of water and wastewater to test ph, temperature, and algae yield in the bioreactors. We have installed ph and temperature probes in the algae reactors that monitor the systems throughout the day. Temperature fluctuations in the algae reactors range from 24 to 38 degrees Celsius and ph ranges from 7.31 to 8.4 through the data in the algal cultivation system. Data analysis is in the preliminary stages, and we will begin algae feeding to cattle during the fall of 2018. The graphs show the daily temperature and ph fluctuations during the data in the algae cultivation system.

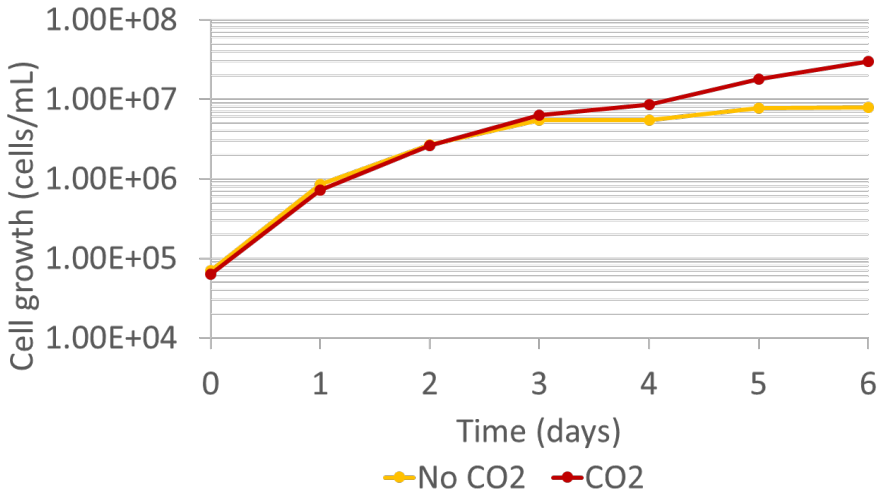


The algae cultivations system at the WCROC Dairy for utilizing dairy wastewater to grow algae.

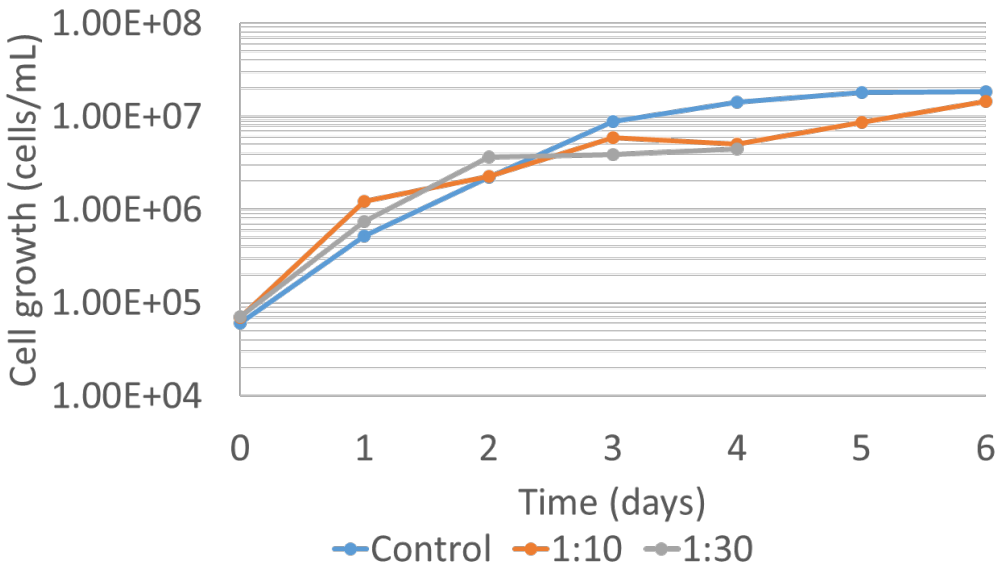
**Activity Status as of *January 1, 2019*:**

We hypothesize that controlling pH with CO2 will increase yield of algae and lower wastewater to potable water ratio will result in higher biomass. This study aims to identify a wastewater dilution capable of producing considerable amounts of algae biomass and also to determine if additional CO2 influences algal biomass productivity. The reactions described in this study occurred in hanging bags of 70L. The treatments were: control (only AM6 media), 1:30 (2.33L of wastewater) and 1:10 (7L of wastewater), and they were made in duplicates. The algae strain used in this study was isolated from the same wastewater lagoon where the wastewater for the dilutions was collected. Temperature and pH were monitored using an Apex system. Daily samples were collected from day 0 to 6 for analysis of cell growth, biomass, ammonium, and nitrate. Harvest occurred on day 4 for 1:30 and on day 6 for the control and 1:10. The biomass was centrifuged for 2 min at 6500xg. The statistical analysis was performed using SAS 9.4. Cell growth was significantly higher ($p < 0.05$) on days 5 and 6 when CO2 was added. Cell growth was not different between treatments until day 4, when the control showed a higher growth than 1:10 and 1:30. On day 5, the control was only higher than 1:10. For biomass yield, there was no significant difference in adding CO2. However, when comparing dilutions, 1:10 showed a significantly lower biomass yield than control on days 0, 1, 4, 5 and, 6. Biomass yield was higher for 1:10 on day 4 compared with 1:30. Bags with CO2 added produced 333% more algae biomass in the control, 14% more in 1:10 and 50% more in 1:30, compared with the bags without additional CO2. Ammonium removal rates varied between 67.1% and 94.4% on both treatments (CO2 added or not). Nitrate removal was higher for 1:30 (more than 90%), followed by 1:10 and control. The 1:10 dilution showed satisfactory algae biomass yield, which may be used for livestock feeding. Biofilm formation was observed, which caused variation in biomass measurements. Furthermore, the nutrient removal rates show that algae are capable of reducing the amount of nutrients in dairy wastewater.

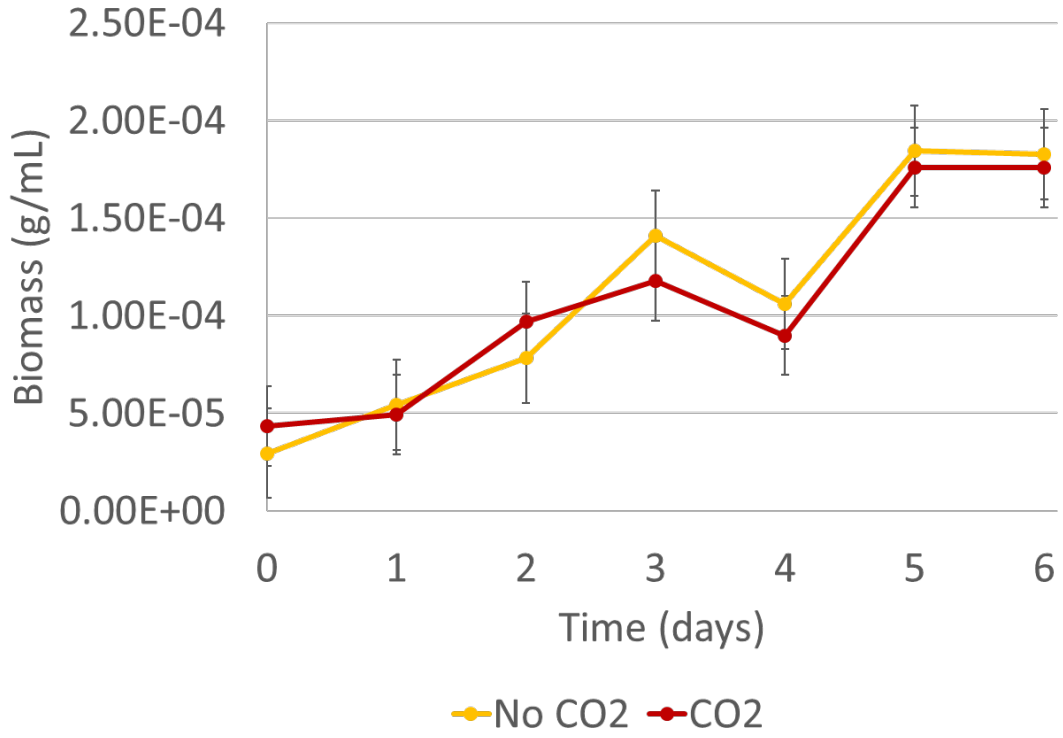
During the summer of 2018, we experienced some difficulties with producing enough algae with our bioreactor system. Initially, we had some contamination in the alae reactor that caused the algae to die for the first run of the study. Additionally, at the 1:40 concentration of wastewater, the algae did not grow as well as expected. There were just too many nutrients in the wastewater for the algae to consume to grow and multiply. We have been pilot testing algae being fed to calves. However, we need to producer more algae to get it to feeding stage for calves. During the winter (December 2018 to April 2019), we are growing algae in an indoor climate controlled greenhouse with adequate sunlight to provide for more growth of algae. Based on growing algae during the winter, we will have enough algae produced during the winter to complete the calf feeding project during the spring of 2019. We will also complete the economic analysis of the algae feeding as well during the spring of 2019.



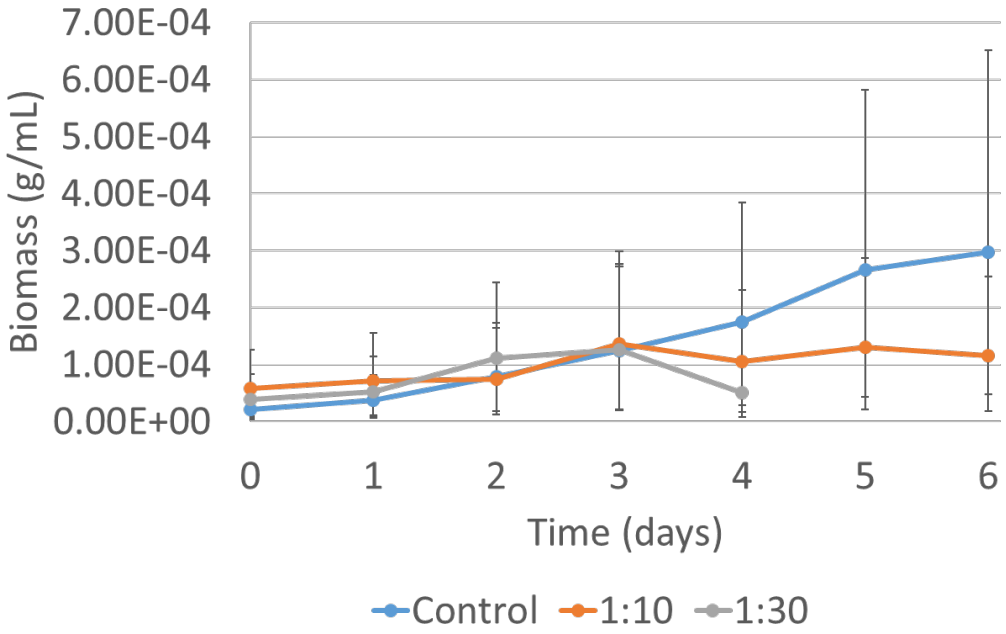

**Algae cell growth for algae growth with $CO_2$ or No $CO_2$**




Algae cell growth for control or 1:10 or 1:30 wastewater dilution rate




Algae biomass yield growth with $CO_2$ or No $CO_2$




Algae biomass yield for control or 1:10 or 1:30 wastewater dilution rate

Table 1 – Wet and dry weights for algae biomass of each bag after harvesting 70L

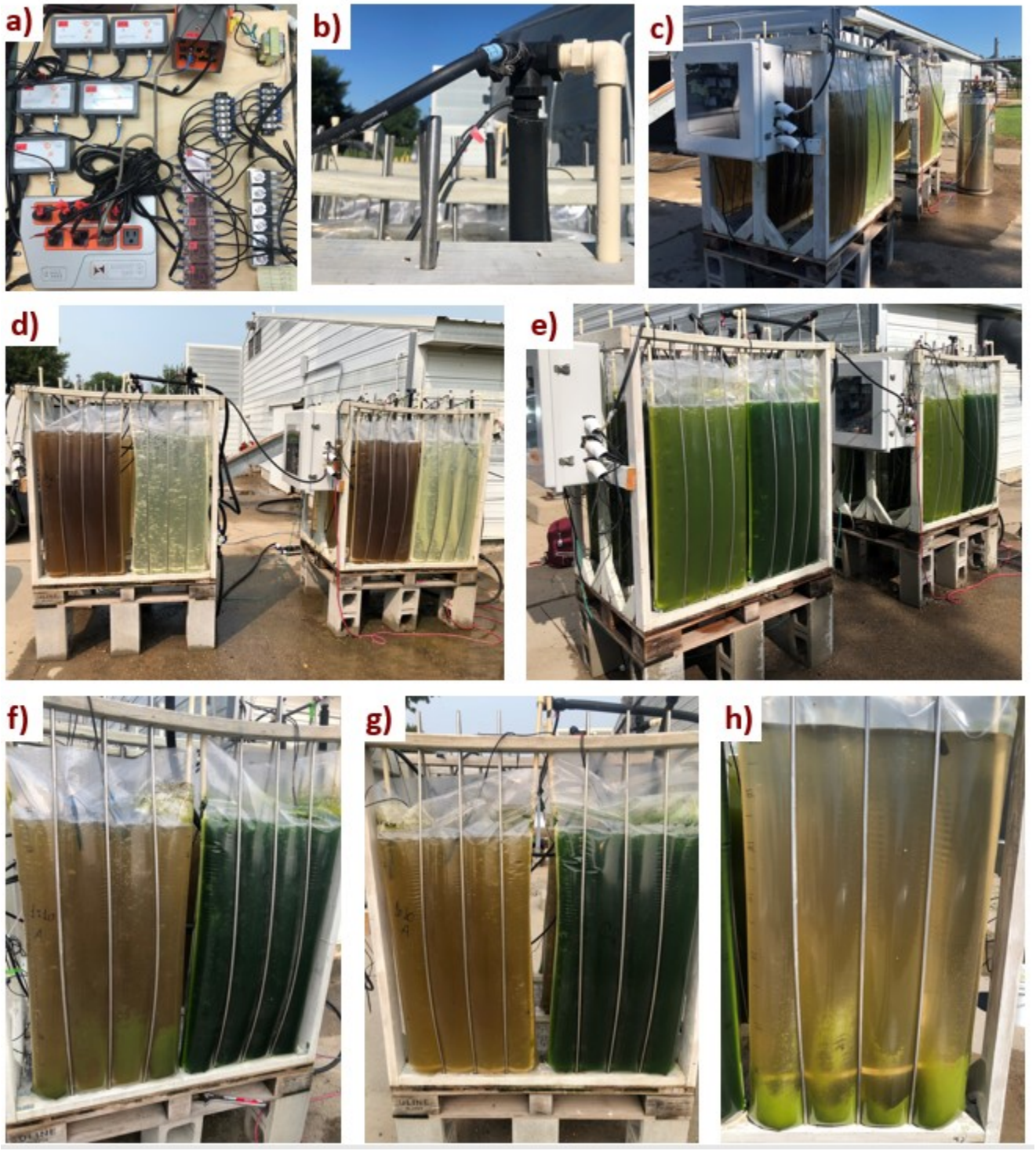
| Added $CO_2$ | | |
|---|---|---|
| **Treatment** | **Wet weight (g/L)** | **Dry weight (g/L)** |
| **Control** | 2.44 | 0.30 |
| **1:10** | 0.85 | 0.08 |
| **1:30** | 0.28 | 0.04 |

| No $CO_2$ | | |
|---|---|---|
| **Treatment** | **Wet weight (g/L)** | **Dry weight (g/L)** |
| **Control** | 0.78 | 0.09 |
| **1:10** | 0.97 | 0.07 |
| **1:30** | 0.15 | 0.02 |

Table 2 – Average consumption of Ammonium and Nitrate on day 0 and day 6 for control and 1:10 and, on day 4 for 1:30.

| Added $CO_2$ | | No $CO_2$ | |
|---|---|---|---|
| Average consumption of Ammonium | | | |
| **Control** | 89.1% | **Control** | 94.4% |
| **1:10** | 72.5% | **1:10** | 91.7% |
| **1:30** | 79.1% | **1:30** | 67.1% |
| **Added $CO_2$** | | **No $CO_2$** | |
| Average consumption of Nitrate | | | |
| **Control** | 73.7% | **Control** | 53.9% |
| **1:10** | 75.2% | **1:10** | 65.0% |
| **1:30** | 90.4% | **1:30** | 92.9% |

Figure 1 – a) Monitoring and control system for temperature and pH; b) Hoses for the input $O_2$ and $CO_2$; c) Experimental setup; d) Experiment at 0 d; e) Experiment at 6 d; f) Example of biofilm formation; g) No biofilm formation; h) Biomass ready to harvest on day 6.



**Final Report Summary:**

## Bioreactor Design, pH and Temperature Monitoring System, and Harvesting Techniques

### Lab-scale Bioreactors

The lab-scale bioreactor is composed of one box made of glass (height = 0.48m, length = 0.76m, width = 0.31m) opened on top, with an acrylic support for eight glass tubes (diameter = 0.075m, height = 0.05m) positioned in parallel to each other (Figure 1). Rubber stoppers were used to keep the system closed. A glass tube (diameter = 3mm) passing through the rubber stopper was used to inject air into the system and $CO_2$, when needed.

The medium used during lab-scale experiments (wastewater, AM6 and DI water) was not sterilized, so there was no need for air filters to be used. Each one of the systems had one blower injecting air at a rate of 0.8 L/min ± 0.1 L/min in each one of the tubes, adjusted by the flowmeters on the top left of Figure 2. The pH and temperature were monitored using the Apex system. Utilizing the Apex system, made it was possible to control the pH changes using a $CO_2$ gas cylinder and maintain pH for the algae to grow.

The $CO_2$ was added without being diluted, but was mixed with air before going into the glass tube and inside the reactor. The pressure was 17kPa (16psi) on the way out of the cylinder. The lights used during the lab-scale experiment were bought from Philips, model F28T5/830 ALTO, warm white, 2900 lumens, and 28 watts. The lab-scale experiments took place at the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) – Soils Management Research, at Morris, Minnesota. Figure 2 is an example of the set up used during the lab-scale experiments.

### Pilot-scale Bioreactors

The pilot-scale bioreactors were designed to have six bags in one system, where all of them could receive approximately the same amount of solar radiation during the day. The structure was made using treated pinewood (height = 1.73m, length = 2.06m and width = 1.22m). Three pars of flexible metal bars (diameter = 0.05m, height = 1.34m), distant 0.03m from each other, were installed to support the shape of the hanging bags (figure 4). PVC tubes (diameter = 0.015m, height = 1.33m, length = 0.52m) were used to blow oxygen into the bags and carbon dioxide, when needed. At the bottom of each PVC tube, twelve holes (diameter = 0.5mm) were drilled for the air to flow, aerating and mixing the whole system.

The bioreactors (pallet 1 and pallet 2) were set up outside of the dairy barn at the West Central Research and Outreach Center (WCROC) in Morris, Minnesota (Figure 4). Each one of the pallets had a box attached in one of the sides, containing the Apex system. A liquid $CO_2$ tank was used as a carbon source and to control the pH when needed. Each one of the bioreactors (two total) had a capacity for six hanging bags with a maximum volume of 80L each.

The airflow was measured using an 8360 VelociCalc® Plus from TSI, and it was flowing in a rate of 4.61 ± 0.09 $m^3$/min from the blower into the bag through the larger black hose (this rate was measured in each one of the bags individually). Figure 5 has the detail of the air flowing system, where the smallest black hose (diameter = 0.48cm) was used to add carbon dioxide, and the larger black hose (diameter = 3.5cm) was responsible for inserting air from the blower into the bag. To not insert 100% $CO_2$ ($CO_2$ was added

only during specific experiments) into the system, ideally the two fluids were mixing while entering together at the beige PVC tube and then flowing into the algal system.

**Temperature and pH Monitoring and Controlling System**

The Apex Fusion® consists of an electronic system created by the Neptune company (California) to monitor different parameters in aquariums. In this project, the Apex Fusion® was used as part of a temperature and pH monitor system and a pH controller system. Using Figure 6 as a reference, the items bought from Neptune are: the energy bar ("b"), the probes' modules ("g"), and the Apex controller ("f"). The main objective of using this system was to use the pH probes from the Apex Fusion® system to determine what was the pH and the temperature in each one of the bags.

Using the pH information, it was possible to program the Apex Fusion® to turn on the solid states relay (Figure 6, "c"), opening a valve from the manifold (Figure 6, "d") and releasing $CO_2$ automatically in each one of the bags when the pH reached a maximum value (8.6) and then closed the valve when the pH reached the minimum value (7.4). In order to make it possible, some electric devices have been added to the system (Figures 6 and 7).

The electric system in Figure 7 was specifically designed to include six valves (because each one of the bioreactors had six bags) using a manifold (VALV). The manifold made it possible to have independent addition of $CO_2$ into the bags. However, the manifold is 24V AC, and the Apex Fusion® system is 120V AC, which means that an extra switch had to be added to the system. In order to make it possible, the solid state relays (RLY) were added to turn on and off the manifold, and they were 120V in one side (to connect to the energy bar) and 24V in the other side (to be able to connect to the manifold). The transformer was added to maintain the manifold energized with 24V in one side, and to connect the manifold to the 24V side of the RLY. All the RLY's, probe modules, the transformer, and the Apex Fusion® controller were connected to the energy bar, and the energy bar was connected to the line.

In summary, the monitoring and controlling system measures temperature and pH and are saved in the Apex Fusion® controller (Figure 6, "f"). When the pH probe has a measurement over 8.6 it will inform the Apex Fusion® controller to unlock the designated switch and turn on the solid state relay (RLY) for the specific bag. The RLY will open one of the manifold valves, and the valve will release $CO_2$ inside the bag until the pH probe gets a measurement below 7.4, then the Apex controller will lock the switch, turning off the RLY and the valve.

**Solar Radiation and PAR Monitoring**

During the lab-scale, in addition to controlling and monitoring the pH, and monitoring the temperature, it was possible to use the Apex Fusion® system to monitor the photosynthetic active radiation (PAR) that the microalgae culture was receiving from the lights. A special probe module (ASM) was added to the system, and a PAR sensor was added to it. The photosynthetic active radiation refers to the visible light that ranges between 400nm and 700nm, and the unit used is $\mu mol/m^2/s$.

During the lab-scale experiments, the PAR sensor was positioned in between tubes, to measure the photosynthetic active radiation that the tubes were receiving and it varied

between 331 and 338 μmol/$m^2$/s. Furthermore, the lights were set up to be on every day between 5 a.m. and 9 p.m. to imitate the duration of the sunlight received by the bags during the pilot-scale when they were outside during the summer.

During the pilot-scale, the bioreactors were positioned outside and exposed to ambient changes between July and August. The orientation of bags was east to west. The solar radiation during pilot-scale was measured using the data from the United States Department of Agriculture – Agricultural Research Service (USDA-ARS), that have permanent experiments going on near Swam Lake, which is approximately 11km from the West Central Research and Outreach Center (WCROC). The information provided by the USDA-ARS was an average of the radiation for the day and ranged between 40.56 and 303.55 W/$m^2$. To be able to compare lab-scale and pilot-scale, an approximate conversion was used, where, for sunlight conversion, 1 μmol/$m^2$/s was equal to 4.57 W/$m^2$ (Langhans, R.W., et al., 1997), so the solar radiation received by the bags on pilot-scale varied between 185.34 and 1387.23 μmol/$m^2$/s. A clear sky during the summer solstice in the northern hemisphere, at noon, would have a maximum photosynthetic active radiation of 2000 μmol/$m^2$/s (Slaterry, R. A., et al, 2017).

**Harvesting Techniques**

The lab-scale experiments were not harvested because of the small amount of biomass produced. In that case, for the lab-scale, only the biomass production per day was evaluated filtering 15ml of the sample through a 0.22μm filter paper. However, for the pilot-scale, two different harvesting techniques had to be used. Furthermore, it was essential to quantify the total biomass produced, especially for the experiments where biofilm formation was observed. The harvesting technique used for the bags with different dilutions (when wastewater was diluted in water) and controls bags (AM6 medium and water) had to be different because of the time management and centrifuge size in use at the laboratory.

The *Chlorella sp.* used in this study had different behaviour when dealing with dairy wastewater than when diluted in artificial medium only. A uniform distribution of microalgae cells was observed in the entire water column (70L) when using AM6 medium and having the blower turned off for more than 24h. However, when using wastewater and water, the microalgae tend to precipitate easily after turning off the blower between 3 and 5 hours.

**Harvesting *Chlorella sp.* from Artificial Medium in Pilot-scale**

When harvesting the biomass from the pilot-scale experiments, the blower was turned off after the last sampling. The microalgae biomass was expected to precipitate, accumulating in the bottom of the 70L bag. However, what was observed for the controls, was that the microalgae would stay distributed in the entire water column and only a minimal amount going to the bottom of the bag. Therefore, the entire bag had to be centrifuged. A milk cream electric centrifugal separator (version 100-18), designed by Motor Sich JSC©, was used to centrifuge the algae biomass. The initial capacity of this product was to separate 100 L in one hour with 75 rpm. However, to separate microalgae, some modifications had to be made, including not using the twelve disks that are supposed to be inside the centrifuge. For this project, the microalgae would stay trapped inside the

centrifuge, and the liquid part would leave the separator system. Because of that, every time that the water leaving the system started to be dark green, the separator was turned off, and the biomass was harvested (Figure 10) from inside the centrifuge system. After collecting the biomass, it was sterilized under 121°C and a pressure of 15 psi, for 30 minutes. The biomass was then kept frozen at -4°C after drying in an incubator for eight days at 65°C.

**Harvesting *Chlorella sp.* from Different Dilutions of Dairy Wastewater in Water in Pilot-scale**

After taking the last sample of each experiment, the blower was turned off, making it possible for the microalgae biomass in the bags containing dairy wastewater, to precipitate after a minimum of 3 hours. In this case, less than five liters of the mixture of water and wastewater containing *Chlorella sp.* biomass had to be centrifuged using a Sorvall Legend XTR Centrifuge, from Thermo Scientific, at 6500*xg* during 2 min. The biomass was collected, sterilized under 121°C and a pressure of 15 psi, during 30 minutes. The biomass was then kept frozen at -4°C after drying in an incubator for eight days at 65°C.

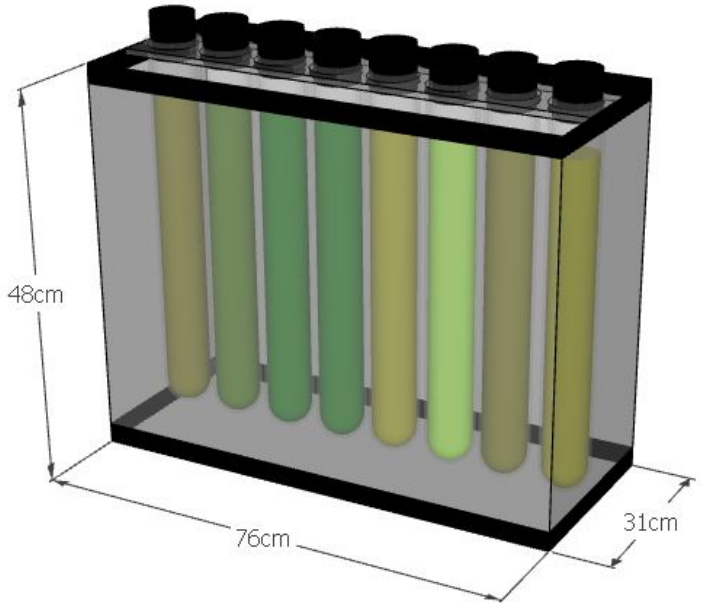



Figure 1. Glass tubes used during the lab-scale experiments.

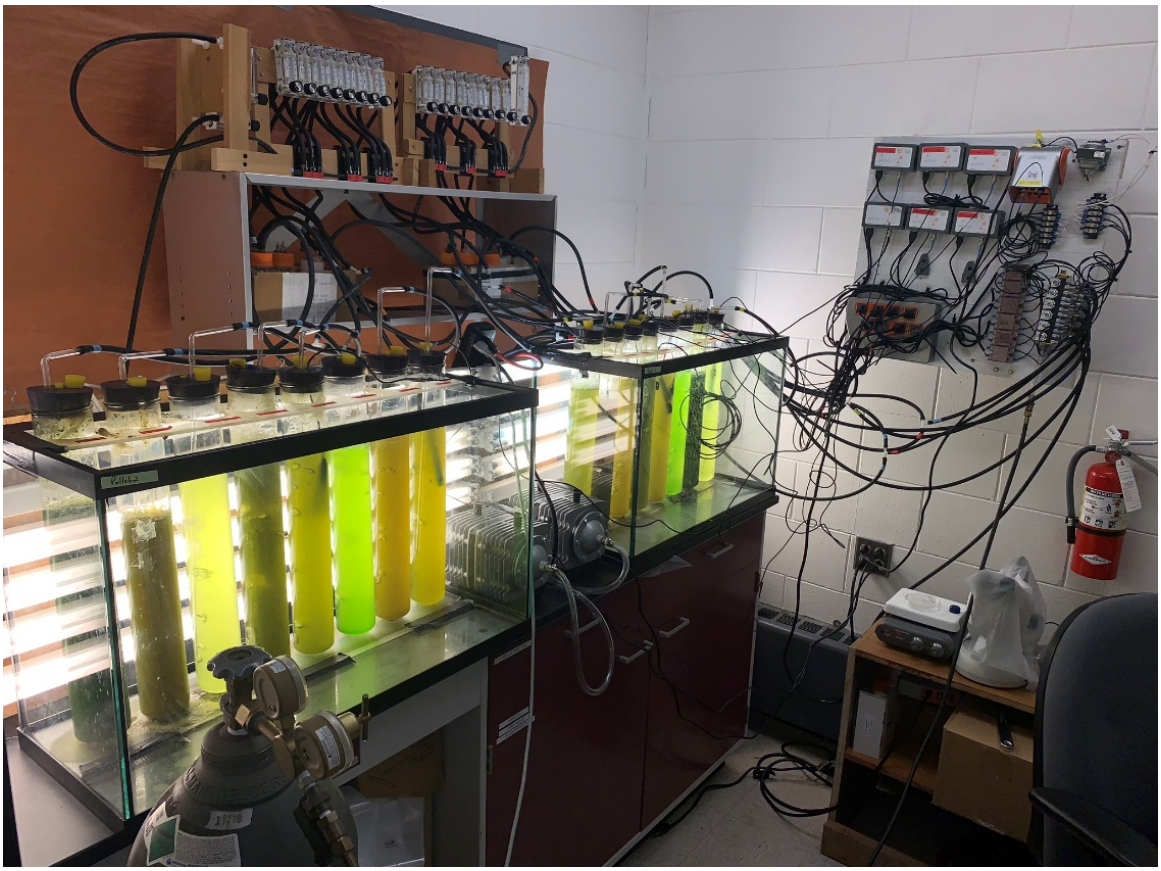


Figure 2. Experimental set up during lab-scale.

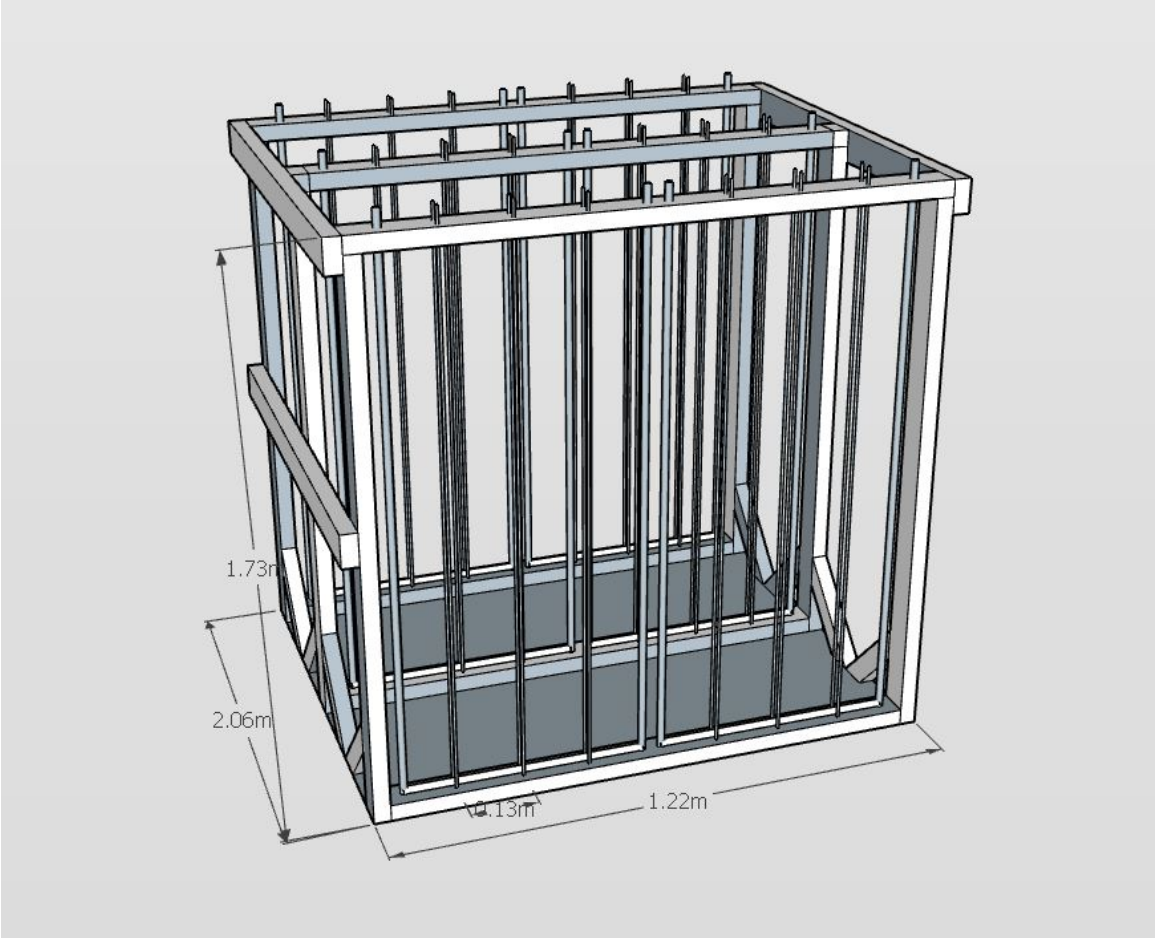


Figure 3. Sketch of the pilot-scale bioreactors.

Figure 4. Pilot-scale set up outside of the dairy barn at WCROC – Morris, Minnesota, during the summer.

Figure 5. Detail on the air and carbon dioxide mixture going into the system.

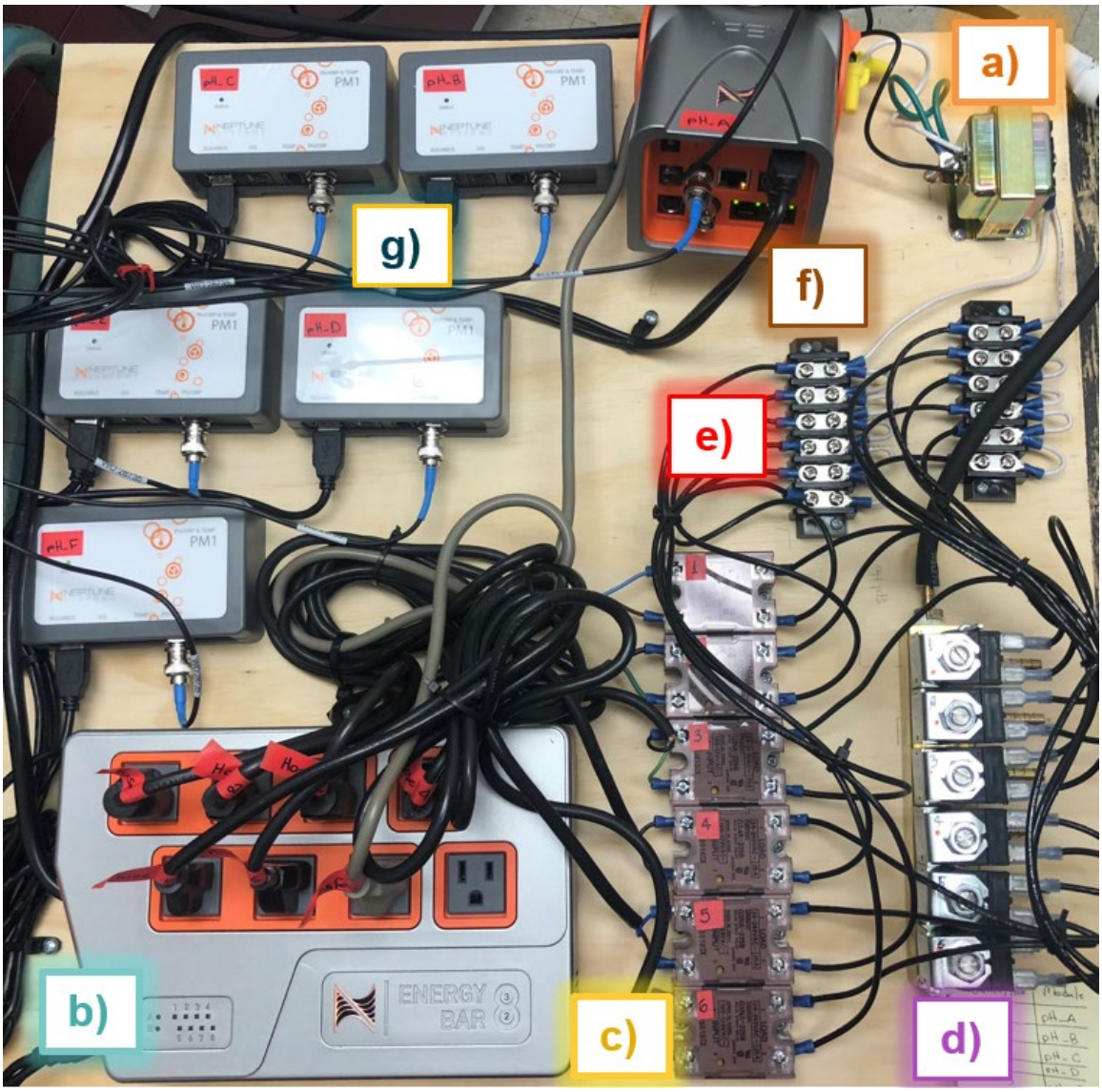


Figure 6. Temperature and pH monitoring and controlling system, where “a” is the transformer, “b” is the energy bar or switch, “c” are the solid state relays, “d” is the manifold or valves, “e” is the energy splitters, “f” is the Apex Fusion® controller, and “g” is the probe modules.

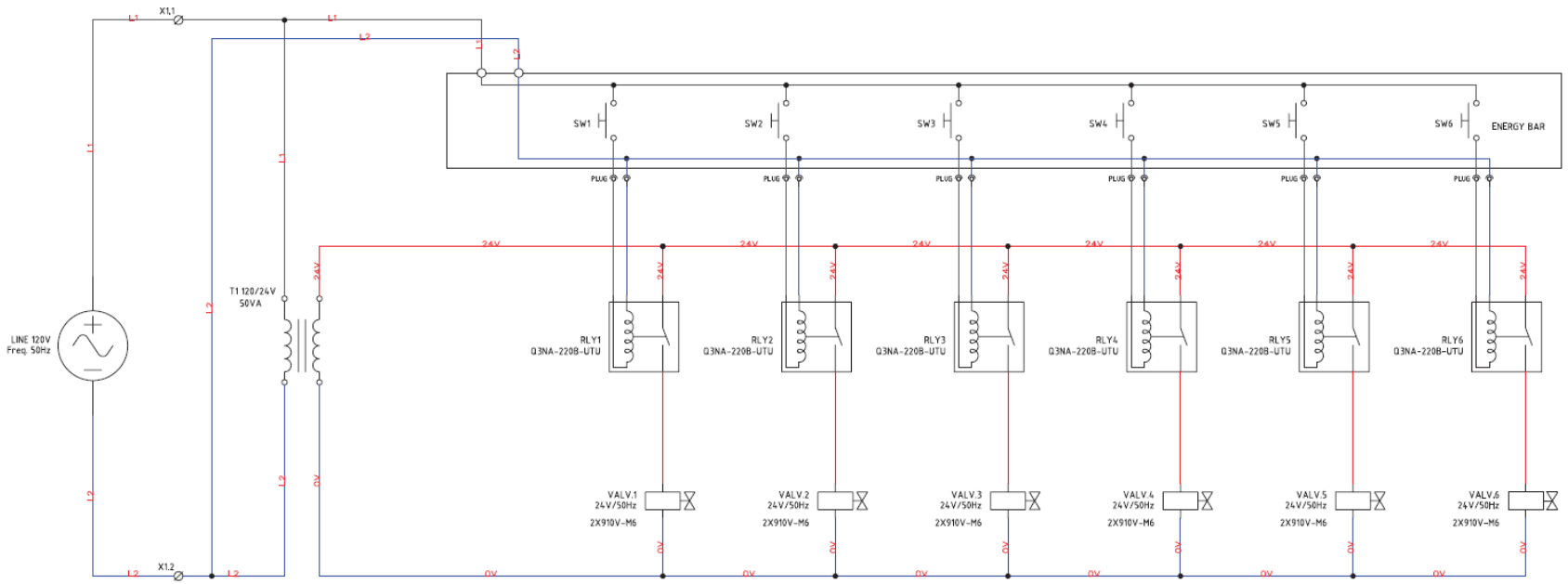



Figure 7. Electrical system designed to use the information collected from the Apex Fusion® to control the pH independently in each one of the bags in the bioreactor. The line (120V) is the voltage provided by the electricity company, T1 is the transformer, SW's are the switches in the energy bar (letter "b" on figure 7), the RLY's are the solid state relays, and the VALV's are the valves in the manifold.

Figure 8. Interfaces of the Apex Fusion® app on the cellphone.

Figure 9. Cream milk electric centrifugal separator (version 100-18), Motor Sich JSC© used to separate the biomass and the liquid part from the bags containing dairy wastewater and water.

Figure 10. *Chlorella sp.* biomass collected inside the cream milk electric centrifugal separator (version 100-18), Motor Sich JSC©.

**Algae cultivation**

The *Chlorella sp.* used in this study was isolated from a dairy wastewater lagoon located at the West Central Research and Outreach Center (WCROC) in Morris, Minnesota – USA (45°35'38.0"N, 95°52'17.4"W), by researchers of the University of Minnesota. The dairy farm at the WCROC milk, approximately, two hundred cows, between conventional and organic, twice a day. The strain was chosen based on its ability to grow fast, to resist high pH, and to not be harmful to the livestock that would eat it. The microalgae were cultivated in BG-11 modified medium (based on Andersen, 2005, pages 435 and 436) which was called AM6 and is shown in table 1. It was used to seed all the different experiments.

The medium used during lab-scale and pilot-scale experiments (dairy wastewater, AM6 and DI water) were not sterilized to see how the microalgae would respond to the toxicity and the interactions with other microorganisms. According to the experimental design, four different ratios would have a better response and balance between cell growth, biomass production and nutrients removal.

In order to find determine the better amount of DW where the microalgae could grow, produce more biomass and to remove the highest amount of nutrients, four different ratios of DW in water were tested, 1:10, 1:20, 1:30 and 1:40. Controls were carried out with AM6 medium. The different ratios experiment had an extra addition of $CO_2$ as an additional source of carbon and to control the pH. A second type of experiment was conducted to evaluate the necessity of adding extra $CO_2$ (to improve the chance of implementing this system in farms and to reduce costs). This second experiment was carried out using the ratios 1:10 and 1:30, and controls. The results of one system with extra $CO_2$ supplementation were compared to the other one without it.

**Dairy wastewater**

The dairy wastewater (DW) used in this study was pumped from the wastewater lagoon at WCROC and reserved in a funnel-shaped tank to settle by gravity between three and five days prior the first use, either for lab or pilot-scale. The DW (table 2) used during the experiments was taken from the top of the tank and used in reactors in different percentages diluted in water. The water used during lab-scale experiments was deionized water (DI) and tap water (with hard water properties) for pilot-scale.

**Lab-scale bioreactors**

It is composed of two boxes made of glass (height = 0.48m, length = 0.76m, width = 0.31m) opened on top, with an acrylic support for eight glass tubes (diameter = 0.075m, height = 0.05m) positioned in parallel to each other. Rubber stoppers were used to keep the system closed. A glass tube (diameter = 3mm) passing through the rubber stopper was used to inject air into the system and $CO_2$ when needed. Each one of the systems had one blower injecting air at a rate of 0.8 L/min ± 0.1 L/min in each one of the tubes, adjusted by flowmeters. $CO_2$ was used to control the pH and as a carbon source in some of the experiments.

The $CO_2$ was added without being diluted, but it was mixed with air before going into the glass tube and inside the reactor. The pressure was 17kPa (16psi) in the way out

of the cylinder. The lights used during the lab-scale experiment were bought from Philips, model F28T5/830 ALTO, warm white, 2900 lumens, and 28 watts. The lab-scale experiments took place at the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) – Soils Management Research, at Morris, Minnesota.

**Pilot-scale photobioreactors**

The pilot-scale photobioreactors (PBR) were designed to have six bags in one system, where all of them could receive approximately the same amount of solar radiation during the day. The structure was made using treated pinewood (height = 1.73m, length = 2.06m and width = 1.22m). Three pars of flexible metal bars (diameter = 0.05m, height = 1.34m), distant 0.03m from each other, were installed to support the shape of the hanging bags. PVC tubes (diameter = 0.015m, height = 1.33m, length = 0.52m) were used to blow oxygen into the bags and carbon dioxide, when needed. At the bottom of each PVC tube, twelve holes (diameter = 0.5mm) were drilled for the air to flow, aerating and mixing the whole system.

The PBR's (pallet 1 and pallet 2) were set up outside of the dairy barn at the West Central Research and Outreach Center (WCROC) in Morris, Minnesota. Each one of the pallets had a box attached in one of the sides, containing a system to monitor pH and temperature, and control the pH. A liquid $CO_2$ tank was used as a carbon source and to control the pH when needed. Each one of the bioreactors (two total) had a capacity for six hanging bags with a maximum volume of 80L each, but all experiments were conducted in 70L.

The airflow was measured using an 8360 VelociCalc® Plus from TSI, and it was flowing in a rate of 4.61 ± 0.09 $m^3$/min from the blower into the bag through the larger black hose (this rate was measured in each one of the bags individually). The air flowing system was composed of a norprene hose (diameter = 0.48cm) to add carbon dioxide, and a rubber hose (diameter = 3.5cm) to insert air from the blower into the bag. To not insert 100% $CO_2$ ($CO_2$ was added only during specific experiments) into the system, ideally the two fluids were mixing while entering together with the PVC tube and then flowing into the algal system.

**Temperature and pH monitoring and controlling system**

The Apex Fusion® consists of an electronic system created by the Neptune company (from California) to monitor different parameters in aquariums. During this study, the Apex Fusion® was used as part of a temperature and pH monitor system, and a pH controller system. The items bought from Neptune are: an energy bar, probes' modules, and an Apex controller. The main objective of using this system was to use the pH probes from the Apex Fusion® system to determine what was the pH and the temperature in each one of the bags. Using the pH information, it was possible to program the Apex Fusion® to turn on a solid states relay, opening a valve from a manifold and releasing $CO_2$ automatically in each one of the bags when the pH reached a maximum value (8.6) and then closed the valve when the pH reached the minimum value (7.4). The pH values could go further down than 7.4 because there was a delay between the measurement and closing the valve.

In summary, what the monitoring and controlling system does is: the temperature and pH probes collect the measurements, and they stay saved in the Apex Fusion® controller, when the pH probe has a measurement over 8.6 it will inform the Apex Fusion® controller to unlock one the designated switch and turn on the solid state relay (RLY) for the specific bag. The RLY will open one of the manifold valves, and the valve will release $CO_2$ inside the bag until the pH probe gets a measurement below 7.4, then the Apex controller will lock the switch, turning off the RLY and the valve. To monitor the temperature, a temperature probe was positioned inside a random glass tube for each one of the aquariums during the lab-scale experiments, and for the pilot-scale, each one of the bags had its individual temperature probe.

During lab-scale the temperature inside the glass tubes varied between 22.8°C and 29.1°C. In the pilot-scale experiments, the temperature variation was between 18.2°C and 40°C.

**Light monitoring: PAR and solar radiation**

During the lab-scale, in addition to control and monitor the pH, and monitor the temperature, it was possible to use the Apex Fusion® system to monitor the photosynthetic active radiation (PAR) that the microalgae culture was receiving from the lights. The PAR sensor was positioned in between tubes, to measure the photosynthetic active radiation that the tubes were receiving and it varied between 331 and 338 µmol/$m^2$/s. Furthermore, the lights were set up to be on every day between 5 a.m. and 9 p.m. to imitate the duration of the sunlight received by the bags during pilot-scale.

Through pilot-scale experiments, the bioreactors were positioned outside, exposed to ambient changes between July and August 2018. The orientation of the bags was east to west. The solar radiation during pilot-scale was measured using the data from the United States Department of Agriculture – Agricultural Research Service (USDA-ARS), that have permanent experiments going on near Swam Lake, which is approximately 11km from the WCROC, where the PBR's used for the wastewater treatment were located. The information provided by the USDA-ARS was an average of the radiation for the day and ranged between 40.56 and 303.55 W/$m^2$. To be able to compare lab-scale and pilot-scale, an approximate conversion was used, where, for sunlight conversion, 1 µmol/$m^2$/s was equal to 4.57 W/$m^2$ (Langhans, R.W., et al., 1997), it means that the solar radiation received by the pilot-scale PBR's varied between 185.34 and 1387.23 µmol/$m^2$/s.

**Cells count and dry weight biomass estimation**

The hemocytometer protocol (Absher, 1973), using a Neubauer chamber, was used to determine the cell density (cells/L). The cells density was determined by Equation 1, where SC is the average cells per small square, D is the dilution factor, and V is the volume of a small square in mL (in this case, $V = 0.0001mL$).

$$Cell\ density = \frac{SC*D}{V} \quad \text{(Eq. 1)}$$

For the biomass estimation, a 0.22µm nitrocellulose membrane was left to for at least one hour, weighted, then a sample of 15mL was filtered through it using a vacuum pump. The filter was then put back to dry at 65°C until the weight was constant. The

difference in the membrane weight estimated the dry weight biomass (g/L) on that day for each one of the treatments.

**Nitrate, Ammonium and Phosphate concentrations**

In order to determine the daily concentration of nitrate, ammonium and phosphate, 30mL of the sample was filtered using a 0.22µm nitrocellulose membrane (Merck Millipore Ltd.) in a 500 mL filtration unit (Nalgene), and a vacuum pump. The same sample was used to determine the concentrations of nitrate and ammonium (15mL). It was stored in a refrigerator at 8°C after 0.3 mL of concentrated sulfuric acid was added to the sample. The phosphate sample (15mL) was stored at -4°C after being filtered. These samples were analyzed using Lanchat.

**Harvesting techniques**

The lab-scale experiments were not harvested because of the small amount of biomass produced. In that case, for the lab-scale, only the biomass production per day was evaluated filtering 15ml of the sample through a 0.22µm filter paper. However, for the pilot-scale, two different harvesting techniques had to be used.

**Harvesting *Chlorella sp.* from artificial medium in pilot-scale**

When harvesting the biomass from the pilot-scale experiments, the blower was turned off after the last sampling. The microalgae biomass was expected to precipitate after three to five hours, accumulating in the bottom of the 70L bag. However, what was observed for the controls, was that the microalgae would stay distributed in the entire water column, having only a minimal amount going to the bottom. Because of that, the entire bag had to be centrifuged. In order to make the process more efficient and less time consuming, a cream milk electric centrifugal separator (version 100-18), designed by Motor Sich JSC©, was used. The initial capacity of this product is to separate 100 L in one hour, with 75 rpm. However, to separate microalgae, some modifications had to be made, including not using the twelve disks that are supposed to be inside the centrifuge. For this project, the microalgae would stay trapped inside the centrifuge, and the liquid part would leave the separator system. Because of that, every time that the water leaving the system started to be dark green, the separator was turned off, and the biomass was harvested from inside the centrifuge system. After collecting the biomass, it was sterilized under 121°C and a pressure of 15 psi, for 30 minutes. The biomass was then kept frozen at -4°C after drying in an incubator for eight days at 65°C.

**Harvesting *Chlorella sp.* from different ratios of dairy wastewater in water in pilot-scale**

After taking the last sample of each experiment, the blower was turned off, making it possible for the microalgae biomass in the bags containing dairy wastewater, to precipitate after a minimum of 3 hours. In this case, less than five liters of the mixture of water and wastewater containing *Chlorella sp.* biomass had to be centrifuged using a Sorvall Legend XTR Centrifuge, from Thermo Scientific, at 6500*xg* during 2 min. The biomass was collected, sterilized under 121°C and a pressure of 15 psi, during 30 minutes.

The biomass was then kept frozen at -4°C after drying in an incubator for eight days at 65°C.

**Data analysis of the different ratios of DW in water for lab and pilot-scale**

The ratios experiment during lab-scale was one week long (two replicates total) with duplicates of five different ratios (control, 1:10, 1:20, 1:30, and 1:40). The same experiment was conducted in pilot-scale (three replicates, one week each) with duplicates of five different ratios (two controls, 1:10, 1:20, 1:30, and 1:40). During both scales, data was collected from day 0 through 6 for the ratios 1:30 and 1:40, and from day 0 to day 7 for control, 1:10, and 1:20. All the ratios in this experiment were supplemented with an extra addition of $CO_2$.

Day 0 to 6 data were analyzed using a mixed model in SAS (SAS Institute Inc.) with fixed effects of day, ratio, and the day and ratio interaction. Covariates of temperature, pH, and solar radiation were included if F-test was meaningful ($P < 0.05$). Random effects were replicate, bag, and replicate and pallet interaction. The repeated effect was day specified by the first-order autoregressive covariance structure. The P-values were adjusted using the Tukey procedure. A different model was built to analyze day 7 for the ratios 1:10, 1:20, and control. However, it was analyzed with the same technique as day 0 through 6. The data were log transformed and back transformed to be reported.

**Data analysis of the different ratios of DW in water for lab and pilot-scale when investigating the necessity of $CO_2$ supplementation**

When the addition of $CO_2$ was investigated, the experiments in lab-scale were one week long (two replicates) with duplicates of six different ratios (control, 1:10, 1:30, controlN[1], 1:10N, and 1:30N). Throughout pilot-scale, an one week experiment was run with duplicates of each one of the six ratios (control, 1:10, 1:30, controlN, 1:10N, and 1:30N). Either in lab or pilot-scale, data for the control, controlN, 1:10, and 1:10N was collected from day 0 to day 6. Although, for ratios 1:30 and 1:30N, data was collect between day 0 and day 4.

Using SAS (SAS Institute Inc.), day 0 to 4 data were analyzed using a mixed model with fixed effects of day, ratio, and the day and ratio interaction for all the different ratios. Covariates of temperature, pH, and solar radiation were included if F-test was meaningful ($P < 0.05$). Random effects were replicate, bag, and replicate and pallet interaction. The repeated effect was day specified by the first-order autoregressive covariance structure. P-values were adjusted using the Tukey procedure. A different model was built to analyze days 5 and 6 for the ratios control, controlN, 1:10, and 1:10N. However, the same model used for day 0 through 4 was to analyze the data for these last two experimental days. The data were log transformed and back transformed to be reported.

**Finding a ratio of dairy wastewater in water to optimize nutrients removal and microalgae biomass production**

**Lab-scale**

During lab-scale, cell density was observed to be higher in the control (Figure 1), followed by ratios 1:10 and 1:20, respectively (Table 3). Temperature and day were significant effects ($P < 0.05$) on the cell density during this experiment. However, in the

first six days of the experiment, only the control and ratio 1:40 were significantly different, while on day 7 control and 1:20 were significantly different. Temperature and dilution were not significant ($P > 0.05$) effects for the ratios harvested on day 7. Delgadillo-Mirquez et al. (2016) also ran experiments in lab-scale (200 mL) with medium where the microalgae was mixed with bacteria cultures, and the temperature was a significant effect on cell growth, as found in the present study. However, the authors also found that the temperature influenced the biomass productivity, phosphate removal, and ammonium removal, what was not found in the models of this study in lab-scale.

The biomass for lab-scale had a considerable variation between experiments on day 7 (Figure 2). On the harvesting day, control had the highest cumulative production, followed by the ratio 1:10 and 1:20 (Table 4). During lab-scale, the only effects influencing the biomass productivity were day, ratio and the interaction between day and ratio. Before harvesting the ratios 1:30 and 1:40, on day 6, the only significant different comparison was between 1:40 and the control. On day 7, when the other ratios and the control were harvested, there was no significant difference between the control and the 1:10, which suggests that 1:10 is a promising ratio to produce biomass to carry feeding studies with ruminants, or to produce biofertilizer while removing nutrients from wastewater. Åkerström et al. (2014), cultivated *Chlorella sp.* using different dilutions of sludge liquor in municipal wastewater, and the lowest concentration was 12%, very close to the ratio 1:10 used in this study. The authors harvested $0.96 \pm 0.18$ g/L of dry weight biomass after ten days of treatment, which is close to the dry weight produced during this study for the ratio 1:10 ($1.575 \pm 0.599$ g/L).

The daily phosphate removal rates are presented in Figure 3 and Table 5. Except for day 0, the controls were significantly different from all the ratios in the experiment. The ratios were not different from each other on any experimental day. On day 6, control and 1:10 were significantly different from all the other treatments. For the ratios and the control harvested on day 7, no difference was observed in the phosphate removal rate. Considering that, the lowest ratio between DW and water, 1:10, is also promising to remove considerable amounts of phosphate from dairy wastewater, 73.13% in this study.

Figure 4 is presenting the removal rates of $NH_4^+$ in lab-scale, where 1:10 was significantly different from ratios 1:30 and 1:40, on day 0. The ratios 1:30 and 1:40 were harvested on day 6 for this experiment, and there was no difference in the ammonium levels between any of the other ratios or the control. However, treatments harvested on day 7 were all different from each other. Throughout this experiment, control removed 99.21% of the $NH_4^+$ , while the ratios 1:10 and 1:20 removed 99.53% and 99.37%, respectively (Table 6).

Figure 5 shows the nitrate removal rates for the lab-scale experiment. 1:30 and 1:40 were harvested on day 6, where all the tubes showed no difference on the nitrate concentrations, and the same was observed for the tubes harvested on day 7 (Table 7). Temperature, day (0 - 6), ratio, and the interaction between day and ratio were significant variables for the nitrate removal. No significant difference was observed for any of the effects on the tubes harvested on day 7. The highest nitrate removal rates were found for the control (97.03%), 1:10 (74.96%), and 1:20 (36.35%), at the last day of the experiment.

**Pilot-scale**

Cell density during pilot-scale was observed to be higher in the control (Figure 6), followed by ratios 1:10 and 1:20. Day 0 was not significant ($P > 0.05$) different in any of the treatments regarding cell density (Table 8), meaning that the bags had a similar number of cells/L at each of the three different batches in this experiment. Day (0 - 6), ratio, and the interaction between day and ratio had a significant effect on cell density. In this case, cell density is dependent on the temperature and solar radiation. For the ratios that were harvested on day 7, temperature and solar radiation were not significant and the only variable significant for the model was ratio.

When running the experiment in pilot-scale and trying to find the best ratio between DW and water, again the control bags with AM6 artificial medium were performing better than the bags with DW in water (Table 9). There was no difference in the initial biomass weight between bags on day 0. However, all the bags had different biomass yield on the last experimental day when comparing to the control (Figure 7). Control produced an estimate of 0.64g/L (0.39 -1.05g/L) of biomass, while 1:10 (DW in water) produced 0.33g/L (0.22-0.50 g/L) in the last day. Day (0-6), ratio, and the interaction between day and ratio were significant variables for biomass productivity. In this case, biomass productivity is dependent on the pH and solar radiation. For the ratios that were harvested on Day 7, pH and solar radiation were not significant, and the only variable significant for the model was ratio.

Daily phosphate ($PO_4^{3-}$) removal rates are presented in Figure 8 and Table 10. The controls were significantly different from all the ratios every day of the experiment because the AM6 medium contains a high amount of prosperous in its recipe. The ratios were not significantly different from each other on any day. Coincidentally with the last day of the log phase for cell growth on ratios 1:10, 1:30, and 1:40, the day with less amount of phosphate dissolved in the DW/water was day 4. After day 4, the cells started to deplete in number, and the quantity of phosphate started to go up again. It is important to emphasize that the DW was used without being sterilized, meaning that all the bacteria and pathogens that are found in this type of effluent were not killed before the experiment started. These organisms may influence the fluctuation of phosphate concentration. Day (0 - 6), ratio, and the interaction between day and ratio had an effect on $PO_4^{3-}$ removal. In this case, phosphate removal is dependent on temperature. For the ratios that were harvested on day 7, temperature was not significant and the only variable significant for the model was ratio. Whitton et al. (2016) reported a low phosphate removal rate, ranging from 12.5 to 19.6%, while in this study, 1:10 removed 27.05% and 1:30 21.54%. Also, the authors present results where *Chlorella vulgaris* had an increase in the phosphorous concentration after ten days of remediation trials. Moreover, comparing the cell growth (Figure 6) to the phosphate removal (Figure 8), the cells start their stationary phase between the third and fourth day, exactly when the phosphate concentration goes down and confirming the phosphate as one of the main nutrients that algae need to grow and regulate their metabolism (Powell et al., 2008; Kim et al., 2012; Razzak, et al., 2013; Ramaraj et al., 2015).

Figure 9 shows the removal rates of Ammonium ($NH_4^+$), where the controls were different from all the DW/water ratios every day of the experiment. However, the different ratios were not different from each other on any day. All the ratios and control harvested

had very high removal rates, dropping the levels close to zero in all three different weeks. Control removed 98.12%, while the ratios 1:10 and 1:30 removed 97.55% and 95.53%, respectively (Table 11). Day (0 - 6) and ratio were significant variables for Ammonium removal. In this case, $NH_4^+$ removal is dependent on solar radiation. For the ratios harvested on day 7, solar radiation was not significant, and ratio was the only variable significant for the model. Whitton et al. (2016) performed similar experiments to the ones presented in this study, where they had batch reactions for 10 days using dilutions of wastewater in artificial medium (BG11) and a mix of different microalgae, including *Chlorella*. They found ammonium removal rates above 99% in liquid phase reactions.

Nitrate removal rates are shown in Figure 10 and Table 12. Control was lower during the length of the study, but none of the ratios were significantly different from each other on any day. All bags, except for the ratio 1:30, had the same behavior with respect to nitrate removal rates. A decrease was observed after day 3 in the bags mentioned above (when the cells were still in log phase - Figure 6). The highest removal rates were observed for the control (84.11%), 1:10 (39.27%), and 1:20 (34.96%), on the last day of the experiment. Day (0 - 6), ratio, and the interaction between day and ratio had an effect on nitrate removal rates. For the ratios harvested on day 7, ratio was the only variable significant for the model. Considering the balance between N:P in this reaction, it is important to compare Figures 8, 9, and 10, where a decrease in the phosphate concentration occurs in the first three days, when the levels of ammonium and nitrate maintain close to the initial amount, being used only after the fourth day and going close to zero in most of the ratios, including the control. These fluctuations were noticed by many authors that have tested different N:P ratios and its variation in different experimental time (Beuckels et al., 2015; Choi & Lee, 2015; Åkerström et al., 2016).

## Testing the necessity of extra addition of $CO_2$ as a carbon source and to control pH when using different ratios of dairy wastewater in water

### Lab-scale

Due to the high costs of adding extra $CO_2$ to systems that may be applied in farms, where people can clean the wastewater and produce livestock feed *in-situ*, this experiment aimed to test the ability of the *Chlorella sp.* to grow and remove nutrients from DW, without adding extra $CO_2$ when running the reactions using PBR's.

Regarding the production of microalgae biomass during lab-scale temperature was the only significant effect ($P < 0.05$) between days (0-4). Moreover, on day 4, when the ration 1:30 was harvested, there was no significant difference in the biomass productivity between the different dilutions (Table 13 and Figure 11). Furthermore, on days 5 and 6, the temperature was not significant for the model anymore, and $CO_2$ continued not to make a difference in biomass productivity. In lab-scale the proportion of biomass production in the treatments with extra $CO_2$ is higher when placing the three treatments together (Figure 12), being significant different in the last two days of experiment. However, between day 0 and 4 no significant difference was observed in the biomass productivity (Table 14).

The higher phosphate concentration were observed in the ratio 1:30 for both experiments, and when extra $CO_2$ was added (removal rate as high as 96.11%) and when it was not (removal rate as high as 98.61%). Due to the up and downs that make it hard to

interpret the results (Table 15), the phosphate data were not analyzed as the other data were. For example, the results for the controls', that started with a low level and for both experiments they went up and reached the higher pick on day 3. After that, the levels went down again but went back up on the last day of the experiment. The same trend is not noticed in the other ratios that had a high value on day 0 and continue to go down in the following days. When considering only the extra addition or not of $CO_2$, the tubes that did not receive carbon dioxide had a significant lower concentration of the gas than the tubes with extra amounts of $CO_2$.

For the first four days of the experiment, the nitrate concentration were significantly different between the control and the ratios 1:10, and 1:30, but it was not different between the two ratios (Table 16). Furthermore, during the last two days of experiments, no significant difference was found in the results between the control and 1:10. Temperature, ratio, and the interactions between day and $CO_2$, and day and ratio were significant effects in the nitrate removal rate, from day 0-4 (Figure 13). Figure 14 shows the results regarding the extra addition of $CO_2$, where no difference was found in the first four days for the nitrate removal rates. However, for day 5 and 6, all the effects mentioned before were not significant, but $CO_2$ was (p-value = 0.0421). Even though the tubes with extra $CO_2$ seemed to have higher nitrate removal rates in the final day, the tubes without extra gas removed higher rates throughout the experiment, 78.66% without extra $CO_2$ and 75.18% with extra $CO_2$ (Table 17).

Ammonium concentration from day 0 to 4 were significantly different only for the ratio 1:10 and 1:10N. For the last two days of experiments, there was no significant difference between the dilutions (Table 18 and Figure 15). Furthermore, during the first four days of the experiment, temperature, the different ratios, and the interaction between the ration and addition of $CO_2$ were significant effects on the model. However, none of the effects listed above were significant during days 5 and 6 of this experiment. Figure 16 and Table 19 show the data regarding the influence of the extra addition of $CO_2$ in the ammonium concentration, and there was no significant difference in none of the experimental days. On the last experimental day, the dilutions without extra $CO_2$ removed up to 96.56% of the initial ammonium concentration, while the tubes with extra $CO_2$ addition removed up to 97.74%. Wang et al. (2010), seemed to not have added $CO_2$ during their nine-day experiments involving four different wastewater from the same treatment plan, and the authors could remove 74.7% of the $NH_4^+$ from the wastewater used in this experiment, 90.6% of the $PO_4^{3-}$, and up to 62.5% of the $NO_3^-$. Liu et al. (2017), found that *Chlorella vulgaris* take long period of time to deplete ammonium from wastewater when no extra $CO_2$ is added to the system. For example, they found that for the microalgae to deplete $NH_4^+$ close to zero, would take three days for concentrations of 1%, 5%, and 10% if $CO_2$, three and a half days for 20% of $CO_2$, and five days and a half when not mixing $CO_2$ in the aeration system. It confirms the results for ammonium concentration found in the study presented in this thesis.

**Pilot-scale**

Cell density had the higher concentration in the control and ratio 1:10 that had the extra $CO_2$ (Figure 17 and Table 20). Day and the interaction between day and ration were the only significant effects for this model during day 0-4. Although, they were not significant ($P > 0.05$) on the last two days of the experiment when the $CO_2$ was the only

significant effect influencing in the cells' density. *Chlorella sp.* is known as a species that has its growth limited when $CO_2$ levels exceed 10% (v/v) (Silva & Pirt, 1984; Lee & Tay, 1991, Cheng et al., 2006). However, Klinthong et al. (2015) stated that some *Chlorella sp.* can grow without added $CO_2$, in levels of up to 15%. Moreover, some species of that microalgae can grow with the addition of $CO_2$ in concentrations of up to 100% and tolerate it, confirming the findings of the study presented in this thesis, where it is confirmed that *Chlorella sp.* biomass can be produced by mixing 100% $CO_2$ in different treatments, including the ones using DW in PBR's.

Regarding the biomass productivity (Figure 18), the higher amounts were produced by the controls and followed by the ratios 1:10. The data (Table 21) analysis showed no significant difference between the ratios on day 0 through 4, but the day and the interaction between Day and ratio were significant effects in this model. For days 5 and 6, the only significant effect was the different ratios. Therefore, $CO_2$ did not affect biomass productivity during this experiment (Table 22 and Figure 19). However, what was noticed during this entire experiment, was the formation of biofilm in the bag's walls, which did not allow the proper sampling for cells and biomass findings. Because of that, when the harvest occurred for each one of the bags, the higher biomass weight was harvested from the control with the extra addition of $CO_2$, and the amount collected was significantly different from the ratios 1:10 and 1:30 in the same conditions. However, after harvesting the bags where the extra $CO_2$ was not added, there was no significant difference between the control and the ratio 1:10, but both of them were different from the ratio 1:30 (Table 23).

Figure 20 is showing the trends for phosphate removal in the different ratios when $CO_2$ was and was not added. Also, the results on Table 24 show that all the ratios that had extra addition of carbon dioxide removed higher rates of phosphate than the ratios without extra addition. The maximum removal rates were registered on day 4 for all the bags, having 1:30 as the ratio with higher removal rates, either with extra $CO_2$ (98.29%) or not (93.05%), followed by 1:10 (82.80% with extra $CO_2$ and 72.11% without extra amount of the gas), and the controls (45.68% with extra $CO_2$ and 15.79% without additional amount of the gas). From day 0 to 4 of the experiment, instead, the day, ratio, and the interaction between day and extra $CO_2$ were. The same was noticed for the model used for days 5 and 6, but the only significant effect was the different ratios. Moreover, the same tendency was found during the experiment to find the best ratios to carry this study, shown in Figure 8. The extra addition of $CO_2$ was not a significant effect in any day of the experiment, as shown in Figure 21 (Table 25).

For this experiment, nitrate removal rates showed to be dependent on the day, ratio, and the interaction between day and ratio, and on days 0 and 4. Although, the nitrate removal was dependent on the ratio only, for days 5 and 6. Day 4 was the day that the ratios 1:10's and 1:30's, had the maximum removal rates during the experiment. The controls removed the maximum nitrate amounts on the last day of the experiment (Figure 22). During this experiment, the ratio 1:10 without extra addition of $CO_2$ had a removal rate 69.78% in the last day, and the control with the extra addition of $CO_2$ removed 73.66% on day 6 (Table 26). Furthermore, there was no significant difference between the ratios and the extra addition of $CO_2$ in any day of this experiment regarding the nitrate removal rates, but overall the ratios and the control with the additional amount of the gas removed higher rates of nitrate (Figure 23 and Table 27).

Initially, the amount of ammonium was higher in the controls comparing to the ratios because of the type of medium used (AM6) during the experiments. However, at the harvesting days, all the bags had a similar concentration of ammonium (Figure 24) where the estimates were below 1ppm. The control without extra $CO_2$ had the highest ammonium removal rate, 94.47%, followed by the ratio 1:10, also without extra $CO_2$, which removed 92.19% throughout the experiment (Table 28). The bags with an extra addition of $CO_2$ showed to overall remove less ammonium (Figure 25 and Table 28), but adding extra $CO_2$ showed to not be a significant effect in any of the experimental days for ammonium removal.

**Biomass characteristics**

Table 30 presents the biochemical composition of the biomass produced during this entire, what was found to have high protein content (49.2%), low fat (2.32%), no mycotoxins, and good concentration of various minerals, such as calcium, iron, and zinc. This composition gives to the biomass produced during this study a high chance to be used as livestock feed, if they accept to eat this product. In animal feeding, microalgae can provide essential nutrients, minerals, vitamins, fatty acids and a high protein content, which is difficult to find all in the same organism (Madeira, M. S. et al., 2017; Kotrbacek, V. et al., 2015; Christaki, E. et al., 2011).

**Conclusions**

The lab-scale study showed promising results of biomass production and nutrient removal, being able to grow considerable amount of biomass in the different treatments (control: 1.315 ± 0.240 g/L; 1:10: 1.575 ± 0.599 g/L; 1:20: 1.005 ± 0.301 g/L; 1:30: 0.985 ± 0.370 g/L; 1:40: 0.800 ± 0.329 g/L) and to remove expressive nutrients levels, such as 73.13% of the phosphate, 99.53% of the ammonium, and 74.96% of the nitrate in the ratio 1:10. Considering the results, the same experiment was set up in pilot-scale (70L for each treatment) having control (0.752 ± 0.397 g/L) and the 1:10 (0.434 ± 0.355 g/L) as the best biomass producers, while control removed 98.12% and the 1:10 removed 97.55% of the ammonium, the control removed 84.11% of the nitrate and 1:10 removed 39.27%. Also, the controls could remove 9.85% of the phosphate, when 1:10 removed 27.05%. Being the lowest ratio between DW and water, 1:10 was the most efficient treatment in this study, using more dairy wastewater, producing the highest amount of biomass between the different DW/water ratios, and removing high rates of phosphate, ammonium, and nitrate. Ratio 1:30 showed to be an alternative for short periods of time where the goal would be removing nutrients (not significant different from any of the removal rates of 1:10 in this study) but not producing biomass.

The PBR designed for this study was meant to be used on farms, where the producers could recycle the wastewater and produce enriched biomass to use in their properties. To be able to do that, an experiment comparing three different treatments (control, 1:10, and 1:30) with and without the addition of extra $CO_2$ was carried out. The treatments 1:30 and 1:30N were harvested on the fourth day, where they could remove a high rate of nutrients, but the biomass productivity was very low. Control, controlN, 1:10, and 1:10N were harvested on the sixth day, producing higher amounts of biomass than the treatments 1:30 and 1:30N. However, in pilot-scale, none of the treatments were

significantly different from each other, considering the nutrients ($NH_4^+$, $PO_4^{3-}$, $NO_3^-$) removal rates, or biomass, when adding or not, extra $CO_2$.

| Component | Concentration (g/L) | Molar Mass (g/mol) | Concentration in Final Medium (M)[1] |
|---|---|---|---|
| **$NaNO_3$** | 0.25 | 84.99 | $2.94 \times 10^{-3}$ |
| **$NH_4Cl$** | 0.05 | 53.49 | $9.34 \times 10^{-4}$ |
| **$MgSO_4.7H_2O$** | 0.075 | 246.47 | $3.04 \times 10^{-4}$ |
| **$CaCl_2.2H_2O$** | 0.025 | 147.01 | $1.70 \times 10^{-4}$ |
| **NaCl** | 0.025 | 58.44 | $4.28 \times 10^{-4}$ |
| **$(NH_4)_5[Fe(C_6H_4O_7)_2]$** | 0.01 | 261.98 | $3.82 \times 10^{-5}$ |
| **$K_2HPO_4$** | 0.025 | 174.20 | $1.44 \times 10^{-4}$ |
| **$Na_2CO_3$** | 0.025 | 105.99 | $2.36 \times 10^{-4}$ |
| **Trace elements solution** | 1mL/L | - | - |

| Trace Elements Solution | |
|---|---|
| **Component** | **Quantity used (g/L)** |
| **$H_3BO_3$** | 0.6 |
| **$MnCl_2.4H_2O$** | 0.25 |
| **$ZnCl_2$** | 0.02 |
| **$CuCl_2$** | 0.015 |
| **$Na_2MoO_4.2H_2O$** | 0.015 |
| **$CoCl_2.6H_2O$** | 0.015 |
| **$NiCl_2.6H_2O$** | 0.01 |

| | |
|---|---|
| **$V_2O_5$** | 0.002 |
| **KBr** | 0.01 |

[1] Molarity = Concentration / Molar Mass

**Table 2**. Raw dairy wastewater properties.

| Parameters | Concentration (mg/L) | Method |
|---|---|---|
| **Biochemical oxygen demand (BOD)** | 498 | SM 5210 B-(2011) |
| **Chemical oxygen demand (COD)** | 3552 | SM 5220 B-(2011) |
| **Ammoniacal Nitrogen** | 301 | SM 4500 NH3 C-(1997) |
| **Nitrate/Nitrite Nitrogen** | 0 | EPA 353.2 |
| **Carbon (total)** | 1630 | SM 5310 B-(2011) |
| **Total Phosphorus** | | |

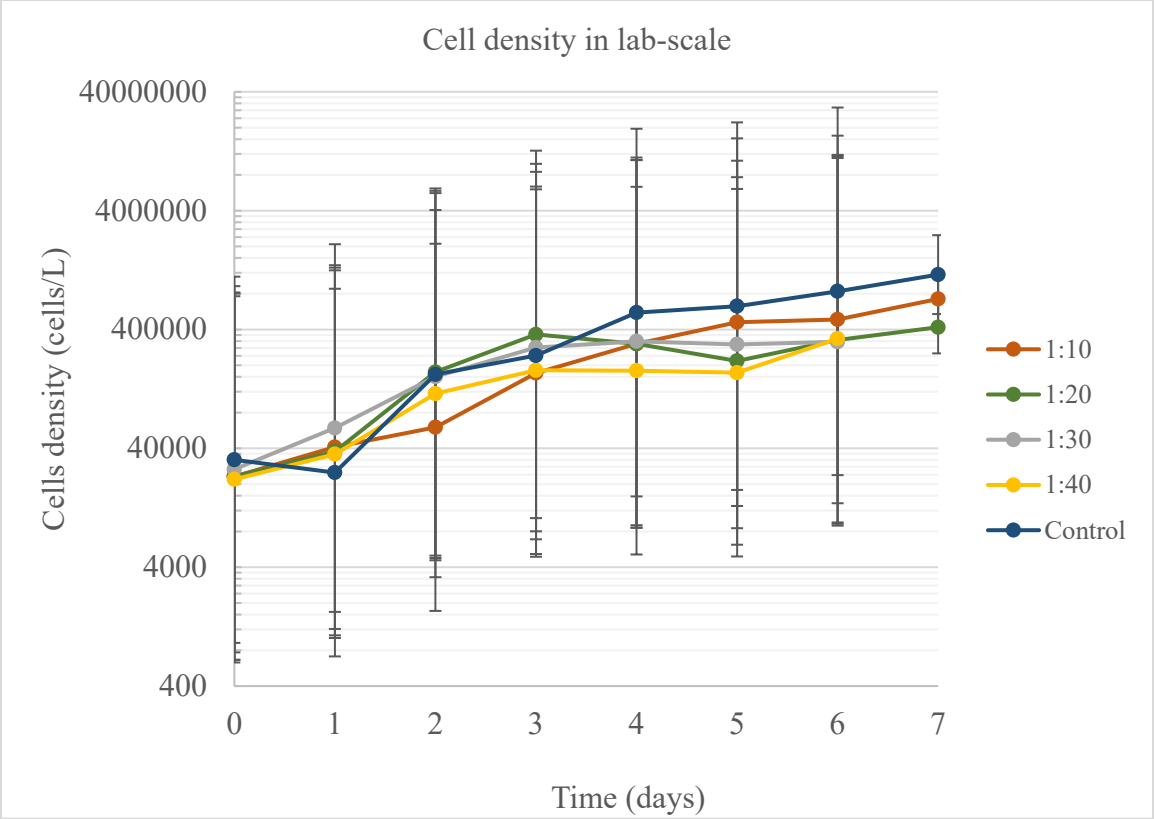



**Figure 1.** Cell density in lab-scale during the experiment to find the best ratio of DW in water.

**Table 3.** Least square means for cell density in lab-scale during the experiment to find the best ratio of DW in water (n = 20).

| | Estimate | | | | |
|---|---|---|---|---|---|
| | Ratio | | | | |
| **Day** | **1:10** | **1:20** | **1:30** | **1:40** | **Control** |
| **0** | 23161.1 | 23344.8 | 26684.3 | 21954.6 | 32087.8 |
| **1** | 41016.5 | 37662.9 | 59309.8 | 35765.2 | 25030.0 |
| **2** | 60158.0 | 175681.8 | 160448.7 | 115581.3 | 167666.1 |
| **3** | 172203.1 | 364043.9 | 282047.2 | 181612.4 | 241573.7 |
| **4** | 302952.0 | 302830.9 | 318134.6 | 180327.5 | 555558.8 |
| **5** | 461312.0 | 217668.8 | 299607.9 | 173343.4 | 629216.0 |
| **6** | 486275.1 | 327102.8 | 315284.2 | 335014.7 | 838801.9 |
| **7** | 723625.9 | 418540.9 | | | 1159535.2 |

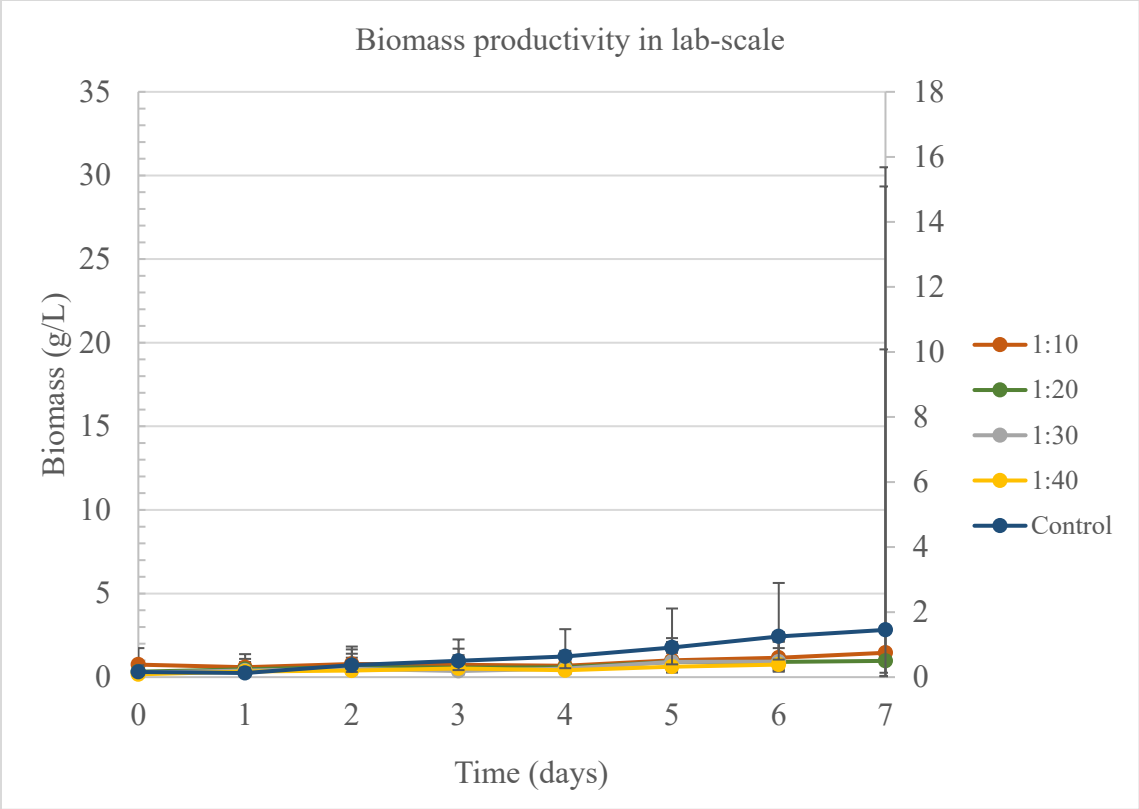



**Figure 2.** Biomass productivity per day of experiment on lab-scale during the experiment to find the best ratio of DW in water. Primary vertical axis (on the left) is correspondent to the results for ratios 1:10, 1:20, 1:30, and 1:40. Secondary vertical axis (on the right) is correspondent to the results for the control.

**Table 4.** Least square means for biomass productivity in lab-scale during the experiment to find the best ratio of DW in water (n = 20).

| | Estimate | | | | |
|---|---|---|---|---|---|
| | Ratio | | | | |
| **Day** | **1:10** | **1:20** | **1:30** | **1:40** | **Control** |
| **0** | 0.750 | 0.327 | 0.272 | 0.167 | 0.159 |
| **1** | 0.594 | 0.471 | 0.391 | 0.338 | 0.127 |
| **2** | 0.790 | 0.605 | 0.503 | 0.392 | 0.368 |
| **3** | 0.735 | 0.568 | 0.369 | 0.520 | 0.501 |
| **4** | 0.679 | 0.582 | 0.507 | 0.409 | 0.637 |
| **5** | 1.008 | 0.908 | 0.908 | 0.626 | 0.912 |
| **6** | 1.163 | 0.914 | 0.926 | 0.750 | 1.251 |
| **7** | 1.457 | 0.973 | | | 1.453 |

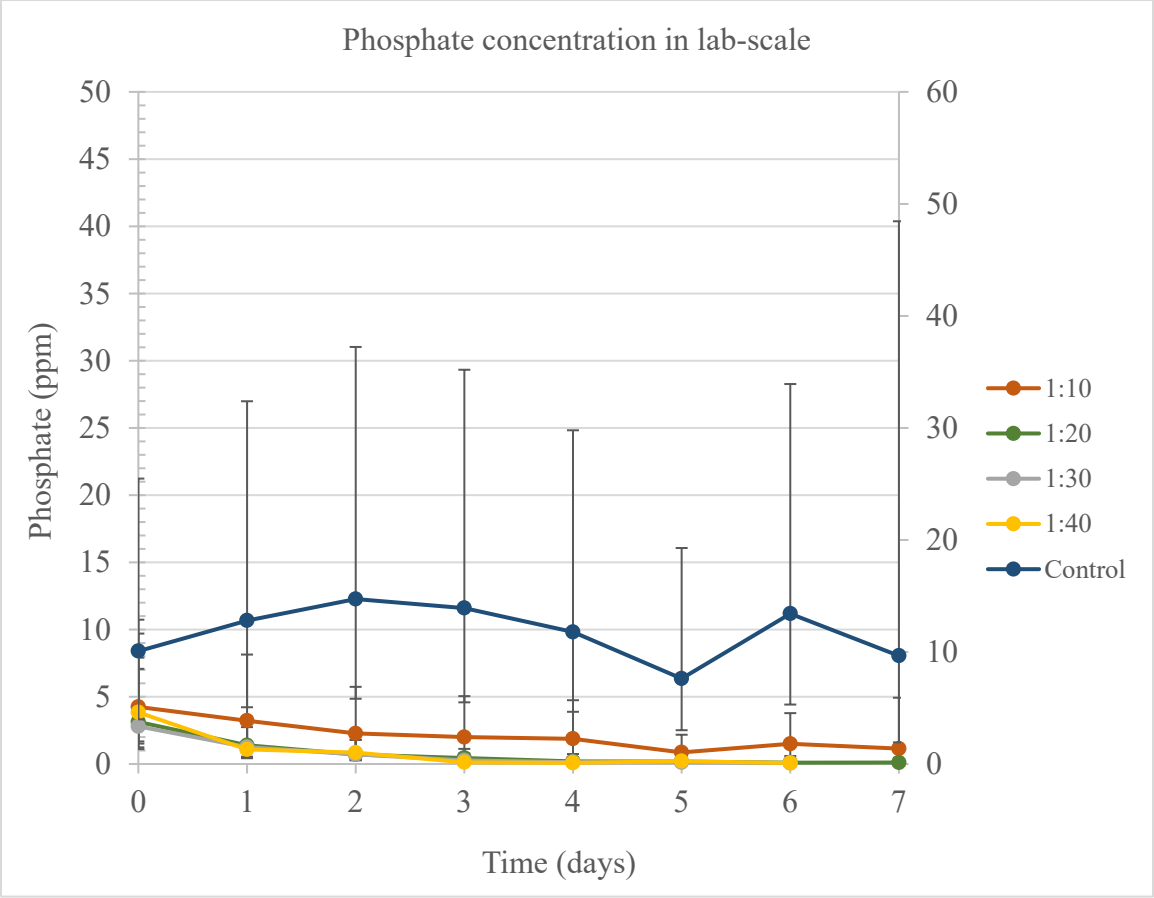



**Figure 3.** Daily phosphate concentration for the lab-scale during the different ratios of DW in water experiments. The primary vertical axis (on the left) is correspondent to the results for the ratios 1:10, 1:20, 1:30, and 1:40. The secondary vertical axis (on the right) is correspondent to the results for the control.

**Table 5.** Least square means for the phosphate concentration in lab-scale during the experiment to find the best ratio of DW in water (n = 20).

| | Estimate | | | | |
|---|---|---|---|---|---|
| | Ratio | | | | |
| **Day** | **1:10** | **1:20** | **1:30** | **1:40** | **Control** |
| **0** | 4.246 | 3.129 | 2.799 | 3.837 | 10.077 |
| **1** | 3.219 | 1.390 | 1.253 | 1.084 | 12.806 |
| **2** | 2.270 | 0.708 | 0.715 | 0.843 | 14.724 |
| **3** | 1.996 | 0.444 | 0.264 | 0.126 | 13.920 |
| **4** | 1.876 | 0.197 | 0.138 | 0.081 | 11.781 |
| **5** | 0.861 | 0.176 | 0.119 | 0.208 | 7.626 |
| **6** | 1.496 | 0.098 | 0.073 | 0.072 | 13.415 |
| **7** | 1.141 | 0.106 | | | 9.663 |

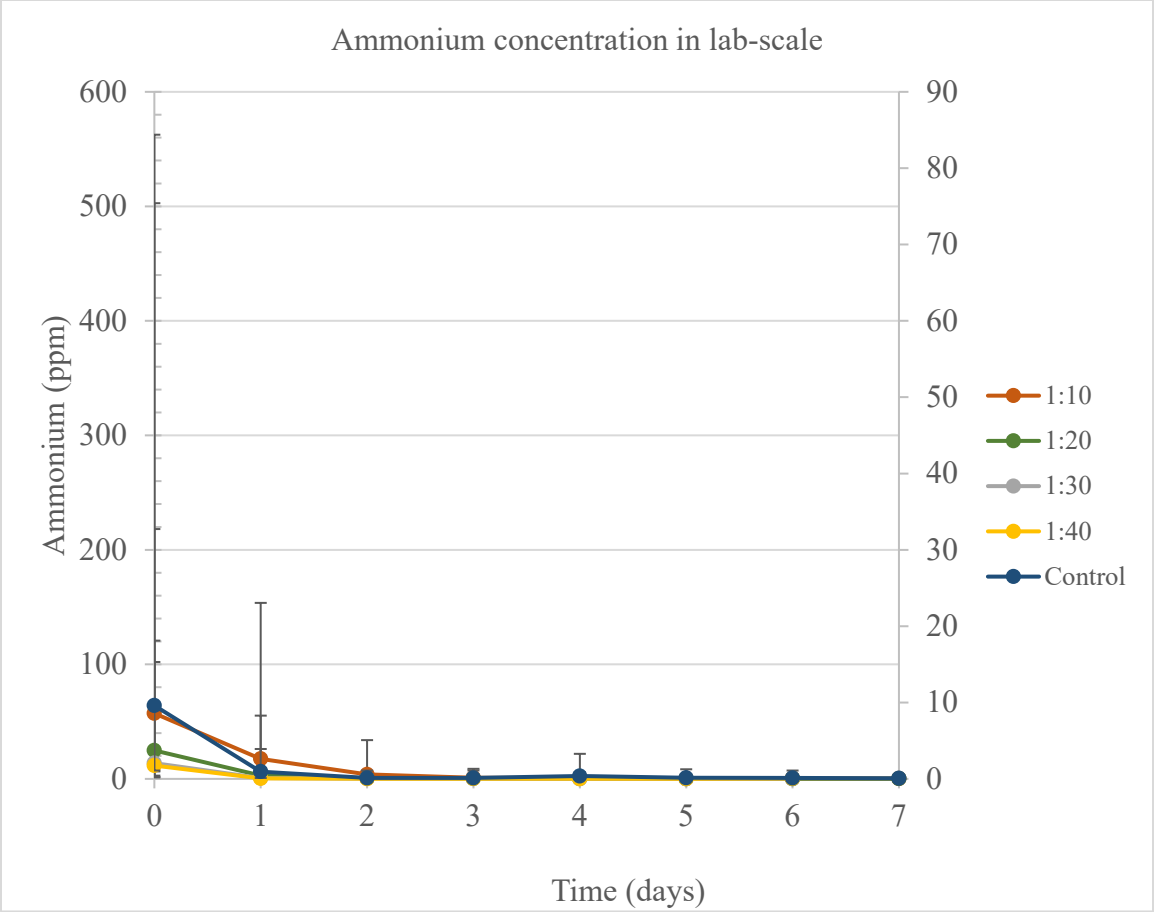



**Figure 4.** Ammonium concentration for the lab-scale experiment when evaluating the better ratio of DW in water. The primary vertical axis (on the left) is correspondent to the results for ratios 1:10, 1:20, 1:30, and 1:40. The secondary vertical axis (on the right) is correspondent to the results for the control.

**Table 6.** Least square means for the Ammonium concentration in lab-scale during the experiment to find the best ratio of DW in water (n = 20).

| | Estimate | | | | |
|---|---|---|---|---|---|
| | Ratio | | | | |
| **Day** | **1:10** | **1:20** | **1:30** | **1:40** | **Control** |
| **0** | 57.237 | 24.836 | 13.745 | 11.621 | 9.607 |
| **1** | 17.498 | 2.971 | 0.729 | 0.406 | 0.943 |
| **2** | 3.853 | 0.542 | 0.188 | 0.118 | 0.124 |
| **3** | 1.000 | 0.250 | 0.171 | 0.181 | 0.125 |
| **4** | 0.657 | 0.262 | 0.138 | 0.120 | 0.373 |
| **5** | 0.206 | 0.134 | 0.113 | 0.089 | 0.143 |
| **6** | 0.234 | 0.131 | 0.341 | 0.350 | 0.125 |
| **7** | 0.269 | 0.157 | | | 0.075 |

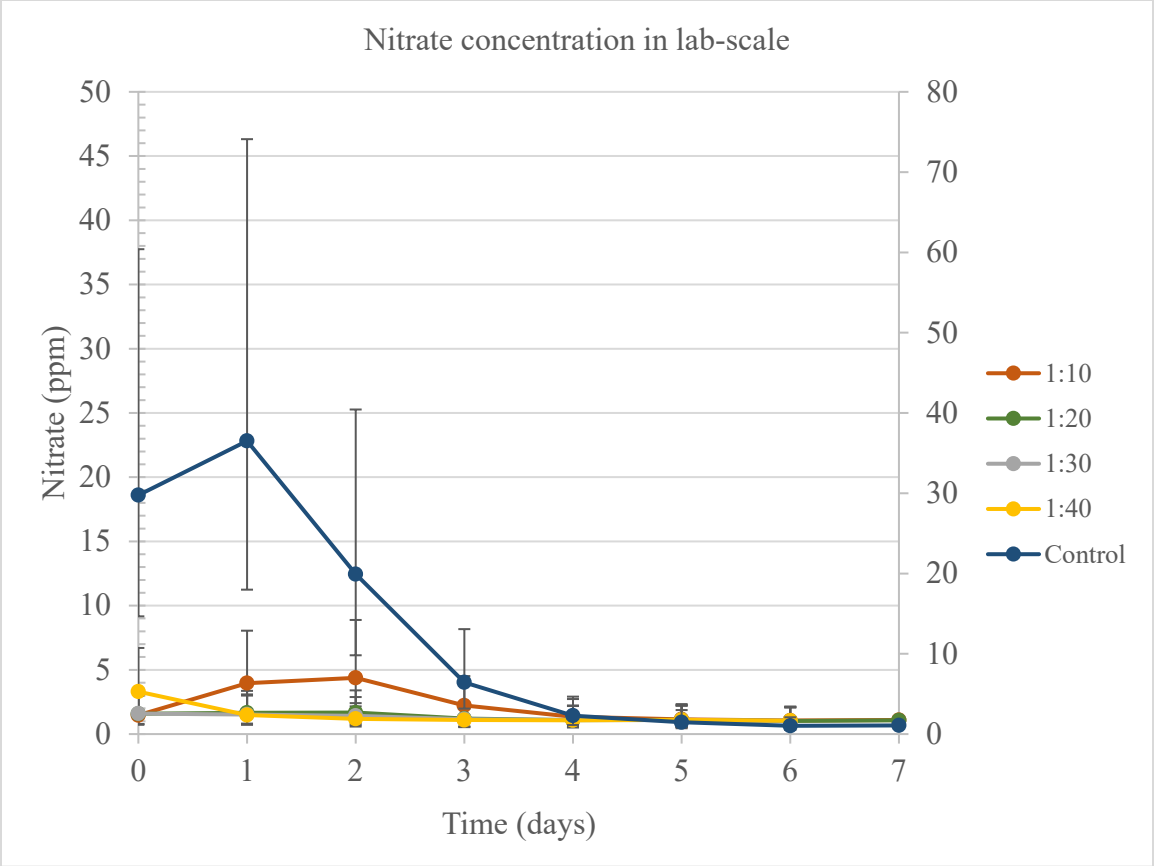



**Figure 5.** Nitrate concentration for the lab-scale experiment when evaluating the better ratio of DW in water. The primary vertical axis (on the left) is correspondent to the results for ratios 1:10, 1:20, 1:30, and 1:40. The secondary vertical axis (on the right) is correspondent to the results for the control.

**Table 7.** Least square means for the Nitrate concentration in lab-scale during the experiment to find the best ratio of DW in water (n = 20).

| | Estimate | | | | |
|---|---|---|---|---|---|
| | Ratio | | | | |
| **Day** | **1:10** | **1:20** | **1:30** | **1:40** | **Control** |
| **0** | 1.469 | 1.580 | 1.596 | 3.304 | 29.761 |
| **1** | 3.967 | 1.658 | 1.522 | 1.477 | 36.514 |
| **2** | 4.378 | 1.677 | 1.420 | 1.187 | 19.922 |
| **3** | 2.225 | 1.202 | 1.163 | 1.095 | 6.441 |
| **4** | 1.344 | 1.086 | 1.092 | 1.076 | 2.298 |
| **5** | 1.150 | 1.107 | 1.085 | 1.104 | 1.474 |
| **6** | 1.058 | 1.021 | 1.030 | 1.035 | 1.032 |
| **7** | 1.096 | 1.067 | | | 1.085 |

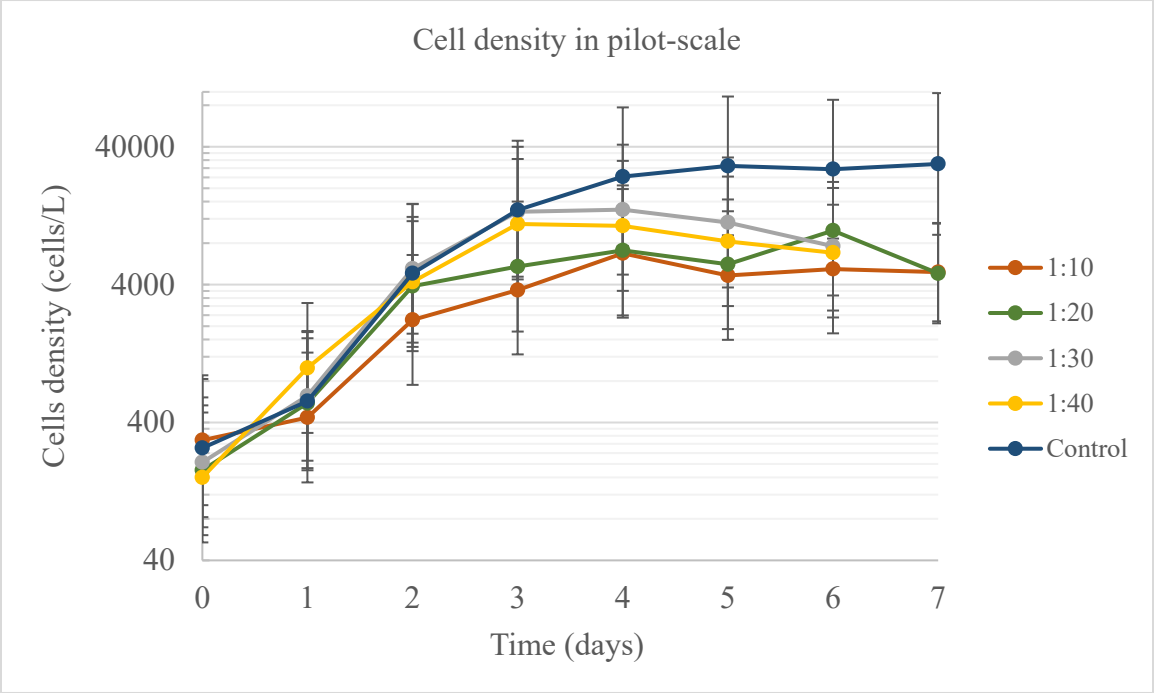



**Figure 6.** Cell density in pilot-scale during the experiment to find the best ratio of DW in water.

**Table 8.** Least square means for cell density in pilot-scale during the experiment to find the best ratio of DW in water (n = 36).

| | Estimate | | | | |
|---|---|---|---|---|---|
| | Ratio | | | | |
| **Day** | **1:10** | **1:20** | **1:30** | **1:40** | **Control** |
| **0** | 297.54 | 180.20 | 205.68 | 159.77 | 261.14 |
| **1** | 434.87 | 552.33 | 624.12 | 994.60 | 571.09 |
| **2** | 2218.85 | 3900.05 | 5214.74 | 4187.02 | 4826.34 |
| **3** | 3652.42 | 5408.05 | 13512.15 | 11015.35 | 13894.45 |
| **4** | 6744.24 | 7082.94 | 13992.05 | 10673.77 | 24280.89 |
| **5** | 4655.69 | 5619.76 | 11297.59 | 8244.86 | 29052.06 |
| **6** | 5190.81 | 9862.00 | 7594.24 | 6817.48 | 27505.60 |
| **7** | 4928.28 | 4820.07 | | | 30017.77 |

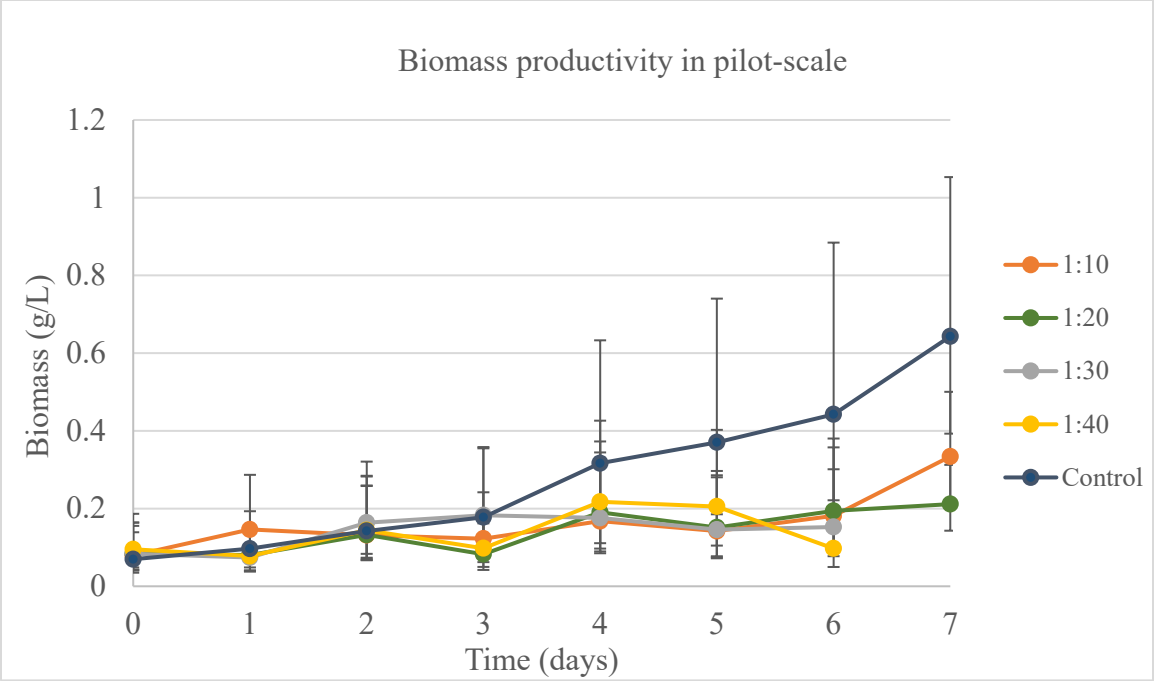



**Figure 7.** Biomass productivity per day of experiment on pilot-scale during the experiment to find the best ratio of DW in water.

**Table 9.** Least square means for biomass productivity during the experiment to find the best ratio of DW in water (n = 36).

| | Estimate | | | | |
|---|---|---|---|---|---|
| | Ratio | | | | |
| **Day** | **1:10** | **1:20** | **1:30** | **1:40** | **Control** |
| **0** | 0.0793 | 0.0835 | 0.0838 | 0.0951 | 0.0697 |
| **1** | 0.1463 | 0.0805 | 0.0741 | 0.0778 | 0.0968 |
| **2** | 0.1316 | 0.1323 | 0.1637 | 0.1441 | 0.1423 |
| **3** | 0.1226 | 0.0826 | 0.1828 | 0.0978 | 0.1776 |
| **4** | 0.1677 | 0.1902 | 0.1758 | 0.2174 | 0.3166 |
| **5** | 0.1422 | 0.1514 | 0.1458 | 0.2053 | 0.3704 |
| **6** | 0.1811 | 0.1940 | 0.1527 | 0.0974 | 0.4425 |
| **7** | 0.3341 | 0.2114 | | | 0.6432 |

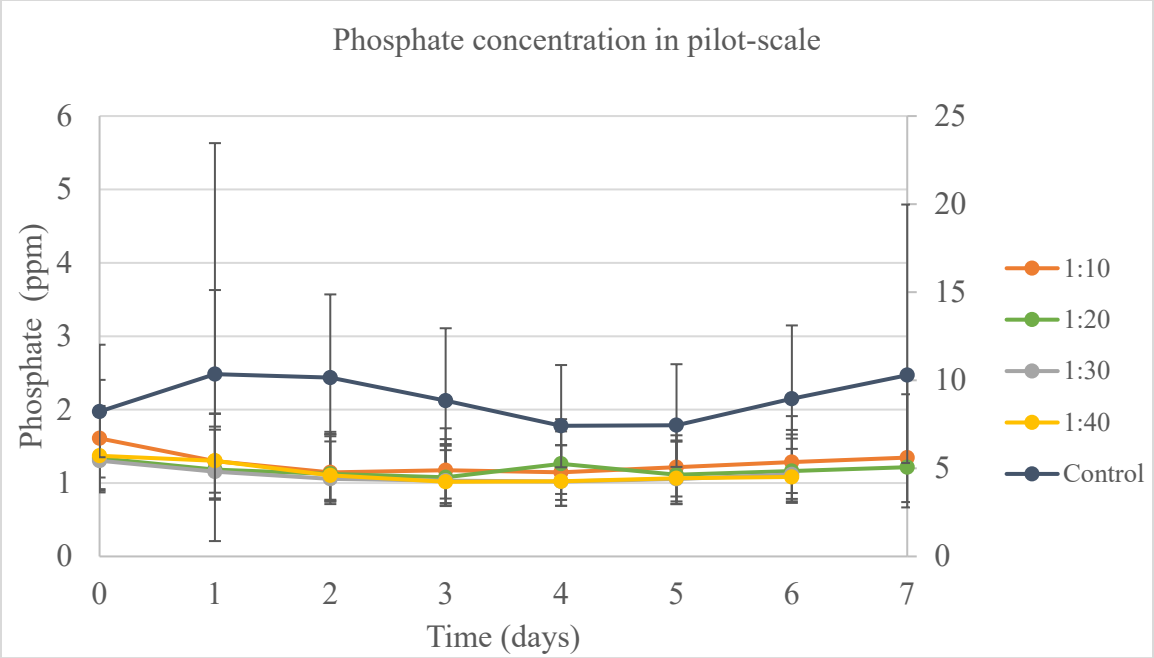



**Figure 8.** Daily phosphate concentration for the pilot-scale during the different ratios of DW in water experiments. The primary vertical axis (on the left) is correspondent to the results for ratios 1:10, 1:20, 1:30, and 1:40. The secondary vertical axis (on the right) is correspondent to the results for the control.

**Table 10.** Least square means for the phosphate concentration during the experiment to find the best ratio of DW in water (n = 36).

| | Estimate | | | | |
|---|---|---|---|---|---|
| | Ratio | | | | |
| **Day** | **1:10** | **1:20** | **1:30** | **1:40** | **Control** |
| **0** | 1.608 | 1.338 | 1.304 | 1.371 | 8.227 |
| **1** | 1.298 | 1.183 | 1.155 | 1.303 | 10.348 |
| **2** | 1.145 | 1.125 | 1.057 | 1.104 | 10.151 |
| **3** | 1.173 | 1.078 | 1.033 | 1.017 | 8.841 |
| **4** | 1.145 | 1.262 | 1.021 | 1.023 | 7.416 |
| **5** | 1.215 | 1.112 | 1.055 | 1.065 | 7.448 |
| **6** | 1.285 | 1.163 | 1.117 | 1.082 | 8.950 |
| **7** | 1.346 | 1.215 | | | 10.292 |

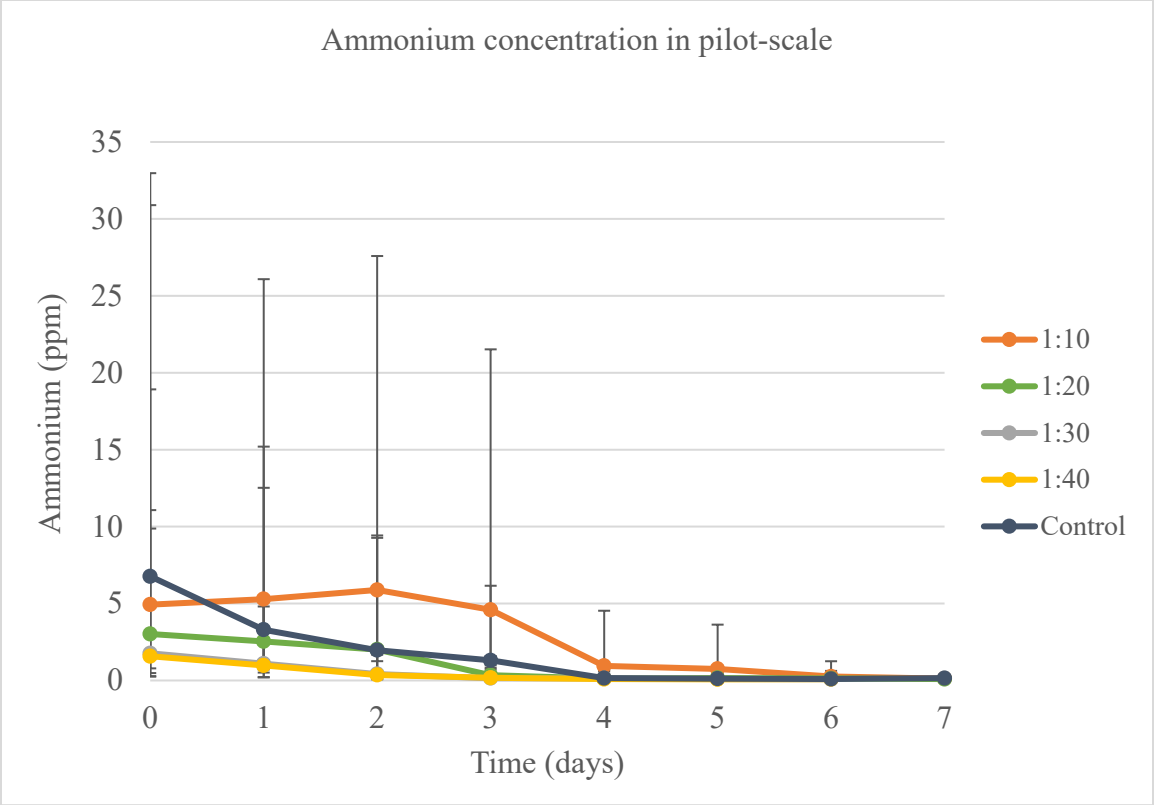



**Figure 9.** Ammonium concentration for the pilot-scale experiment when evaluating the better ratio of DW in water.

**Table 11.** Least square means for the Ammonium concentration during the experiment to find the best ratio of DW in water (n = 36).

| | Estimate | | | | |
|---|---|---|---|---|---|
| | Ratio | | | | |
| **Day** | **1:10** | **1:20** | **1:30** | **1:40** | **Control** |
| **0** | 4.933 | 3.021 | 1.768 | 1.577 | 6.771 |
| **1** | 5.288 | 2.539 | 1.097 | 0.975 | 3.300 |
| **2** | 5.885 | 2.011 | 0.422 | 0.357 | 1.970 |
| **3** | 4.593 | 0.339 | 0.151 | 0.174 | 1.307 |
| **4** | 0.951 | 0.127 | 0.140 | 0.094 | 0.155 |
| **5** | 0.754 | 0.161 | 0.090 | 0.075 | 0.120 |
| **6** | 0.259 | 0.133 | 0.079 | 0.089 | 0.096 |
| **7** | 0.121 | 0.092 | | | 0.156 |

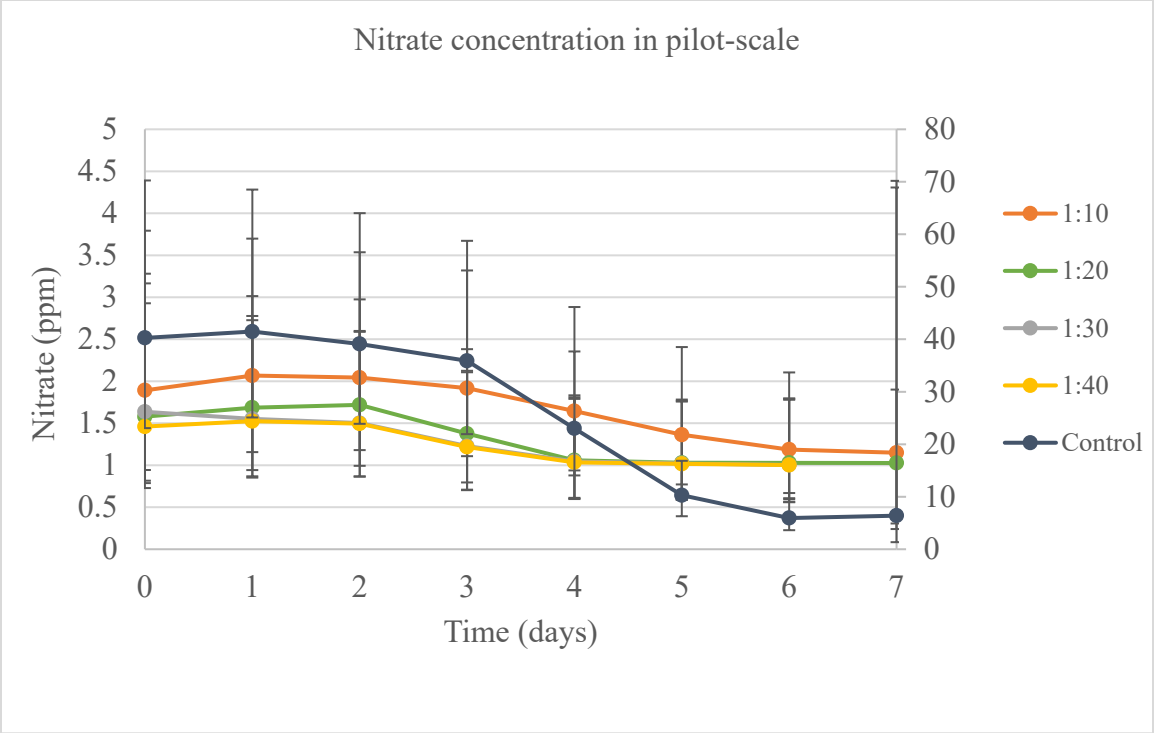



**Figure 10.** Nitrate concentration for the pilot-scale experiment when evaluating the better ratio of DW in water. The primary vertical axis (on the left) is correspondent to the results for ratios 1:10, 1:20, 1:30, and 1:40. The secondary vertical axis (on the right) is correspondent to the results for the control.

**Table 12.** Least square means for the Nitrate concentration during the experiment to find the best ratio of DW in water (n = 36).

| | Estimate | | | | |
|---|---|---|---|---|---|
| | Ratio | | | | |
| **Day** | **1:10** | **1:20** | **1:30** | **1:40** | **Control** |
| **0** | 1.892 | 1.579 | 1.636 | 1.461 | 40.262 |
| **1** | 2.068 | 1.686 | 1.553 | 1.525 | 41.475 |
| **2** | 2.043 | 1.719 | 1.502 | 1.496 | 39.111 |
| **3** | 1.918 | 1.376 | 1.228 | 1.218 | 35.895 |
| **4** | 1.645 | 1.059 | 1.045 | 1.034 | 23.019 |
| **5** | 1.363 | 1.030 | 1.021 | 1.016 | 10.293 |
| **6** | 1.187 | 1.028 | 1.004 | 1.003 | 5.940 |
| **7** | 1.149 | 1.027 | | | 6.396 |

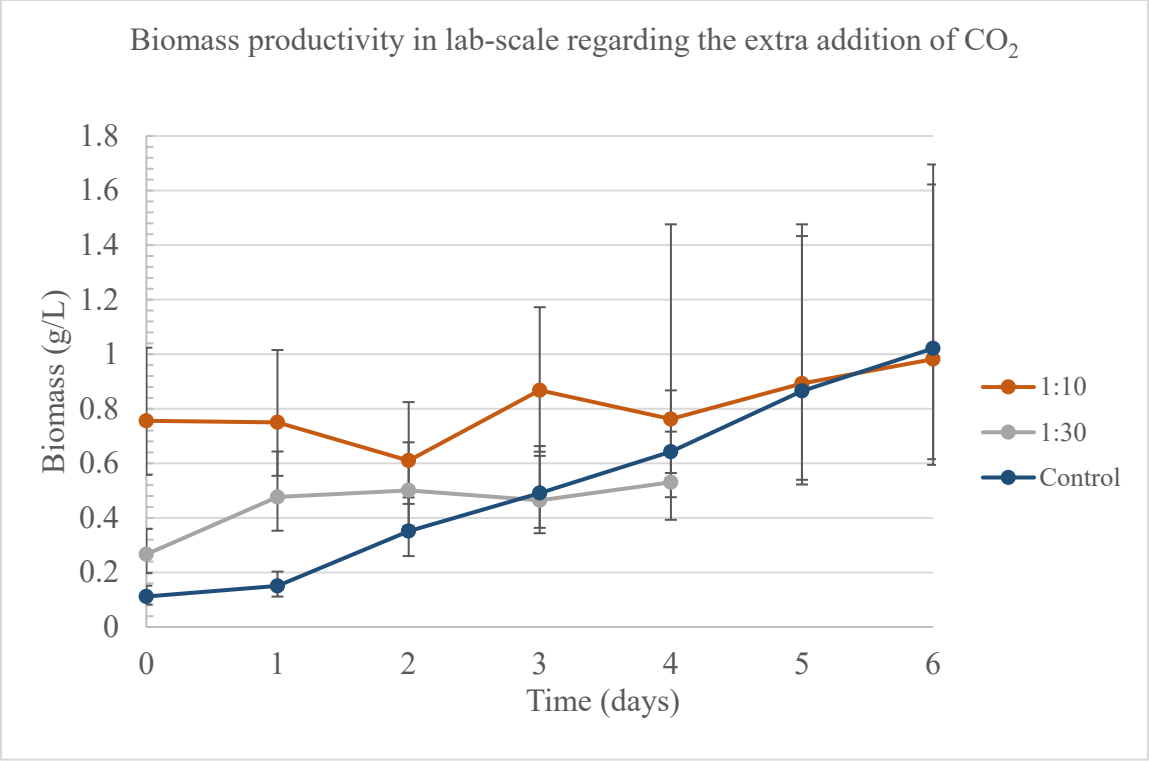



**Figure 11.** Biomass productivity for the lab-scale experiment when evaluating the significance in the extra addition of $CO_2$ using three different ratios.

**Table 13.** Least square means for biomass productivity in lab-scale when evaluating the significance in the extra addition of $CO_2$ using three different ratios (n = 24).

| | Estimate | | | Standard Error | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ratio | | | Lower | | | Upper | | |
| Day | 1:10 | 1:30 | Control | 1:10 | 1:30 | Control | 1:10 | 1:30 | Control |
| **0** | 0.756 | 0.266 | 0.112 | 0.198 | 0.069 | 0.029 | 0.268 | 0.094 | 0.040 |
| **1** | 0.750 | 0.477 | 0.150 | 0.196 | 0.124 | 0.039 | 0.265 | 0.167 | 0.053 |
| **2** | 0.610 | 0.501 | 0.351 | 0.159 | 0.130 | 0.091 | 0.215 | 0.176 | 0.124 |
| **3** | 0.868 | 0.464 | 0.491 | 0.225 | 0.121 | 0.127 | 0.304 | 0.163 | 0.172 |
| **4** | 0.762 | 0.530 | 0.643 | 0.198 | 0.138 | 0.167 | 0.714 | 0.186 | 0.225 |
| **5** | 0.893 | | 0.865 | 0.353 | | 0.343 | 0.584 | | 0.568 |
| **6** | -6.926 | | 1.021 | -7.520 | | 0.406 | 8.548 | | 0.674 |

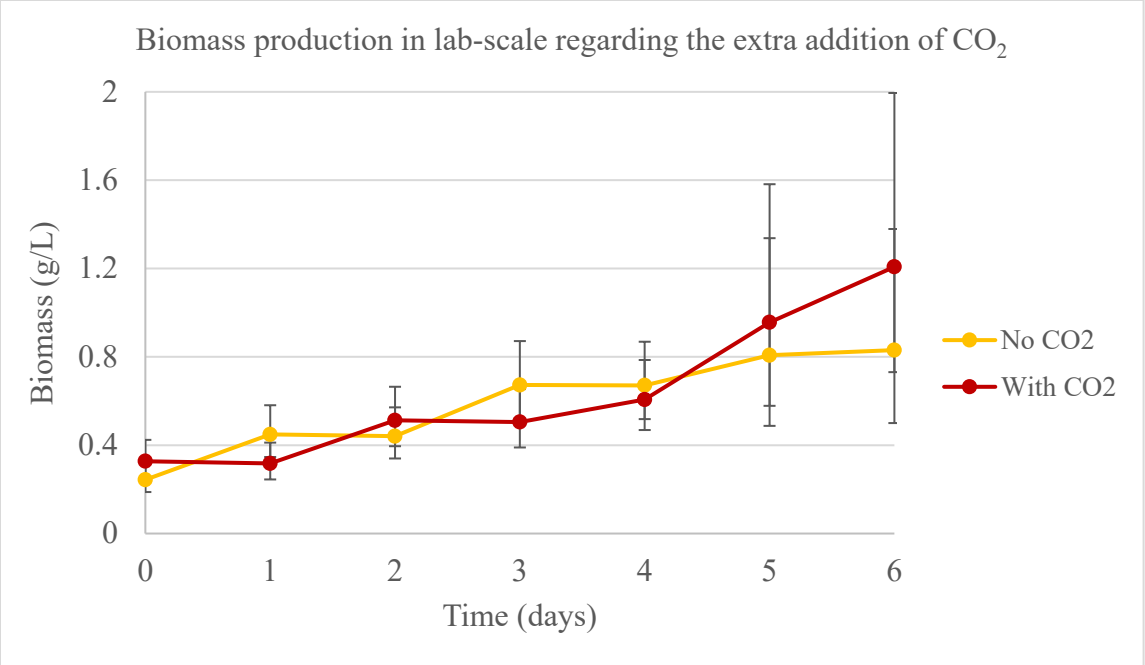



**Figure 12.** Biomass productivity for the lab-scale experiment when evaluating the significance of adding extra $CO_2$.

**Table 14.** Least square means for biomass productivity in lab-scale when evaluating the significance of adding extra $CO_2$ (n = 24).

| | Estimate | | Standard Error | | | |
|---|---|---|---|---|---|---|
| | Ratio | | Lower | | Upper | |
| Day | No | Yes | No | Yes | No | Yes |
| **0** | 0.244 | 0.327 | 0.056 | 0.075 | 0.072 | 0.097 |
| **1** | 0.449 | 0.318 | 0.102 | 0.073 | 0.132 | 0.094 |
| **2** | 0.441 | 0.513 | 0.101 | 0.117 | 0.131 | 0.152 |
| **3** | 0.673 | 0.505 | 0.153 | 0.115 | 0.199 | 0.149 |
| **4** | 0.671 | 0.607 | 0.153 | 0.138 | 0.198 | 0.179 |
| **5** | 0.808 | 0.956 | 0.320 | 0.378 | 0.530 | 0.625 |
| **6** | 0.831 | 1.208 | 0.330 | 0.477 | 0.548 | 0.787 |

**Table 15.** Means for phosphate concentration in lab-scale for experiment 1 and 2, when evaluating the significance in the extra addition of $CO_2$ (n = 24).

| Experiment 1 | | | | | | |
|---|---|---|---|---|---|---|
| | Extra $CO_2$ | | | No extra $CO_2$ | | |
| **Day** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** |
| **0** | 5.75 | 5.34 | 12.49 | 4.41 | 4.34 | 11.53 |
| **1** | 6.01 | 3.26 | 15.26 | 6.37 | 2.84 | 14.80 |
| **2** | 6.81 | 1.41 | 21.23 | 6.79 | 1.44 | 17.45 |
| **3** | 5.88 | 1.49 | 20.85 | 5.49 | 0.61 | 24.18 |
| **4** | 3.72 | 0.48 | 23.54 | 3.11 | 0.06 | 24.69 |
| **5** | 3.99 | | 19.70 | 1.45 | | 9.87 |
| **6** | 3.54 | | 20.71 | 1.64 | | 20.65 |
| **Experiment 2** | | | | | | |
| | Extra $CO_2$ | | | No extra $CO_2$ | | |
| **Day** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** |
| **0** | 12.57 | 5.92 | 33.73 | 7.68 | 7.28 | 32.79 |
| **1** | 6.98 | 2.01 | 43.06 | 1.81 | 2.84 | 37.74 |
| **2** | 3.90 | 1.54 | 43.15 | 2.65 | 1.27 | 43.24 |
| **3** | 2.82 | 0.26 | 38.47 | 1.63 | 0.94 | 15.47 |
| **4** | 4.00 | 0.23 | 29.91 | 1.40 | 0.94 | 41.62 |
| **5** | 2.41 | | 25.64 | 1.99 | | 39.35 |
| **6** | 2.95 | | 35.37 | 4.34 | | 38.83 |

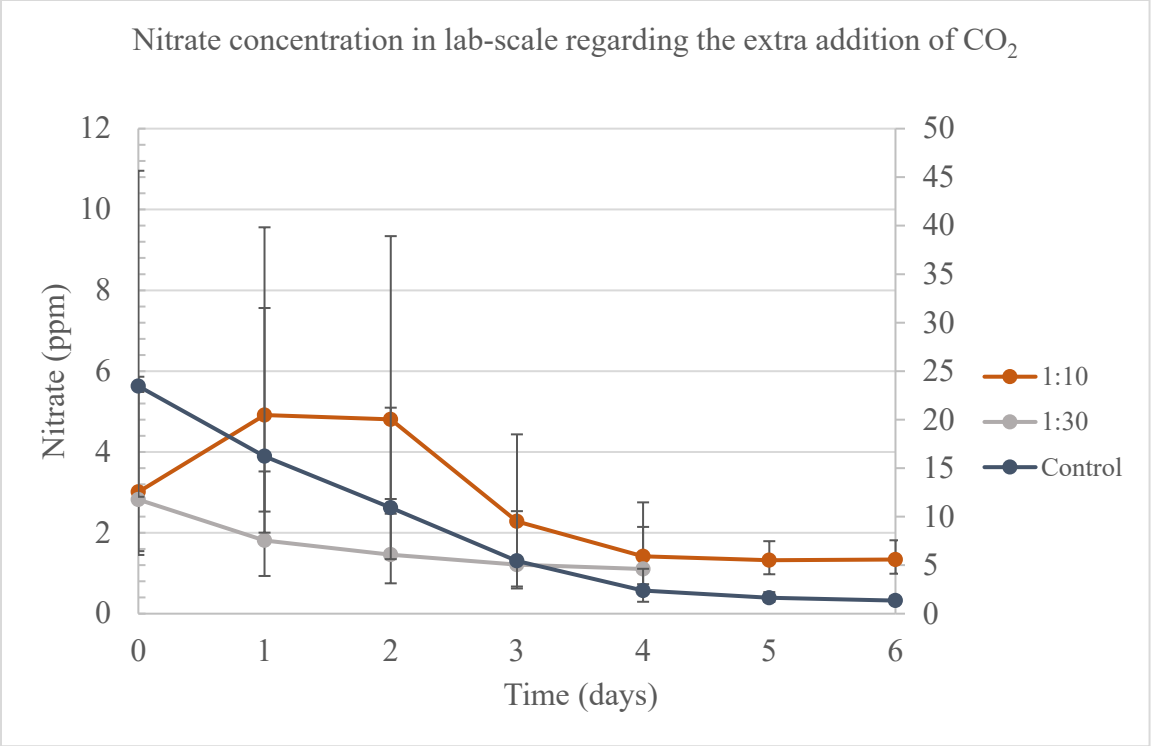



**Figure 13.** Results for the nitrate concentration for the lab-scale experiment when evaluating the significance in the extra addition of $CO_2$ using three different ratios. The results correspondent to the control is found on the secondary axis (on the right), and the results for the ratios 1:10 and 1:30, are on the primary axis (on the left).

**Table 16.** Least square means for nitrate concentration in lab-scale when evaluating the significance in the extra addition of $CO_2$ using three different ratios (n = 24).

| | Estimate | | | Standard Error | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ratio | | | Lower | | | Upper | | |
| Day | 1:10 | 1:30 | Control | 1:10 | 1:30 | Control | 1:10 | 1:30 | Control |
| **0** | 3.012 | 2.823 | 23.458 | 1.464 | 1.370 | 11.407 | 2.850 | 2.662 | 22.201 |
| **1** | 4.914 | 1.812 | 16.223 | 2.388 | 0.879 | 7.871 | 4.645 | 1.707 | 15.287 |
| **2** | 4.806 | 1.460 | 10.926 | 2.333 | 0.709 | 5.305 | 4.535 | 1.377 | 10.311 |
| **3** | 2.283 | 1.212 | 5.440 | 1.108 | 0.588 | 2.640 | 2.153 | 1.142 | 5.129 |
| **4** | 1.418 | 1.105 | 2.382 | 0.688 | 0.536 | 1.156 | 1.336 | 1.041 | 2.245 |
| **5** | 1.322 | | 1.639 | 0.347 | | 0.432 | 0.471 | | 0.586 |
| **6** | 1.340 | | 1.349 | 0.351 | | 0.357 | 0.476 | | 0.485 |

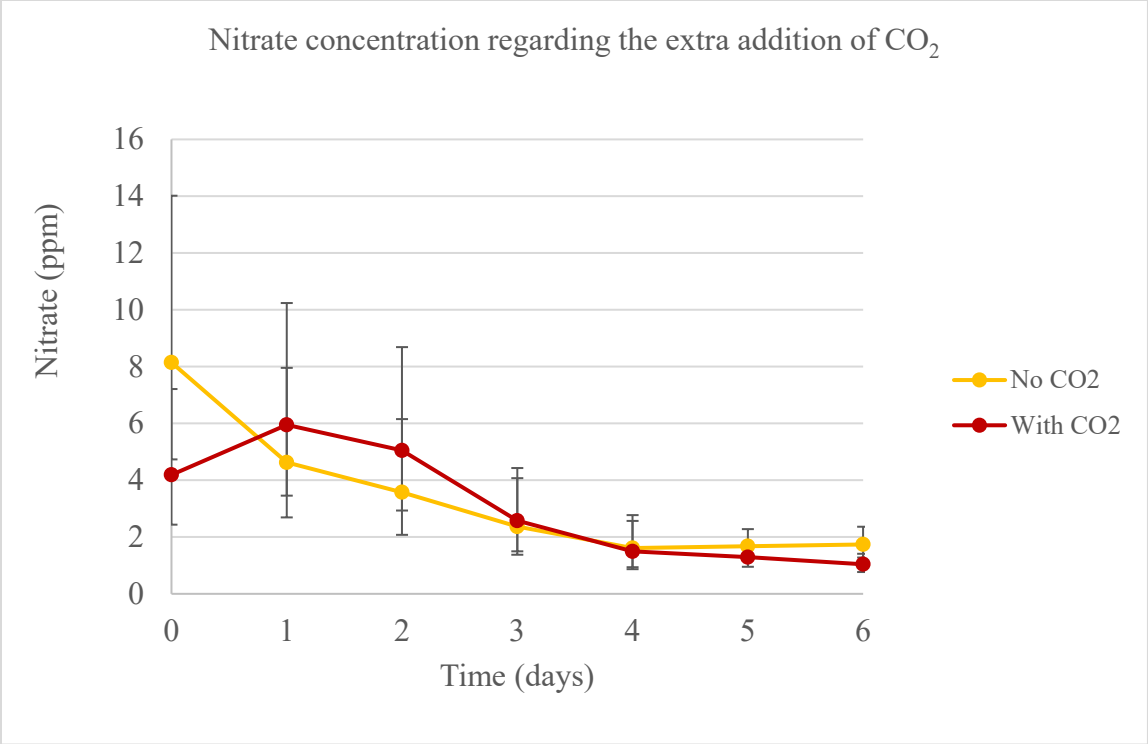



**Figure 14.** Nitrate concentration for the lab-scale experiment when evaluating the significance of adding extra $CO_2$.

**Table 17.** Least square means for nitrate concentration in lab-scale when evaluating the significance of adding extra $CO_2$ (n = 24).

| | Estimate | | Standard Error | | | |
|---|---|---|---|---|---|---|
| | Ratio | | Lower | | Upper | |
| **Day** | **No** | **Yes** | **No** | **Yes** | **No** | **Yes** |
| **0** | 8.146 | 4.190 | 3.412 | 1.755 | 5.872 | 3.021 |
| **1** | 4.627 | 5.949 | 1.936 | 2.492 | 3.330 | 4.290 |
| **2** | 3.574 | 5.048 | 1.497 | 2.115 | 2.578 | 3.639 |
| **3** | 2.368 | 2.575 | 0.991 | 1.078 | 1.705 | 1.855 |
| **4** | 1.612 | 1.493 | 0.675 | 0.625 | 1.160 | 1.074 |
| **5** | 1.678 | 1.291 | 0.442 | 0.339 | 0.600 | 0.460 |
| **6** | 1.738 | 1.040 | 0.459 | 0.273 | 0.625 | 0.370 |

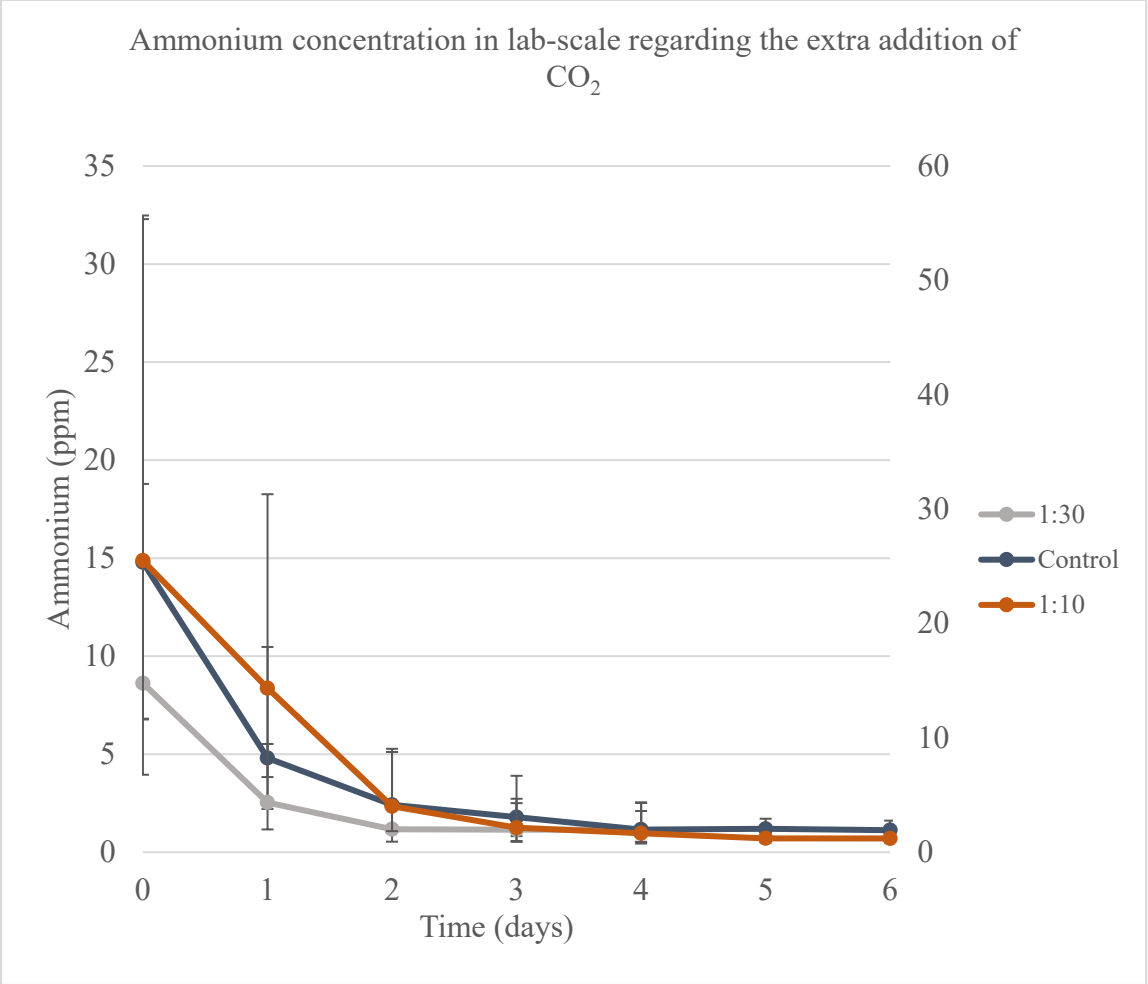



**Figure 15.** Ammonium concentration for the lab-scale experiment when evaluating the significance in the extra addition of $CO_2$ using three different ratios. The primary axis (on the left) is correspondent to the results for control and 1:30, while the secondary axis (on the right) corresponds to the results for 1:10.

**Table 18.** Least square means for ammonium concentration in lab-scale when evaluating the significance in the extra addition of $CO_2$ using three different ratios (n = 24).

| | Estimate | | | Standard Error | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ratio | | | Lower | | | Upper | | |
| Day | 1:10 | 1:30 | Control | 1:10 | 1:30 | Control | 1:10 | 1:30 | Control |
| **0** | 25.498 | 8.618 | 14.788 | 13.821 | 4.665 | 8.016 | 30.181 | 10.170 | 17.507 |
| **1** | 14.345 | 2.534 | 4.805 | 7.772 | 1.371 | 2.600 | 16.961 | 2.988 | 5.666 |
| **2** | 4.018 | 1.174 | 2.419 | 2.176 | 0.635 | 1.310 | 4.745 | 1.386 | 2.856 |
| **3** | 2.144 | 1.149 | 1.788 | 1.161 | 0.622 | 0.968 | 2.531 | 1.355 | 2.110 |
| **4** | 1.653 | 1.152 | 1.157 | 0.895 | 0.624 | 0.631 | 1.950 | 1.359 | 1.389 |
| **5** | 1.213 | | 1.192 | 0.366 | | 0.360 | 0.525 | | 0.516 |
| **6** | 1.203 | | 1.124 | 0.363 | | 0.340 | 0.520 | | 0.488 |

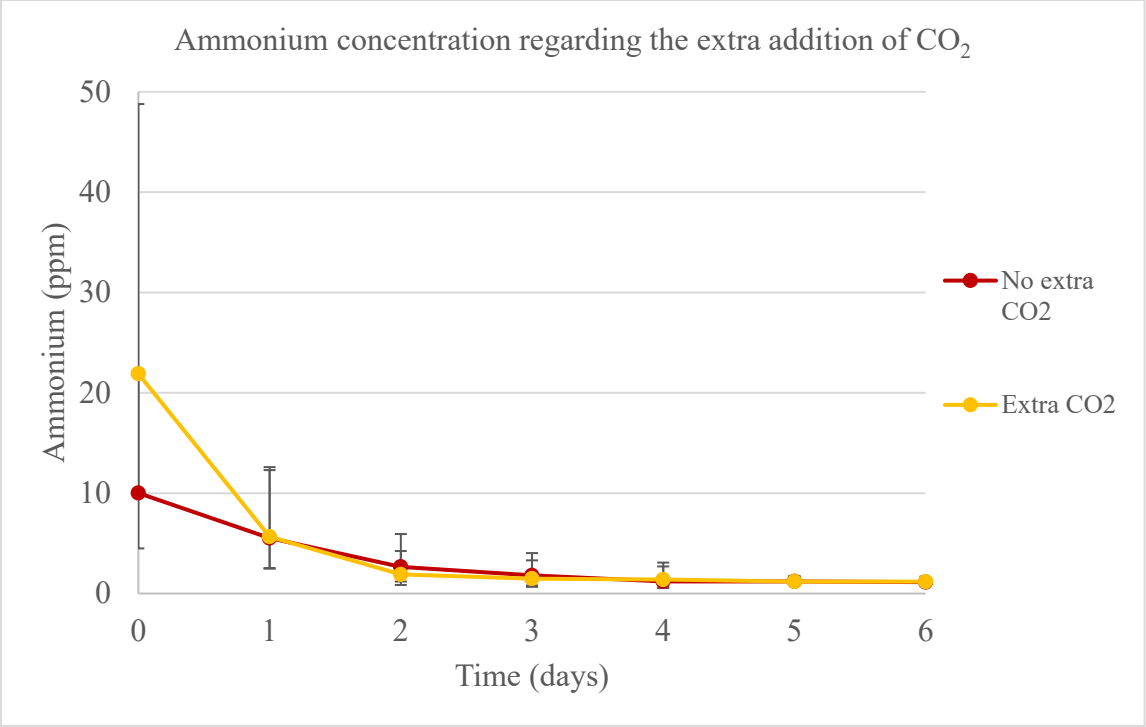



**Figure 16.** Ammonium concentration for the lab-scale experiment when evaluating the significance of adding extra $CO_2$.

**Table 19.** Least square means for ammonium concentration in lab-scale when evaluating the significance of adding extra $CO_2$ (n = 24).

| | Estimate | | Standard Error | | | |
|---|---|---|---|---|---|---|
| | Ratio | | Lower | | Upper | |
| **Day** | **No** | **Yes** | **No** | **Yes** | **No** | **Yes** |
| **0** | 10.014 | 21.907 | 5.517 | 12.069 | 12.286 | 26.877 |
| **1** | 5.522 | 5.658 | 3.043 | 3.117 | 6.781 | 6.941 |
| **2** | 2.663 | 1.903 | 1.467 | 1.048 | 3.266 | 2.334 |
| **3** | 1.810 | 1.485 | 0.997 | 0.818 | 2.222 | 1.822 |
| **4** | 1.213 | 1.397 | 0.668 | 0.764 | 1.489 | 1.688 |
| **5** | 1.207 | 1.198 | 0.365 | 0.362 | 0.523 | 0.518 |
| **6** | 1.137 | 1.189 | 0.344 | 0.359 | 0.494 | 0.513 |

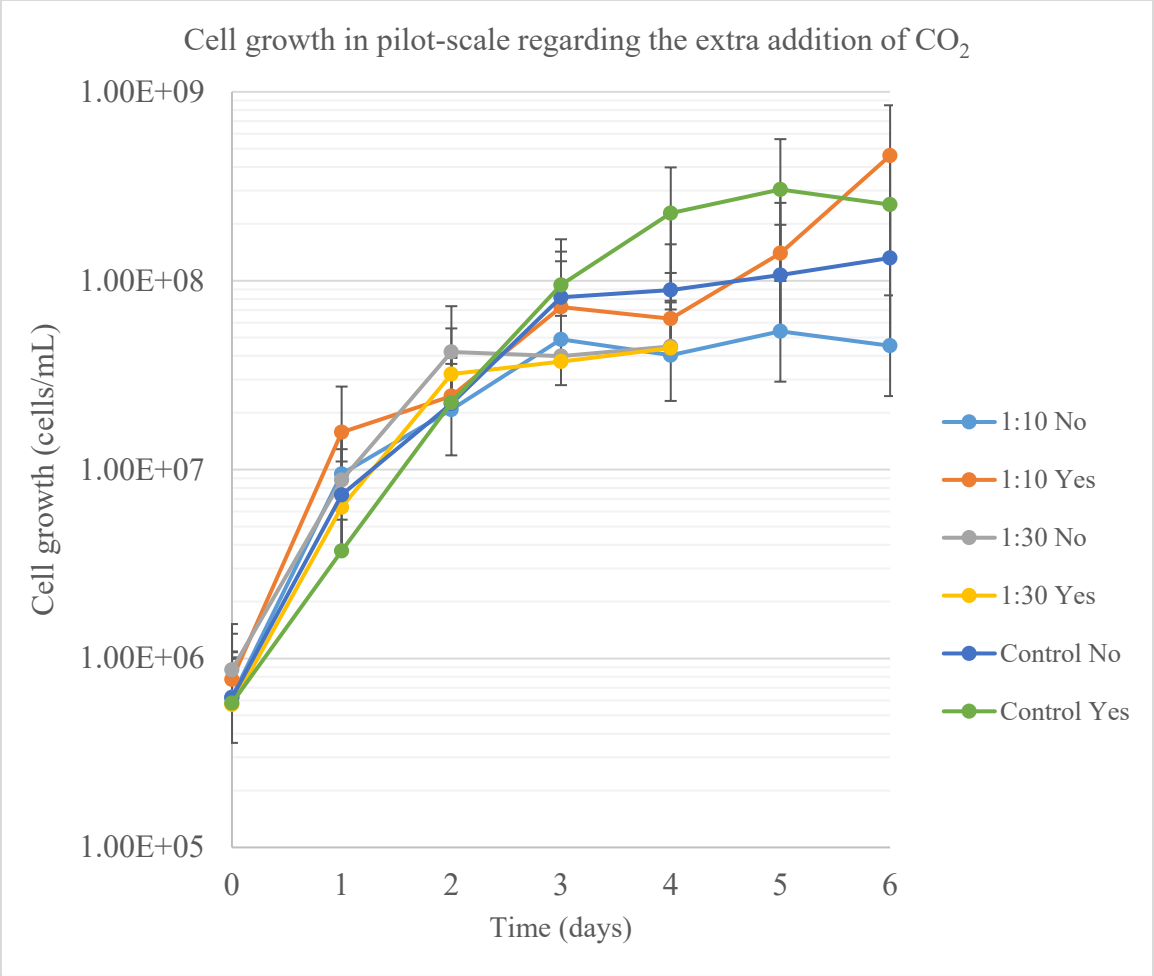



**Figure 17.** Cell density for the pilot-scale experiment when evaluating the significance on adding extra $CO_2$ using three different ratios.

**Table 20.** Least square means for cell density in pilot-scale when evaluating the significance on adding extra $CO_2$ using three different ratios (n = 12).

| | Estimate for the ratios with extra $CO_2$ | | | Standard Error for the ratios with extra $CO_2$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ratio | | | Lower | | | Upper | | |
| **Day** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** |
| **0** | 774598 | 570062 | 580938.4 | -97188 | -48406 | 223328 | 578084 | 425438 | 433555 |
| **1** | 1.6E+07 | 6324870 | 3707988 | 6960406 | -1E+06 | -2E+06 | 1.2E+07 | 4719154 | 2767276 |
| **2** | 2.4E+07 | 3.2E+07 | 22648405 | -2E+07 | 9670175 | 1.1E+07 | 1.8E+07 | 2.4E+07 | 1.7E+07 |
| **3** | 7.3E+07 | 3.7E+07 | 94925222 | 3.3E+07 | -4E+07 | 6.7E+07 | 5.4E+07 | 2.8E+07 | 7.1E+07 |
| **4** | 6.3E+07 | 4.4E+07 | 2.28E+08 | 1.8E+07 | -5E+07 | 2E+08 | 4.7E+07 | 3.3E+07 | 1.7E+08 |
| **5** | 1.4E+08 | | 3.04E+08 | 1.4E+08 | | 2.7E+08 | 1.2E+08 | | 2.6E+08 |
| **6** | 4.6E+08 | | 2.53E+08 | 4.6E+08 | | 2.3E+08 | 3.9E+08 | | 2.1E+08 |
| | **Estimate for the ratios without extra $CO_2$** | | | **Standard Error for the ratios with extra $CO_2$** | | | | | |
| | **Ratio** | | | **Lower** | | | **Upper** | | |
| **Day** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** |
| **0** | 624496 | 871787 | 618467.5 | -150102.542 | 301725 | 37529.2 | 466062.073 | 650615 | 461563 |
| **1** | 9486687 | 8802972 | 7348450 | -6276691.69 | 2478103 | 3640462 | 7079926.66 | 6569670 | 5484158 |
| **2** | 2.1E+07 | 4.2E+07 | 22360353 | -3711376.03 | 9957706 | -288052 | 15511218.7 | 3.1E+07 | 1.7E+07 |
| **3** | 4.9E+07 | 4E+07 | 81670221 | -23684150.2 | 2551381 | -1E+07 | 36493331.3 | 3E+07 | 6.1E+07 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **4** | 4E+07 | 4.5E+07 | 89263212 | -22615735.2 | 897837 | -1E+08 | 30106152.1 | 3.4E+07 | 6.7E+07 |
| **5** | 5.4E+07 | | 1.07E+08 | -85882552.6 | | -2E+08 | 45748190.9 | | 9.1E+07 |
| **6** | 4.5E+07 | | 1.32E+08 | -414292601 | | -1E+08 | 38369092.3 | | 1.1E+08 |

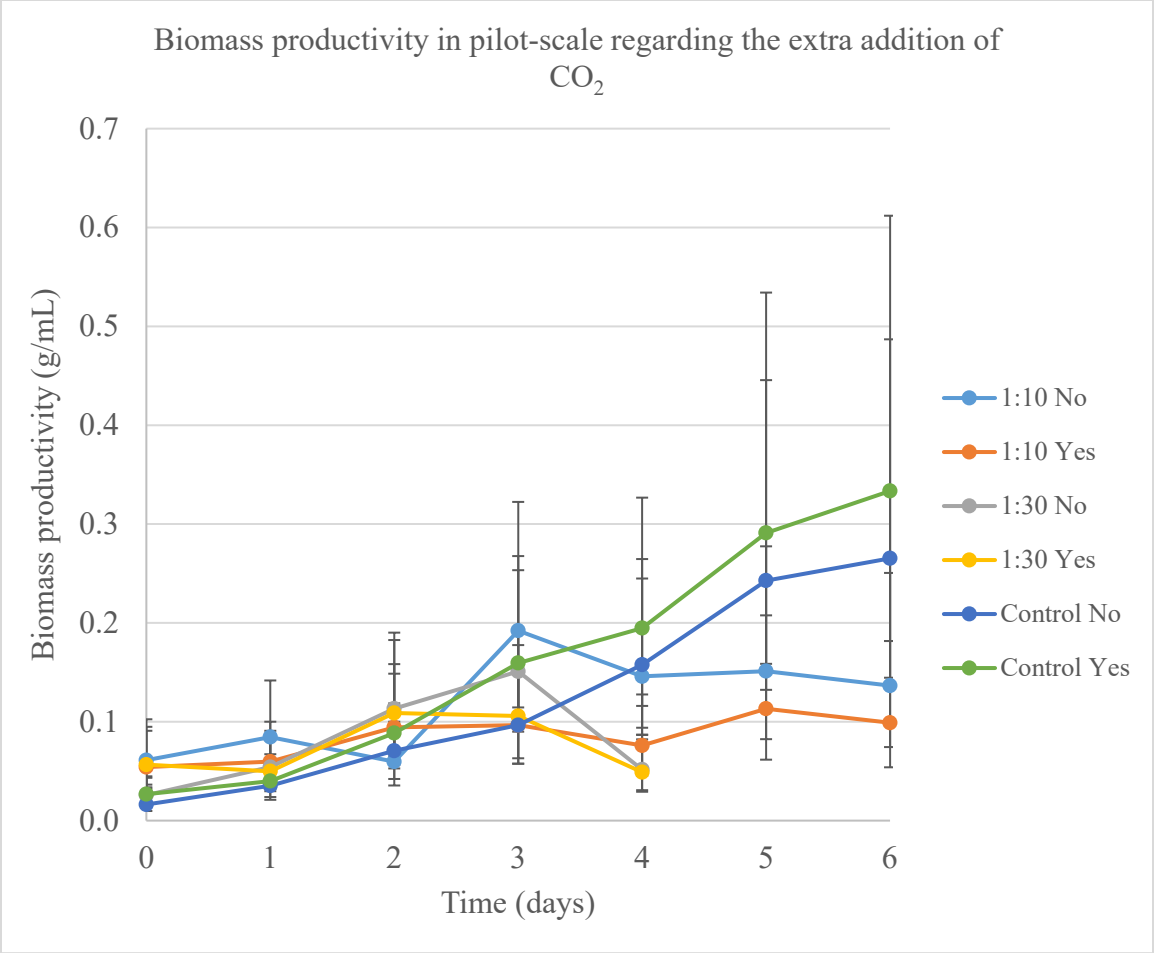



**Figure 18.** Biomass productivity for the pilot-scale experiment when evaluating the significance in the extra addition of $CO_2$ using three different ratios.

**Table 21.** Least square means for biomass productivity in pilot-scale when evaluating the significance in the extra addition of $CO_2$ using three different ratios (n = 12).

| | Estimate for the ratios with extra $CO_2$ | | | Standard Error for the ratios with extra $CO_2$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ratio | | | Lower | | | Upper | | |
| **Day** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** |
| **0** | 0.054 | 0.057 | 0.027 | 0.022 | 0.023 | 0.011 | 0.037 | 0.038 | 0.018 |
| **1** | 0.060 | 0.050 | 0.040 | 0.024 | 0.020 | 0.016 | 0.040 | 0.034 | 0.027 |
| **2** | 0.094 | 0.109 | 0.089 | 0.038 | 0.044 | 0.036 | 0.064 | 0.074 | 0.060 |
| **3** | 0.097 | 0.106 | 0.159 | 0.039 | 0.043 | 0.064 | 0.066 | 0.072 | 0.108 |
| **4** | 0.076 | 0.049 | 0.195 | 0.031 | 0.020 | 0.079 | 0.052 | 0.033 | 0.132 |
| **5** | 0.113 | | 0.291 | 0.051 | | 0.132 | 0.095 | | 0.243 |
| **6** | 0.099 | | 0.333 | 0.045 | | 0.152 | 0.083 | | 0.279 |
| | **Estimate for the ratios without extra $CO_2$** | | | **Standard Error for the ratios with extra $CO_2$** | | | | | |
| | Ratio | | | Lower | | | Upper | | |
| **Day** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** |
| **0** | 0.061 | 0.026 | 0.016 | 0.025 | 0.010 | 0.007 | 0.041 | 0.018 | 0.011 |
| **1** | 0.084 | 0.054 | 0.035 | 0.034 | 0.022 | 0.014 | 0.057 | 0.037 | 0.024 |
| **2** | 0.060 | 0.113 | 0.071 | 0.024 | 0.046 | 0.029 | 0.040 | 0.077 | 0.048 |
| **3** | 0.192 | 0.151 | 0.097 | 0.078 | 0.061 | 0.039 | 0.130 | 0.102 | 0.066 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **4** | 0.146 | 0.052 | 0.158 | 0.059 | 0.021 | 0.064 | 0.099 | 0.035 | 0.107 |
| **5** | 0.151 | | 0.243 | 0.069 | | 0.111 | 0.126 | | 0.203 |
| **6** | 0.137 | | 0.265 | 0.062 | | 0.121 | 0.114 | | 0.222 |

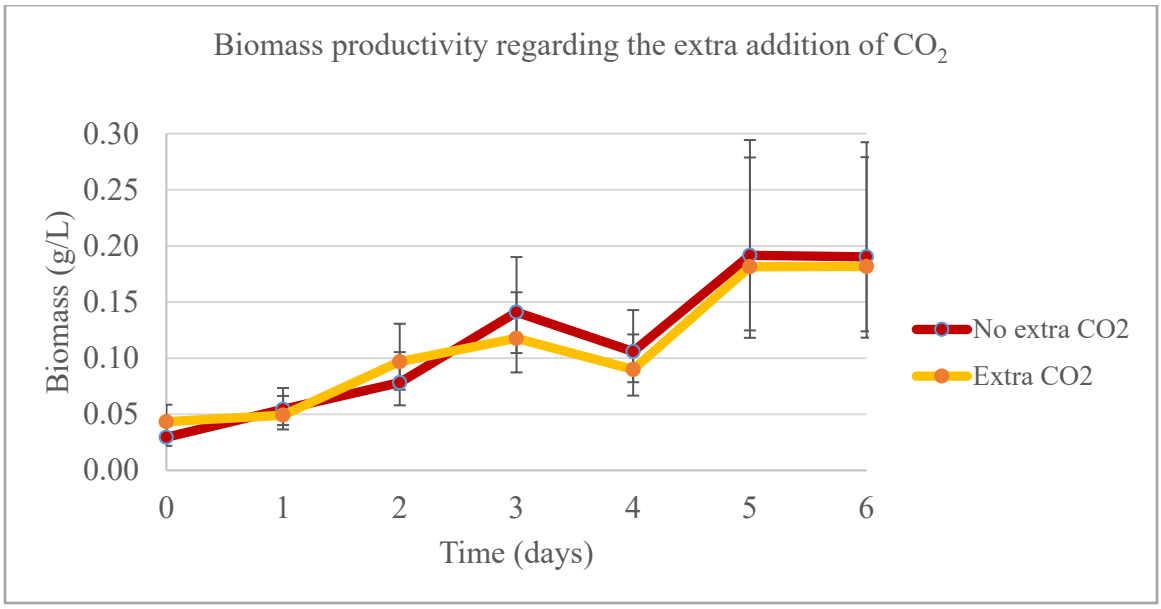



**Figure 19.** Biomass productivity for the pilot-scale experiment when evaluating the significance of adding extra $CO_2$.

**Table 22.** Least square means for biomass productivity in pilot-scale when evaluating the significance of adding extra $CO_2$ (n = 12).

| | Estimate | | Standard Error | | | |
|---|---|---|---|---|---|---|
| | Ratio | | Lower | | Upper | |
| **Day** | **No** | **Yes** | **No** | **Yes** | **No** | **Yes** |
| **0** | 0.030 | 0.043 | 0.008 | 0.011 | 0.010 | 0.015 |
| **1** | 0.054 | 0.049 | 0.014 | 0.013 | 0.019 | 0.017 |
| **2** | 0.078 | 0.097 | 0.020 | 0.025 | 0.027 | 0.034 |
| **3** | 0.141 | 0.118 | 0.036 | 0.030 | 0.049 | 0.041 |
| **4** | 0.106 | 0.090 | 0.027 | 0.023 | 0.037 | 0.031 |
| **5** | 0.192 | 0.181 | 0.067 | 0.063 | 0.103 | 0.097 |
| **6** | 0.190 | 0.182 | 0.066 | 0.063 | 0.102 | 0.097 |

**Table 23.** Biomass weight when the entire bag with microalgae was harvested in the last day of experiment for each of the ratios and controls.

| **Biomass weight when extra $CO_2$ was added** | | | |
|---|---|---|---|
| **Treatment** | **Wet weight (g)** | **Dry weight (g)** | **Dry mass (%)** |
| **Control** | 341.13 | 42.31 | 12.40 |
| **1:10** | 119.58 | 10.60 | 8.86 |
| **1:30** | 39.40 | 5.07 | 12.87 |
| **Biomass weight when no extra $CO_2$ was added** | | | |
| **Treatment** | **Wet weight (g)** | **Dry weight (g)** | **Dry mass (%)** |
| **Control** | 109.68 | 12.85 | 11.71 |
| **1:10** | 136.37 | 10.07 | 7.38 |
| **1:30** | 20.36 | 2.93 | 14.39 |

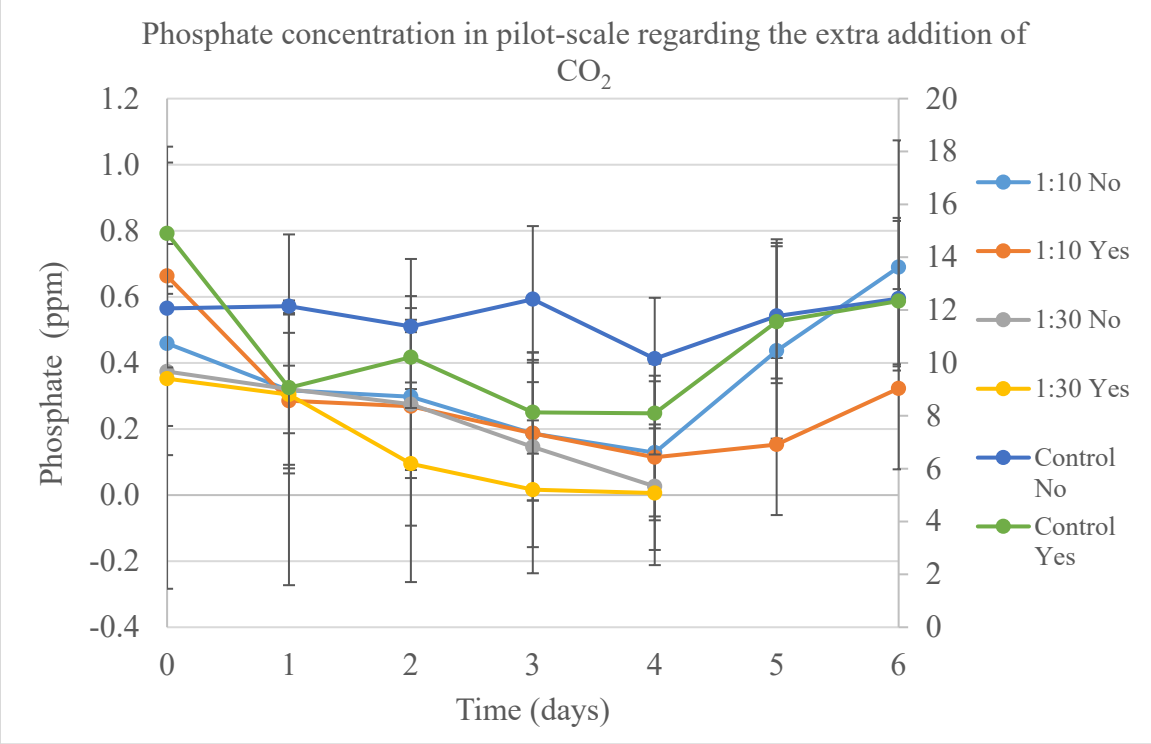



**Figure 20.** Phosphate concentration for the pilot-scale experiment when evaluating the significance in the extra addition of $CO_2$ using three different ratios. The primary axis (on the left) correspond to the results of the ratios, and the secondary axis (on the right) correspond to the results for the controls.

**Table 24.** Least square means phosphate concentration in pilot-scale when evaluating the significance in the extra addition of $CO_2$ using three different ratios (n = 12).

| | Estimate for the ratios with extra $CO_2$ | | | Standard Error for the ratios with extra $CO_2$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ratio | | | Lower | | | Upper | | |
| Day | 1:10 | 1:30 | Control | 1:10 | 1:30 | Control | 1:10 | 1:30 | Control |
| 0 | 0.663 | 0.352 | 14.900 | 0.285 | 0.231 | 2.720 | 0.343 | 0.279 | 3.283 |
| 1 | 0.286 | 0.304 | 9.062 | 0.220 | 0.223 | 1.722 | 0.265 | 0.269 | 2.076 |
| 2 | 0.269 | 0.095 | 10.214 | 0.217 | 0.187 | 1.919 | 0.262 | 0.226 | 2.315 |
| 3 | 0.187 | 0.016 | 8.130 | 0.203 | 0.174 | 1.563 | 0.245 | 0.210 | 1.885 |
| 4 | 0.114 | 0.006 | 8.093 | 0.191 | 0.172 | 1.556 | 0.230 | 0.208 | 1.876 |
| 5 | 0.153 | | 11.560 | 0.213 | | 2.325 | 0.262 | | 2.855 |
| 6 | 0.323 | | 12.342 | 0.245 | | 2.471 | 0.301 | | 3.031 |
| | Estimate for the ratios without extra $CO_2$ | | | Standard Error for the ratios with extra $CO_2$ | | | | | |
| | Ratio | | | Lower | | | Upper | | |
| Day | 1:10 | 1:30 | Control | 1:10 | 1:30 | Control | 1:10 | 1:30 | Control |
| 0 | 0.459 | 0.374 | 12.065 | 0.250 | 0.235 | 2.236 | 0.301 | 0.284 | 2.697 |
| 1 | 0.317 | 0.320 | 12.147 | 0.225 | 0.226 | 2.250 | 0.272 | 0.273 | 2.714 |
| 2 | 0.298 | 0.275 | 11.378 | 0.222 | 0.218 | 2.117 | 0.268 | 0.263 | 2.556 |
| 3 | 0.186 | 0.146 | 12.410 | 0.203 | 0.196 | 2.295 | 0.245 | 0.237 | 2.767 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **4** | 0.128 | 0.026 | 10.160 | 0.193 | 0.176 | 1.910 | 0.233 | 0.212 | 2.304 |
| **5** | 0.437 | | 11.775 | 0.266 | | 2.365 | 0.327 | | 2.904 |
| **6** | 0.690 | | 12.434 | 0.313 | | 2.487 | 0.384 | | 3.053 |

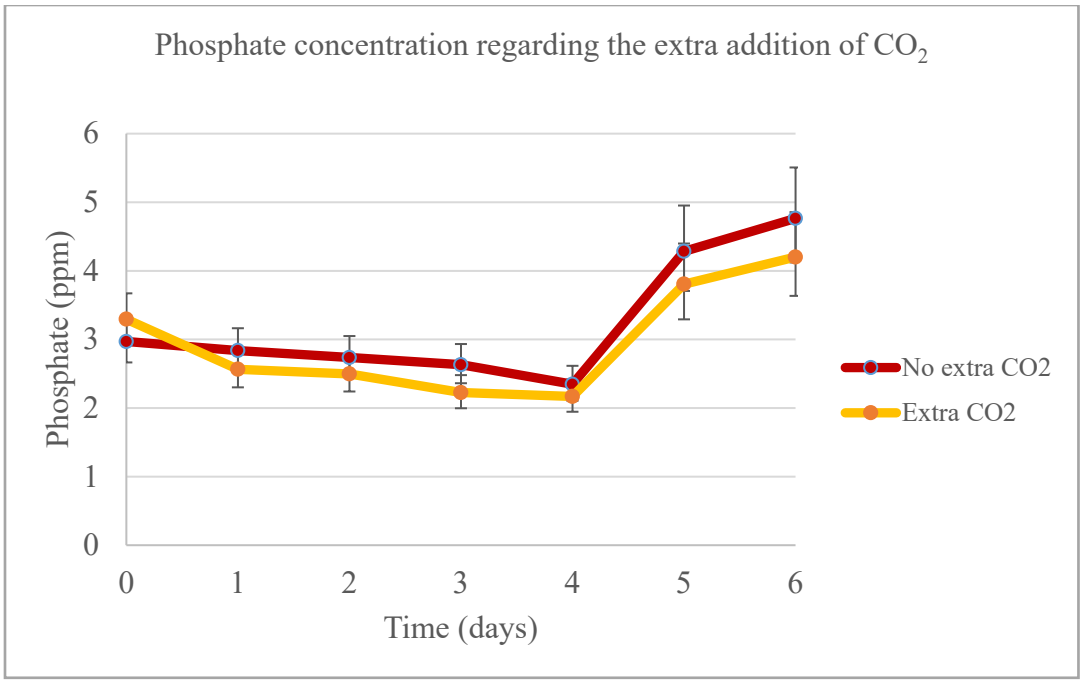



**Figure 21.** Phosphate concentration for the pilot-scale experiment when evaluating the significance on adding extra of $CO_2$.

**Table 25.** Least square means phosphate concentration in pilot-scale when evaluating the significance on adding extra of $CO_2$ (n = 12).

| | Estimate | | Standard Error | | | |
|---|---|---|---|---|---|---|
| | Ratio | | Lower | | Upper | |
| Day | No | Yes | No | Yes | No | Yes |
| **0** | 2.970 | 3.295 | 0.305 | 0.338 | 0.340 | 0.377 |
| **1** | 2.838 | 2.565 | 0.291 | 0.263 | 0.325 | 0.294 |
| **2** | 2.736 | 2.498 | 0.281 | 0.257 | 0.313 | 0.286 |
| **3** | 2.632 | 2.225 | 0.270 | 0.229 | 0.301 | 0.255 |
| **4** | 2.347 | 2.168 | 0.241 | 0.223 | 0.268 | 0.248 |
| **5** | 4.284 | 3.806 | 0.578 | 0.513 | 0.668 | 0.593 |
| **6** | 4.765 | 4.201 | 0.642 | 0.566 | 0.742 | 0.654 |

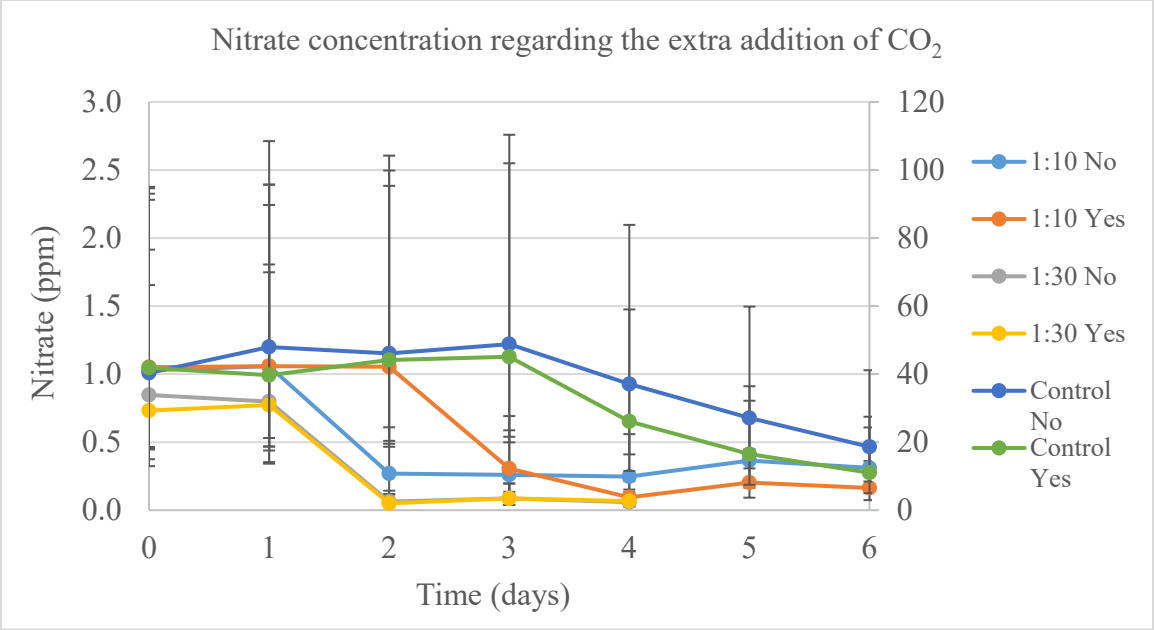



**Figure 22.** Nitrate concentration for the pilot-scale experiment when evaluating the significance of the extra addition of $CO_2$ using three different ratios. The primary axis (on the left) correspond to the results of the ratios, and the secondary axis (on the right) correspond to the results for the controls.

**Table 26.** Least square means for nitrate concentration in pilot-scale when evaluating the significance in the extra addition of $CO_2$ using three different ratios (n = 12).

| | Estimate for the ratios with extra $CO_2$ | | | Standard Error for the ratios with extra $CO_2$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ratio | | | Lower | | | Upper | | |
| **Day** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** |
| **0** | 1.051 | 0.732 | 41.863 | 0.586 | 0.408 | 23.348 | 1.325 | 0.923 | 52.788 |
| **1** | 1.058 | 0.773 | 39.678 | 0.590 | 0.431 | 22.129 | 1.335 | 0.975 | 50.033 |
| **2** | 1.054 | 0.051 | 44.168 | 0.588 | 0.028 | 24.633 | 1.329 | 0.064 | 55.695 |
| **3** | 0.306 | 0.087 | 45.105 | 0.171 | 0.048 | 25.156 | 0.386 | 0.109 | 56.877 |
| **4** | 0.095 | 0.067 | 26.104 | 0.053 | 0.037 | 14.559 | 0.119 | 0.085 | 32.917 |
| **5** | 0.203 | | 16.517 | 0.111 | | 9.034 | 0.245 | | 19.935 |
| **6** | 0.164 | | 11.025 | 0.090 | | 6.030 | 0.198 | | 13.309 |
| | **Estimate for the ratios without extra $CO_2$** | | | **Standard Error for the ratios with extra $CO_2$** | | | | | |
| | **Ratio** | | | **Lower** | | | **Upper** | | |
| **Day** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** |
| **0** | 1.029 | 0.847 | 40.358 | 0.574 | 0.472 | 22.508 | 1.297 | 1.068 | 50.891 |
| **1** | 1.058 | 0.799 | 47.986 | 0.590 | 0.445 | 26.762 | 1.334 | 1.007 | 60.509 |
| **2** | 0.270 | 0.063 | 46.099 | 0.150 | 0.035 | 25.708 | 0.340 | 0.080 | 58.131 |
| **3** | 0.260 | 0.087 | 48.813 | 0.145 | 0.049 | 27.224 | 0.328 | 0.110 | 61.553 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **4** | 0.247 | 0.059 | 37.096 | 0.138 | 0.033 | 20.687 | 0.312 | 0.074 | 46.777 |
| **5** | 0.364 | | 27.123 | 0.199 | | 14.835 | 0.440 | | 32.736 |
| **6** | 0.311 | | 18.644 | 0.170 | | 10.197 | 0.376 | | 22.505 |

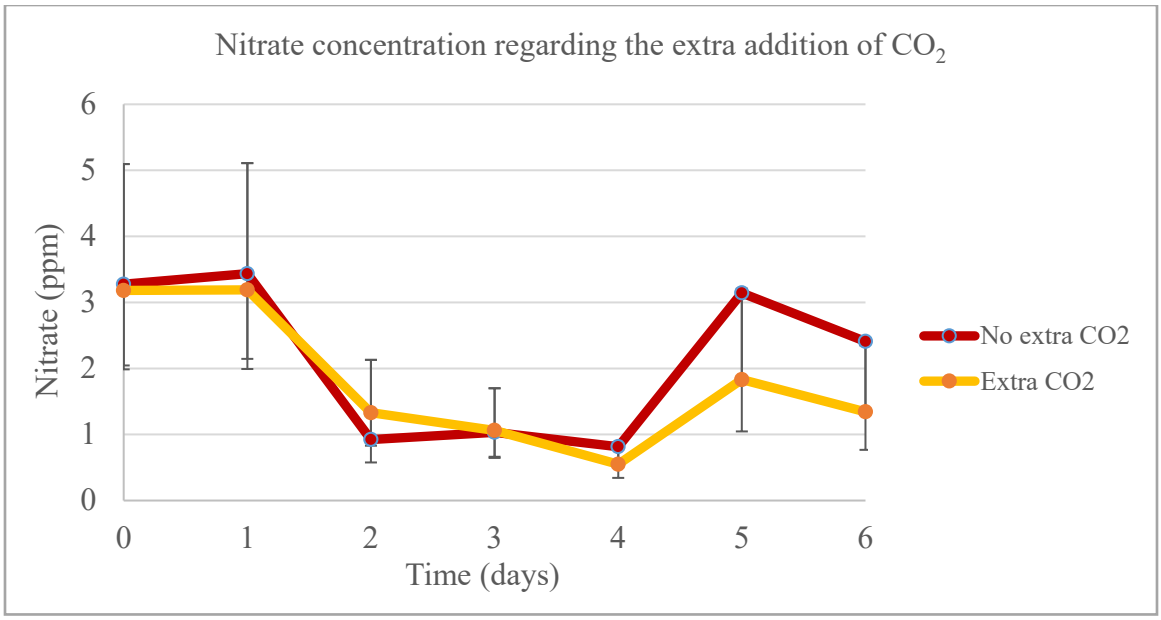



**Figure 23.** Nitrate concentration for the pilot-scale experiment when evaluating the significance of adding extra $CO_2$.

**Table 27.** Least square means for nitrate concentration in pilot-scale when evaluating the significance of adding extra $CO_2$ (n = 12).

| | Estimate | | Standard Error | | | |
|---|---|---|---|---|---|---|
| | Ratio | | Lower | | Upper | |
| Day | No | Yes | No | Yes | No | Yes |
| **0** | 3.276 | 3.181 | 1.231 | 1.195 | 1.819 | 1.914 |
| **1** | 3.436 | 3.190 | 1.291 | 1.198 | 1.674 | 1.919 |
| **2** | 0.924 | 1.330 | 0.347 | 0.500 | 1.207 | 0.800 |
| **3** | 1.034 | 1.062 | 0.388 | 0.399 | 0.667 | 0.639 |
| **4** | 0.815 | 0.549 | 0.306 | 0.206 | 0.065 | 0.331 |
| **5** | 3.144 | 1.832 | 1.348 | 0.785 | 0.063 | 1.375 |
| **6** | 2.409 | 1.343 | 1.033 | 0.576 | -0.058 | 1.008 |

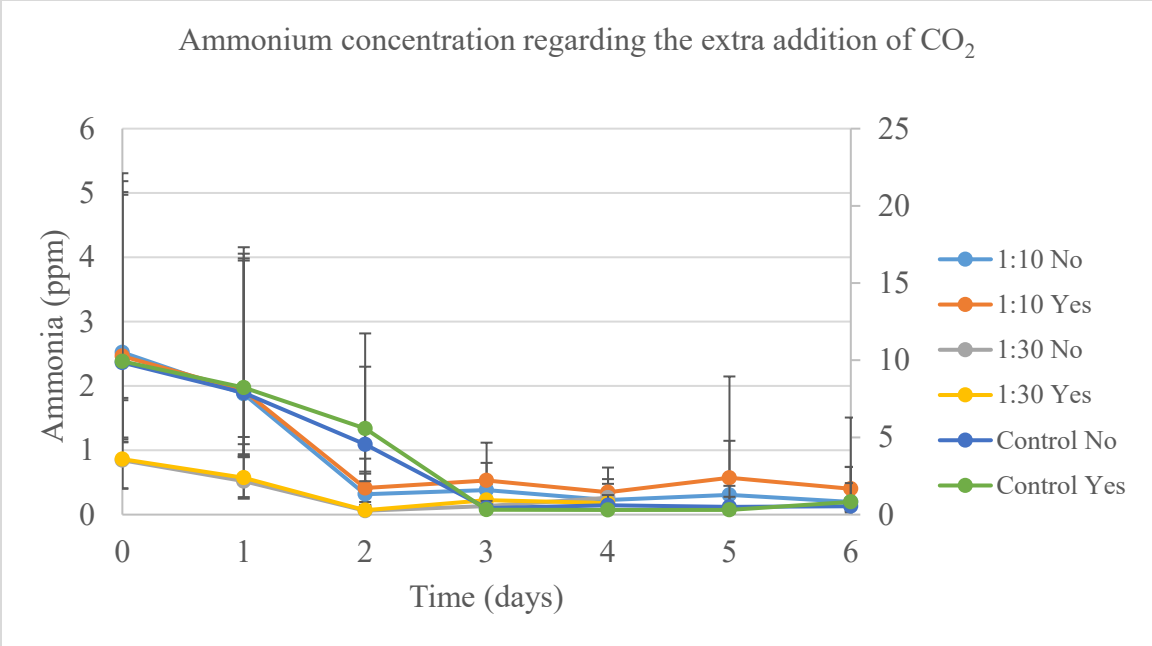



**Figure 24.** Ammonium concentration for the pilot-scale experiment when evaluating the significance in the extra addition of $CO_2$ using three different ratios. The primary axis (on the left) correspond to the results of the ratios, and the secondary axis (on the right) correspond to the results for the controls.

**Table 28.** Least square means for ammonium concentration in pilot-scale when evaluating the significance in the extra addition of $CO_2$ using three different ratios (n = 12).

| | Estimate for the ratios with extra $CO_2$ | | | Standard Error for the ratios with extra $CO_2$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ratio | | | Lower | | | Upper | | |
| **Day** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** |
| **0** | 2.464 | 0.862 | 9.929 | 1.293 | 0.452 | 5.210 | 2.720 | 0.952 | 10.961 |
| **1** | 1.928 | 0.574 | 8.232 | 1.012 | 0.301 | 4.320 | 2.129 | 0.633 | 9.087 |
| **2** | 0.413 | 0.068 | 5.578 | 0.217 | 0.036 | 2.927 | 0.456 | 0.075 | 6.159 |
| **3** | 0.531 | 0.225 | 0.316 | 0.279 | 0.118 | 0.166 | 0.587 | 0.249 | 0.349 |
| **4** | 0.348 | 0.180 | 0.301 | 0.182 | 0.094 | 0.158 | 0.384 | 0.198 | 0.332 |
| **5** | 0.572 | | 0.307 | 0.420 | | 0.225 | 1.575 | | 0.846 |
| **6** | 0.402 | | 0.825 | 0.295 | | 0.605 | 1.107 | | 2.270 |
| | **Estimate for the ratios without extra $CO_2$** | | | **Standard Error for the ratios with extra $CO_2$** | | | | | |
| | **Ratio** | | | **Lower** | | | **Upper** | | |
| **Day** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** | **1:10** | **1:30** | **Control** |
| **0** | 2.522 | 0.845 | 9.846 | 1.324 | 0.444 | 5.166 | 2.784 | 0.933 | 10.870 |
| **1** | 1.877 | 0.520 | 7.888 | 0.985 | 0.273 | 4.139 | 2.073 | 0.574 | 8.709 |
| **2** | 0.318 | 0.061 | 4.553 | 0.167 | 0.032 | 2.389 | 0.351 | 0.067 | 5.027 |
| **3** | 0.382 | 0.132 | 0.422 | 0.201 | 0.069 | 0.221 | 0.422 | 0.146 | 0.466 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **4** | 0.227 | 0.262 | 0.602 | 0.119 | 0.138 | 0.316 | 0.251 | 0.290 | 0.665 |
| **5** | 0.306 | | 0.497 | 0.224 | | 0.364 | 0.842 | | 1.367 |
| **6** | 0.197 | | 0.544 | 0.145 | | 0.399 | 0.542 | | 1.497 |

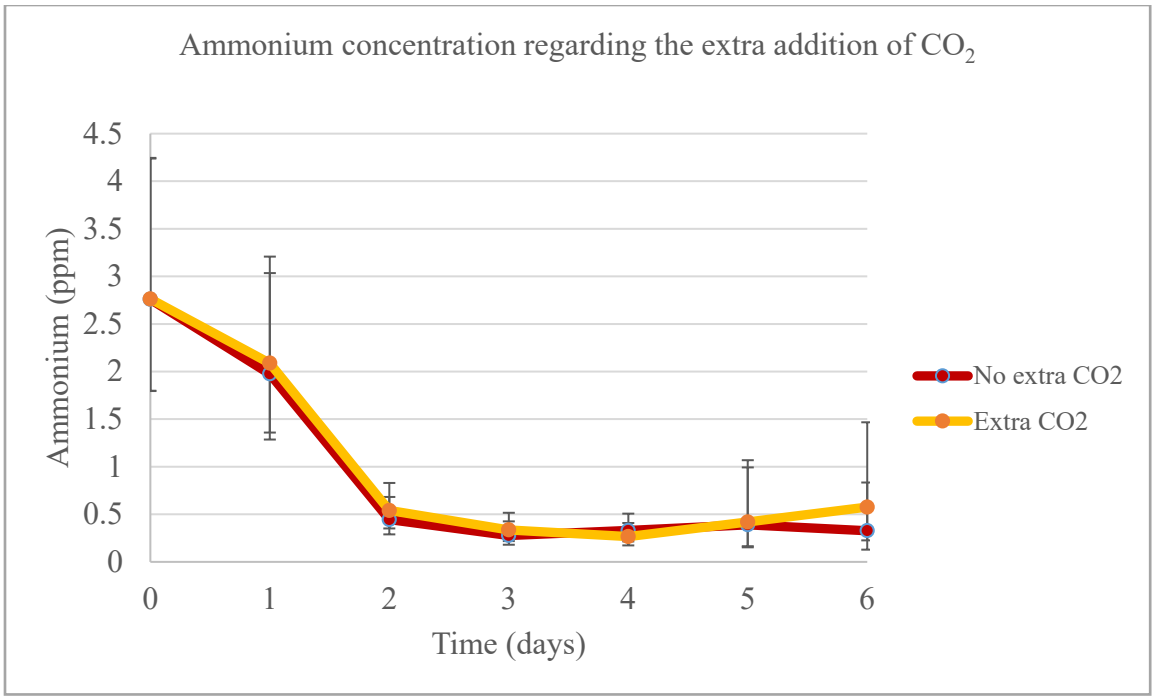



**Figure 25.** Ammonium concentration for the pilot-scale experiment when evaluating the significance of adding extra $CO_2$.

**Table 29.** Least square means for ammonium concentration in pilot-scale when evaluating the significance of adding extra $CO_2$ (n = 12).

| | Estimate | | Standard Error | | | |
|---|---|---|---|---|---|---|
| | Ratio | | Lower | | Upper | |
| Day | No | Yes | No | Yes | No | Yes |
| 0 | 2.759 | 2.763 | 0.963 | 0.965 | 1.480 | 1.482 |
| 1 | 1.975 | 2.088 | 0.689 | 0.729 | 1.060 | 1.120 |
| 2 | 0.444 | 0.540 | 0.155 | 0.188 | 0.238 | 0.290 |
| 3 | 0.277 | 0.336 | 0.097 | 0.117 | 0.149 | 0.180 |
| 4 | 0.330 | 0.266 | 0.115 | 0.093 | 0.177 | 0.143 |
| 5 | 0.390 | 0.419 | 0.237 | 0.255 | 0.603 | 0.649 |
| 6 | 0.327 | 0.576 | 0.199 | 0.350 | 0.507 | 0.891 |

**Table 30**. Biochemical analysis of *Chlorella sp.* biomass produced using dairy effluent.

| Analysis | Dry Weight (DW) | Units | Method |
|---|---|---|---|
| **Protein** | 49.2 | % | AOAC 990.03 |
| **Fat** | 2.32 | % | AOAC 2003.05 |
| **Carbohydrates** | 38.5 | % | Calculated |
| **Fiber** | 6.6 | % | AOCS Ba 6a-05 |
| **Neutral Detergent Fiber** | 32.8 | % | Ankom Technology/AOAC 2001.11 |
| **Acid Detergent Fiber** | 11.6 | % | ANKOM Tech. Method |
| **Ash** | 9.91 | % | AOAC 942.05 |
| **Total digestible nutrients** | 71.6 | % | Calculated |
| **Digestible energy** | 1.43 | % | Calculated |
| **Metabolizable energy** | 1.23 | % | Calculated |
| **Calcium** | 1.17 | % | AOAC 985.01 (mod) |
| **Phosphorus** | 1.70 | % | AOAC 985.01 (mod) |
| **Potassium** | 1.12 | % | AOAC 985.01 (mod) |
| **Sodium** | 0.48 | % | AOAC 985.01 (mod) |
| **Iron** | 5020 | ppm | AOAC 985.01 (mod) |
| **Manganese** | 157 | ppm | AOAC 985.01 (mod) |
| **Zinc** | 46 | ppm | AOAC 985.01 (mod) |

**Calf Algae Feeding**

The microalgae used in this study was isolated from the dairy wastewater lagoon at the University of Minnesota West Central Research and Outreach Center in Morris, Minnesota. The microalgae biomass was produced using hanging bags bioreactor with *Chlorella sp*. to recycle the wastewater produced at the WCROC. The bioreactor's structure was made using treated wood (height = 1.73m, length = 2.06m and width = 1.22m). Three pairs of flexible metal bars (diameter = 5mm, height = 1.34m), 0.03m from each other were installed to support the hanging bags shape. Plastic tubes (diameter = 15mm, height = 1.33m, length = 0.52m) were used to blow oxygen into the bags and add carbon dioxide when needed. The bottom of each plastic tube were drilled to open twelve holes (diameter = 0.5mm) for aeration.

The biomass was produced using 1:10 wastewater and water ratio in order to recycle nutrients from the dairy wastewater lagoon. Using an autoclave the *Chlorella sp*. biomass was sterilized under 121°C and at a pressure of 15 psi, for 30 minutes, in order not to be harmful to the calves involved in this study. After the sterilization, the biomass was kept frozen.

No mycotoxins were found in the biomass, including Aflatoxin (B1, B2, G1 and G2), DON (Vomitoxin), Fumonisin (B1, B2 and B3), Ochratoxin, T-2 toxin, and Zearalenone. Additionally, a heavy metals screening was conducted, and the levels of arsenic, mercury, cadmium, lead, and antimony, were below the maximum content recommended for animal feeding. Moreover, a detailed screening presenting the biochemical biomass content can be found in table 1 conducted at the Midwest Laboratories®, Omaha - Nebraska.

All animal care and management for this specific study was approved by the University of Minnesota Institutional Animal Care and Use Committee (Animal Subjects Code number 1709-35098A). Six calves ranging in age from 12 to 14 weeks (106.71 ± 3.81 kg) old were enrolled in the taste preference study. The calves were used to test their preference for control, 30g/d, or 60g/day of *Chlorella sp.* produced *in situ* using dairy wastewater. In this study, 2mm grains of *Chlorella sp.* were added to calf organic starter grain and hand mixed before feeding the calves.

The experiment was seven days (7d) long. Day one and day two were for adaptation (segment 1) and day three and day four were for data collection (segment 2). During the last three days (segment 3) of the experiment, the primarily consumed treatment was removed, and instead of feeding, the bucket was empty. The last three days were used to determine the second preferred treatment.

All six calves participated in the study at the same time. Calves were housed individually in hutches (2.12m x 1.14m x 1.22m) with outdoor access (17.98$m^2$) under solar panels (approximately 3m out of the ground), with free-choice water. Pinewood shavings were used as bedding inside and outside of the hutches.

Every morning, starting at 0800 h. the feed and orts were weighed using a hanging scale, and recorded. Five buckets (diameter = 28cm, height = 21cm) were placed outside of the hutches. The two buckets at the edges were used to avoid border effects, and the three buckets in the middle received 2.3 kg of feeding grain each. The treatments in each bucket were randomized during the whole experiment. The water bucket was allocated inside the hutch. The calves had five days to adapt to the new surrounds prior the beginning of this study, and they were fed as usual (1.16 kg/d) during this period.

Table 1 shows the nutritional difference between the three different calf starters. The analysis was conducted by the Rock River Laboratory. INC – Watertown, Wisconsin. The crude protein (CP) increased with the addition of *Chlorella sp.* in both treatments that contained the microalgae as a supplement. An increase up to 10.1% on CP content was found when 60g of *Chlorella sp.* powder was added to 2300g of

grain starter. A decrease in the fat content was found when 30g of the microalgae was added to the control, and the starch content decreased in both treatments with *Chlorella sp.* in relation to the control. Furthermore, there was a gradual increase in the mineral content when comparing the control with the treatments containing *Chlorella sp.*

Kendall's coefficient of concordance, W, was calculated to rank the consumption of the treatments from most to least preferred (Nombekela, et al., 1994) using JMP statistical (SAS Institute Inc.). Pairwise comparisons and Tukey adjustment were applied to evaluate the difference between the treatments total intake.

The total dry matter (DM) for the adaptation time (day one and two) for the different treatments is presented on Table 2. For this time period, the control was greatly consumed by the calves, followed by the control with 30g of *Chlorella sp.* and then the treatment with the highest content of microalgae, 60g.

The total dry matter intake (DMI) is in Table 3 for each one of the segments of the study. During the first segment the calves averaged 3.52 ± 1.38 kg of DM/d, 3.88 ± 1.31 kg of DM/d during the second segment, and 3.33 ± 1.87 kg of DM/d in the third segment. Neither for the segment two nor for segment three was the consumption different ($P > 0.05$).

Table 4 has DMI for three and four. The control treatment showed to be the first option on half of the time, being chosen six times out of twelve. The treatment where 30g of microalgae was added to the control was second and the 60g was third. Kendall's coefficient of concordance ($W= 0.12$) between control and 30g, and $W=0.25$ between control and 60g designated disagreement in preference rankings among the heifers in this study.

When ranking the data for the full experiment (table 5), the control was the most preferred treatment. However, the results show that the control was the most preferred treatment for the calves that had the control plus 30g of microalgae as their first choice during the data collection time. Furthermore, the inverse is also true, since the control was the least preferred for the calves that had it as the first choice. Therefore, it was the treatment less consumed because they did not have control as a choice during the last three days of the experiment.

The control was preferred (1.36 times) by the calves the entire experimental time (7 days), but there was no significant difference between control and the other two treatments ($P > 0.05$). However, the difference between the control and the control with 30g of microalgae is not significant ($P > 0.05$). Furthermore, table 7, shows the DMI per calf, indicating that the control was the first choice treatment for only two out of six calves participating in the study.

Previous studies with kelp (Erickson, P. S., et al, 2011; Heins, B. J. & Chester-Jones, H., 2015) showed that calves mainly preferred being fed without the addition of kelp to their grain starter. These results match the results presented in this study when considering total grain consumption. However, the studies with kelp showed that the calves involved almost rejected the treatments with kelp. In this study, most of the calves had a treatment with *Chlorella sp*. as their first choice. Moreover, during the third segment, when the greater consumed treatment was removed, the DMI did not have much fluctuation, where the overall consumption per day was 3.33kg (table 3).

The microalgae cellulosic cell wall is rigid and difficult to be digested, that is the main reason for the difficulty on substituting part or all the animal feeding for algal feeding (Kotrbacek, V. et al., 2015). Improvement on the digestibility was found when a mixture of *Chlorella* and *Scenedesmu sp.* was added to the milk fed to calves at roughly 10% of their body weights when compared to sesame seed oil (Chowdhury, S.A., et al., 1995).

Increasing protein on cattle feeding has a high economic cost, and it is also challenging to do it without decreasing or changing the amounts of minerals in the feeding. However, introducing dry feeding earlier (around three days of age) has been a practice well researched, and has demonstrated many benefits to the heifers. Considering the calves receptiveness to the taste of the *Chlorella sp*. biomass produced in

this study, it is an interesting alternative to be tested in further studies where body changes (weight, size, and other body aspects) could be measured. Moreover, the addition of 60g of microalgae biomass in this study resulted in an increase of 10.1% in the protein content of the starter grain, suggesting that small amounts of *Chlorella sp*. would increase the CP rapidly in the feeding. The production of this microalgae is also an alternative for producers that want to treat dairy effluent and produce high protein feeding (the biomass produced during this study contained 49.2% CP, table 1).

Previous studies with kelp showed adverse taste acceptance by calves and cows, indicating that even if the kelp has high nutrition values, the animals will not eat it because of the taste. However, the results presented in this study indicate that *Chlorella sp.* may be well accepted by calves when used as a supplement in their grain starter, providing a high protein, vitamins, minerals and less fat content when compared to other supplements. There was no difference in any of the segments of this study or the entire period of the study, indicating that the calves would eat any of the treatments without having a taste preference. Furthermore, this study shows that the *Chlorella sp*. biomass could be produced *in situ*, recycling nutrients from the effluent produced during the milking cycle, and help producers to decrease supplement feeding costs.

Table 1. Nutrient composition in different treatment feeds offered to the calves.

| Nutrient (% of DM) | *Chlorella sp.* | Control | 30g of algae | 60g of algae |
|---|---|---|---|---|
| **Crude protein** | 49.2 | 18.60 | 18.95 | 20.48 |
| **Fat** | 2.32 | 6.88 | 6.39 | 6.96 |
| **Starch** | 38.5 | 47.10 | 43.36 | 40.49 |
| **Neutral detergent fiber** | 32.8 | 12.52 | 14.59 | 12.86 |
| **Acid detergent fiber** | 11.6 | 7.67 | 5.51 | 5.21 |
| **Ash** | 9.91 | 7.55 | 6.63 | 8.46 |
| **Calcium** | 1.17 | 0.92 | 0.90 | 1.15 |
| **Phosphorus** | 1.70 | 0.47 | 0.55 | 0.59 |
| **Potassium** | 1.12 | 0.78 | 0.80 | 0.87 |
| **Magnesium** | 157 | 0.18 | 0.18 | 0.21 |
| **Total digestible nutrients** | 71.6 | - | - | - |
| **Digestible energy** | 1.43 | - | - | - |
| **Metabolizable energy** | 1.23 | - | - | - |
| **Sodium** | 0.48 | - | - | - |
| **Iron** | 5020 | - | - | - |
| **Zinc** | 46 | - | - | - |

**Table 2. Results dry matter intake for day one and two for the calf starters offered to calves.**

| | Treatment | | |
|---|---|---|---|
| **Heifer** | Control | 30 g of algae | 60 g of algae |
| **1** | 2.54 | 3.86 | 1.45 |
| **2** | 3.36 | 0.91 | 1.13 |
| **3** | 2.09 | 2.27 | 1.63 |
| **4** | 4.24 | 2.22 | 0.82 |
| **5** | 3.72 | 2.86 | 2.49 |
| **6** | 4.35 | 2.43 | 1.84 |
| **Total** | 20.30 | 14.54 | 9.37 |
| **Mean** | 3.38 | 2.42 | 1.56 |

**Table 3. Average DMI in kg/d during the different segments of the experiment.**

| | | Heifer | | | | | | DMI/d |
|---|---|---|---|---|---|---|---|---|
| **Segment** | Day | 1 | 2 | 3 | 4 | 5 | 6 | |
| **1** | 1 to 2 | 7.50 | 5.19 | 5.72 | 6.96 | 8.67 | 8.24 | 3.52 |
| **2** | 3 to 4 | 7.76 | 6.72 | 6.50 | 7.78 | 7.63 | 10.19 | 3.88 |
| **3** | 5 to 7 | 11.10 | 11.14 | 7.20 | 8.06 | 11.53 | 10.97 | 3.33 |
| **Mean[1]** | | 8.79 | 7.68 | 6.47 | 7.60 | 9.28 | 9.80 | |

**[1]Average consumption over all treatments in the three different segments**

**Table 4. Number of days that the treatment was chosen as first choice by the heifers during the collection data period, days three and four.**

| | Treatment | | |
|---|---|---|---|
| **Heifer** | Control | 30g of algae | 60g of algae |
| **1** | 1 | 0 | 1 |
| **2** | 2 | 0 | 0 |
| **3** | 0 | 1 | 1 |
| **4** | 1 | 1 | 0 |
| **5** | 1 | 1 | 0 |
| **6** | 1 | 1 | 0 |
| **Sum** | 6 | 4 | 2 |
| **Mean[1]** | 1.00 | 0.67 | 0.33 |

**[1]Average per calf**

**Table 5. Ranking of dry matter intake from day 1 to 7 for overall calf starter consumption.**

| | Treatment | | |
|---|---|---|---|
| **Heifer** | Control | 30g of algae | 60g of algae |
| **1** | 3 | 1 | 2 |
| **2** | 3 | 2 | 1 |
| **3** | 1 | 2 | 3 |
| **4** | 1 | 2 | 3 |
| **5** | 1 | 3 | 2 |
| **6** | 1 | 3 | 2 |
| **Sum** | 10 | 13 | 13 |
| **Mean[1]** | 1.67 | 2.17 | 2.17 |

**[1]The average closest to one means that the treatment was the most preferred, and the average closest to 3 means that the treatment was the least preferred.**

**Table 6. DMI for from day 1 through 7 during days 3 to 4 for calf starter grain.**

| | DMI (kg/d) from day 1 to 7 | | | DMI (kg/d) from days 3 to 4 | | |
|---|---|---|---|---|---|---|
| **Heifer** | Control | 30g of algae | 60g of algae | Control | 30g of algae | 60g of algae |
| **1** | 3.8 | 10.5 | 8.8 | 2.8 | 2.2 | 2.2 |
| **2** | 5.1 | 7.6 | 7.8 | 3.9 | 1.6 | 2.1 |
| **3** | 9.6 | 8.6 | 3.0 | 1.7 | 2.4 | 3.0 |
| **4** | 12.2 | 3.8 | 3.3 | 3.1 | 3.4 | 1.3 |
| **5** | 11.4 | 4.4 | 9.4 | 3.1 | 3.4 | 2.3 |
| **6** | 12.2 | 4.3 | 7.8 | 2.6 | 4.1 | 2.4 |
| **Sum** | 54.3 | 39.2 | 40.0 | 17.2 | 17.1 | 13.3 |
| **Mean*** | 9.1 | 6.5 | 6.7 | 2.9 | 2.9 | 2.2 |
| **Mean/d** ** | 1.3 | 0.93 | 0.96 | 1.4 | 1.4 | 1.1 |

***Average per calf**

****Average per calf per day**

Cultivating algae in waste water reduces pollution and provides an economic source of feed ingredient. However, the metabolic events occurred in feeding waste water algae to animals have not been examined in details. In this study, young male mice (n= 8/group) were fed the chows containing 0, 5% , and 10% green algae (Chlorella Vulgaris) grown in waste water from slaughterhouse and dairy processing facilities, respectively, for 21 days. The metabolic effects of waste water algae were investigated by growth performance, blood chemistry, and liquid chromatography-mass spectrometry (LC-MS)-based metabolomics. The results showed that growth performance and blood chemistry, including glucose, triacylglycerol, cholesterol, and blood urea nitrogen, were not significantly affected by either slaughterhouse or dairy algae (Figure 15). Metabolomic analysis of liver, cecum, feces, and urine samples identified the metabolic changes shared by both sources of algae and the source-specific metabolic changes. The levels of B vitamins in urine were significantly increased by both waste water algae treatment. Short-chain fatty acids in feces were only increased by 10% slaughterhouse algae. Muricholic acid and deoxycholic acid in feces were decreased by both 10% slaughterhouse and 10% dairy algae, while lithocholic acid was reduced only by 10% slaughterhouse algae. Liver metabolites including oxidized glutathione, niacinamide and adenosine were only reduced by 5% slaughterhouse algae. Overall, the results suggested that feeding waste water algae in an appropriate include rate is an acceptable practice for animal nutrition.

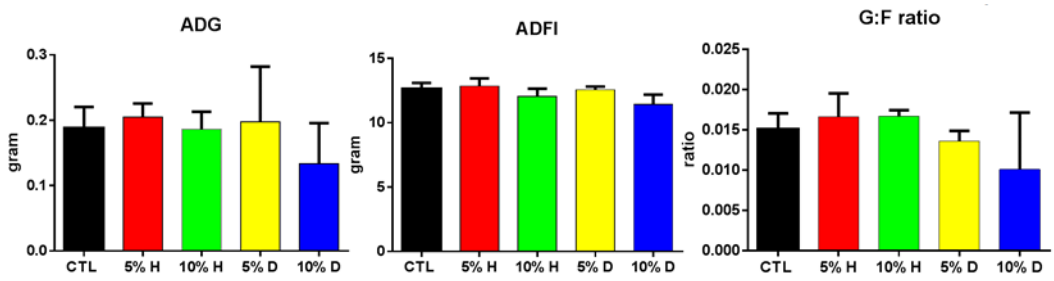



Figure 15 . Effects of algae feeding on growth performance of young mice

**ACTIVITY 3: Educate producers and consumers about technology to recycle nutrients, prevent runoff and add value to nutrients in dairy wastewater.**
**Description:**

We will develop a comprehensive extension program to educate producers, dairy professionals, and other stakeholders on the implementation of dairy wastewater efficiency, through the following activities: 1) Maintaining a web page within the University of Minnesota Dairy Extension websites throughout the project and beyond dedicated to dissemination of electronic information, 2) Disseminate results and educational information via social media (Facebook and YouTube, and 3) Present study results at extension and professional conferences in the state and region. For all outreach activities, we will solicit feedback using standard survey documents, and these surveys will determine the impacts of our activities on audience knowledge and farmers' behaviors related to adopting practices that reduce runoff of dairy wastewater to lakes and streams.

| **Summary Budget Information for Activity 3:** | **ENRTF Budget:** | **$ 12,500** |
|---|---|---|
| | **Amount Spent:** | **$ 788** |
| | **Balance:** | **$ 11,712** |

| **Outcome** | **Completion Date** |
|---|---|
| 1. Conduct workshops, webinars, and a WCROC field day each year of the integrated facility for producers. | 6/30/2019 |
| 2. Prepare 5 Extension factsheets to inform stakeholders of the demonstration sites. | 6/30/2019 |
| 3. Update the WCORC website every 6 months with an update on the dairy wastewater project | 6/30/2019 |

**Activity Status as of *January 1, 2017*:**
Extension and outreach work has not yet begun.

**Activity Status as of *January 1, 2018*:**
At the West Central Research and Outreach Center, we held the 2017 Midwest Farm Energy Conference from June 13 to 14, 2017 in Morris, MN. At the conference Dr. Rob Gardner presented a seminar entitled "Utilizing Dairy Wastewater for Sustainable Production of Energy, Feed, and Food". There he discusses how microalgae are part of the solution for nutrient capture, conversion, and recycling. We also provided a tour to conference participants at the WCROC dairy and introduce the algae project.

**Activity Status as of *July 1, 2018*:**

We have provided many tours to guest and researchers at the WCROC dairy to introduce the algae project. Over 500 people have viewed the algae cultivation system and have provide great feedback on the research project. We will be extension articles within the next 6 months to update on the algae cultivation project, as well as the algae feeding trials.

**Activity Status as of *January 1, 2019*:**
During the Dairy Day at the West Central Research and Outreach Center in August 2018, we introduced the algae project to dairy farmers and showed them the operating biomass production. In addition, during Horticulture night in July 2018, we have a tour of the algae production to over 250 people. Our graduate student on the project presented an abstract at the Algal Biomass Conference in New Orleans, LA on biomass production for livestock. We are planning a session on algae biomass production with wastewater for the Midwest Farm Energy Conference in July 2019.

**Final Report Summary:**

We have provided tours of the algae biomass system at the WCROC to legislators, farmers, and industry representatives. We have also hosted field days that have shown the results and bioreactors to the public as well. Over 2,000 people have viewed the system and have responded with favorable interest in the system. Our graduate student on the project presented an abstract at the Algal Biomass Conference in Denver, CO on biomass production for livestock. Within the next month the website will be updated with the final results of the project and infographics for promotion of the project. An abstract on calf feeding with be presented at the American Dairy Science Association meeting in 2020. This applied algae livestock feeding component is the Master's thesis of Siane Luzzi in the Department of Bioproducts and Biosystems Engineering at the University of Minnesota and she will defend her thesis on August 28, 2019.

**V. DISSEMINATION:**
**Description:**

The most effective way to educate and motivate livestock producers to adopt new technologies is to demonstrate improved profitability and minimize the environmental impact of dairy wastewater. The results from Activity 1 and 2 will be used to demonstrate the potential of the microalgae system. The research and outreach center will be used as the demonstration site to showcase the opportunities to recycle nutrients and clean dairy wastewater, as well as generate new opportunities for the 5,000+ Minnesota dairy and pork producers to utilize a nutrient dense, alternative and sustainable feed ingredient. These activities are well within the capabilities of the WRCOC and the University of Minnesota.

**Status as of *January 1, 2017*:**
Extension and outreach work has not yet begun.

**Status as of *January 1, 2018*:**

Published papers:
Addy, Min M., Faryal Kabir, Renchuan Zhang, Qian Lu, Xiangyuan Deng, Dean Current, Richard Griffith et al. "Co-cultivation of microalgae in aquaponic systems." *Bioresource technology* 245 (2017): 27-34.

Deng, X. Y., Gao, K., Zhang, R. C., Addy, M., Lu, Q., Ren, H. Y., ... & Ruan, R. (2017). Growing Chlorella vulgaris on thermophilic anaerobic digestion swine manure for nutrient removal and biomass production. *Bioresource technology*, *243*, 417-425.

Zhang, R., Anderson, E., Addy, M., Deng, X., Kabir, F., Lu, Q., ... & Ruan, R. (2017). An innovative intermittent-vacuum assisted thermophilic anaerobic digestion process for effective animal manure utilization and treatment. *Bioresource technology*, *244*, 1073-1080.

**Status as of *July 1, 2018*:**

Algae Alchemy. U-M researchers are growing algae for feed in dairy wastewater.
https://www.the-farmer.com/manure/algae-alchemy

Published papers:
Deng, X.-Y., K. Gao, M. Addy, D. Li, R.-C. Zhang, Q. Lu, Y.-W. Ma, Y.-L. Cheng, P. Chen, Y.-H. Liu, and R. Ruan. 2018. Cultivation of Chlorella vulgaris on anaerobically digested swine manure with daily recycling of the post-harvest culture broth. Bioresource Technology 247:716–723. doi:10.1016/j.biortech.2017.09.171.

Lu, Q., P. Chen, M. Addy, R. Zhang, X. Deng, Y. Ma, Y. Cheng, F. Hussain, C. Chen, Y. Liu, and R. Ruan. 2018. Carbon-dependent alleviation of ammonia toxicity for algae cultivation and associated mechanisms exploration. Bioresource Technology 249:99–107. doi:10.1016/j.biortech.2017.09.175.

We will be including more extension information within the next 6 months as production of algae will be completed for feeding to dairy animals.

**Status as of *January 1, 2019*:**

Published papers:

Wang, L., M. Addy, J. Liu, C. Nekich, R. Zhang, P. Peng, Y. Cheng, K. Cobb, Y. Liu, H. Wang, and R. Ruan. 2019. Integrated process for anaerobically digested swine manure treatment. Bioresource Technology 273:506–514. doi:10.1016/j.biortech.2018.11.050.

Zhang, R., E. Anderson, P. Chen, M. Addy, Y. Cheng, L. Wang, Y. Liu, and R. Ruan. 2019. Intermittent-vacuum assisted thermophilic co-digestion of corn stover and liquid swine manure: Salinity inhibition. Bioresource Technology 271:16–23. doi:10.1016/j.biortech.2018.09.071.

Luzzi, S., B. Heins, and R. Gardner. 2018. Algae cultivation processes - ponds, photobioreactors, fermenters; algae for fish and animal feed. Algal Biomass Conference. New Orleans, LA. July 2018

**Final Report Summary:**

Published papers:

Cao, L., Z. Li, S. Xiang, Z. Huang, R. Ruan, and Y. Liu. 2019a. Preparation and characteristics of bentonite–zeolite adsorbent and its application in swine wastewater. Bioresource Technology 284:448–455. doi:10.1016/j.biortech.2019.03.043.

Cao, L., J. Wang, S. Xiang, Z. Huang, R. Ruan, and Y. Liu. 2019b. Nutrient removal from digested swine wastewater by combining ammonia stripping with struvite precipitation. Environ Sci Pollut Res 26:6725–6734. doi:10.1007/s11356-019-04153-x.

Cao, L., J. Wang, T. Zhou, Z. Li, S. Xiang, F. Xu, R. Ruan, and Y. Liu. 2019c. Evaluation of ammonia recovery from swine wastewater via a innovative spraying technology. Bioresource Technology 272:235–240. doi:10.1016/j.biortech.2018.10.021.

Cao, L., T. Zhou, Z. Li, J. Wang, J. Tang, R. Ruan, and Y. Liu. 2018. Effect of combining adsorption-stripping treatment with acidification on the growth of Chlorella vulgaris and nutrient removal from swine wastewater. Bioresource Technology 263:10–16. doi:10.1016/j.biortech.2018.04.094.

Chen, D., Y. Cheng, P. Peng, J. Liu, Y. Wang, Y. Ma, E. Anderson, C. Chen, P. Chen, and R. Ruan. 2019. Effects of intense pulsed light on Cronobacter sakazakii and Salmonella surrogate Enterococcus faecium inoculated in different powdered foods. Food Chemistry 296:23–28. doi:10.1016/j.foodchem.2019.05.180.

Deng, X., K. Gao, M. Addy, P. Chen, D. Li, R. Zhang, Q. Lu, Y. Ma, Y. Cheng, Y. Liu, and R. Ruan. 2018. Growing Chlorella vulgaris on mixed wastewaters for biodiesel f eedstock production and nutrient removal. Journal of Chemical Technology & Biotechnology 93:2748–2757. doi:10.1002/jctb.5634.

Li, K., Q. Liu, F. Fang, R. Luo, Q. Lu, W. Zhou, S. Huo, P. Cheng, J. Liu, M. Addy, P. Chen, D. Chen, and R. Ruan. 2019. Microalgae-based wastewater treatment for nutrients recovery: A review. Bioresource Technology 291:121934. doi:10.1016/j.biortech.2019.121934.

Luo, S., X. Wu, H. Jiang, M. Yu, Y. Liu, A. Min, W. Li, and R. Ruan. 2019. Edible fungi-assisted harvesting system for efficient microalgae bio-flocculation. Bioresource Technology 282:325–330. doi:10.1016/j.biortech.2019.03.033.

Wang, L., M. Addy, J. Liu, C. Nekich, R. Zhang, P. Peng, Y. Cheng, K. Cobb, Y. Liu, H. Wang, and R. Ruan. 2019a. Integrated process for anaerobically digested swine manure treatment. Bioresource Technology 273:506–514. doi:10.1016/j.biortech.2018.11.050.

Wang, L., M. Addy, Q. Lu, K. Cobb, P. Chen, X. Chen, Y. Liu, H. Wang, and R. Ruan. 2019b. Cultivation of Chlorella vulgaris in sludge extracts: Nutrient removal and algal utilization. Bioresource Technology 280:505–510. doi:10.1016/j.biortech.2019.02.017.

Wang, Y., Z. Zeng, X. Tian, L. Dai, L. Jiang, S. Zhang, Q. Wu, P. Wen, G. Fu, Y. Liu, and R. Ruan. 2018. Production of bio-oil from agricultural waste by using a continuous fast microwave pyrolysis system. Bioresource Technology 269:162–168. doi:10.1016/j.biortech.2018.08.067.

Zheng, H., X. Wu, G. Zou, T. Zhou, Y. Liu, and R. Ruan. 2019. Cultivation of Chlorella vulgaris in manure-free piggery wastewater with high-strength ammonium for nutrients removal and biomass production: Effect of ammonium concentration, carbon/nitrogen ratio and pH. Bioresource Technology 273:203–211. doi:10.1016/j.biortech.2018.11.019.

Luzzi, S., B. Heins, and R. Gardner. 2019. Dairy wastewater treatment aiming to produce algae biomass for livestock feed application. Denver, CO July 2019

Three manuscripts are submitted from Luzzi thesis on the production of algae and feeding to dairy animals and will be published in 2020.

**VI. PROJECT BUDGET SUMMARY:**

**A. ENRTF Budget Overview:**

| Budget Category | $ Amount | Overview Explanation |
|---|---|---|
| Personnel: | $ 196,500 | 1 BBE research technician at 20% FTE for 3 years ($20,720); 1 ANSC research technician at 20% FTE for 3 years ($10,000); 2.25 graduate research assistants at 50% FTE each year for 3 years ($175,780) |
| Equipment/Tools/Supplies: | $222,886 | Column, reagents, HPLC vial, chemical standards, biochemical kits for Chi Chen laboratory ($5,000); Supplies for scoping parameters for the photobioreactor system for Roger Ruan laboratory in Bioproducts and BioSystems Engineering; Supplies include bags, tubing, chemicals, racks, pumps, lights ($25,000); Small research facility and vacuum ammonia stripping for both ammonia sulfate production and enhancement of the wastewater process; supplies include piping and mechanisms for data collection for ammonia |

| | | |
|---|---|---|
| | | sulfate production for testing algae strains ($45,000); Algal cultivation system, centrifuge to harvest algae, pumps for moving water and wastewater throughout system at the WCROC Dairy; supplies for the system include Bags, pvc piping, compressor, heat sealer, filters, centrifuge, chemicals, metal racks, pumps, lights, electrical wiring, pH monitoring and control, CO2 sparging equipment and storage at the WCROC, Morris – The Morris system will be made from no parts costing more than $5,000 individually ($159,000); Costs include Extension programming, workshops, field days, factsheets, and dissemination of information at the WCROC ($2,500) |
| Capital Expenditures over $5,000: | $32,000 | Automatic calf feeder and mixer for mixing algae with calf feed for feeding algae as a probiotic to pre-weaned dairy calves |
| **TOTAL ENRTF BUDGET:** | **$451,386** | |

**Explanation of Use of Classified Staff:**

**Explanation of Capital Expenditures Greater Than $5,000:** One automatic calf feeder/mixer and supplies is being purchased and will continue to be used by the University of Minnesota WCROC for the life of the instrument for similar projects and purposes. If the instrument is sold prior to its useful life, proceeds from the sale will be paid back to the Environment and Natural Resources Trust Fund.

**Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 5.575**

**Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation: 3**

**B. Other Funds:**

| Source of Funds | $ Amount Proposed | $ Amount Spent | Use of Other Funds |
|---|---|---|---|
| **Non-state** | | | |
| University of Minnesota (In-kind support) | $234,720 | $0 | The 52% foregone federally negotiated ICR funding constitutes the University of Minnesota cost share to the project. |
| **State** | $0 | $0 | |
| **TOTAL OTHER FUNDS:** | **$234,720** | **$0** | |

**VII. PROJECT STRATEGY:**

**A. Project Partners:**

Bradley Heins, U of MN Dairy Scientist, will serve as PI and project manager. He will be responsible for all reports and deliverables. He will also manage the activities of the dairy production system at the WCROC, conduct feeding trials, and manage the demonstration dairy site. Rob Gardner (U of MN Renewable Energy Scientist) will develop the microalgae system at the WCROC. Roger Ruan and Paul Chen (U of MN Bioproducts and Biosystems engineers) will design and develop integrated system for testing and demonstration. Gerald

Shurson and Pedro Urriola (U of MN Swine Scientists) will be responsible for assisting with livestock feeding trials to demonstrate the nutritional value of microalgae. Chi Chen (U of MN Nutrition Scientist) will analyze the nutrient content of the products to characterize nutritional effects of algae from this system.

**B. Project Impact and Long-term Strategy:**
The overall goal of the project is to develop and demonstrate a technology that will recycle nutrients and add value to nutrients in wastewater from dairy farms in Minnesota to reduce environmental impact. This collaborative project will build on current algal and nutritional activities of the project investigators. The proposed project does not need additional investment other than funding requested from the ENRTF to be completed. Additional long-term funding will be sought to conduct research to integrate this facility within large livestock operations within Minnesota. It may be necessary to acquire federal funding before large scale demonstrations of the integrated facility may be commercialized.

**C. Funding History:**
N/A

**VIII. FEE TITLE ACQUISITION/CONSERVATION EASEMENT/RESTORATION REQUIREMENTS:**
N/A
**A. Parcel List:**
N/A
**B. Acquisition/Restoration Information:**
N/A
**IX. VISUAL COMPONENT or MAP(S):**

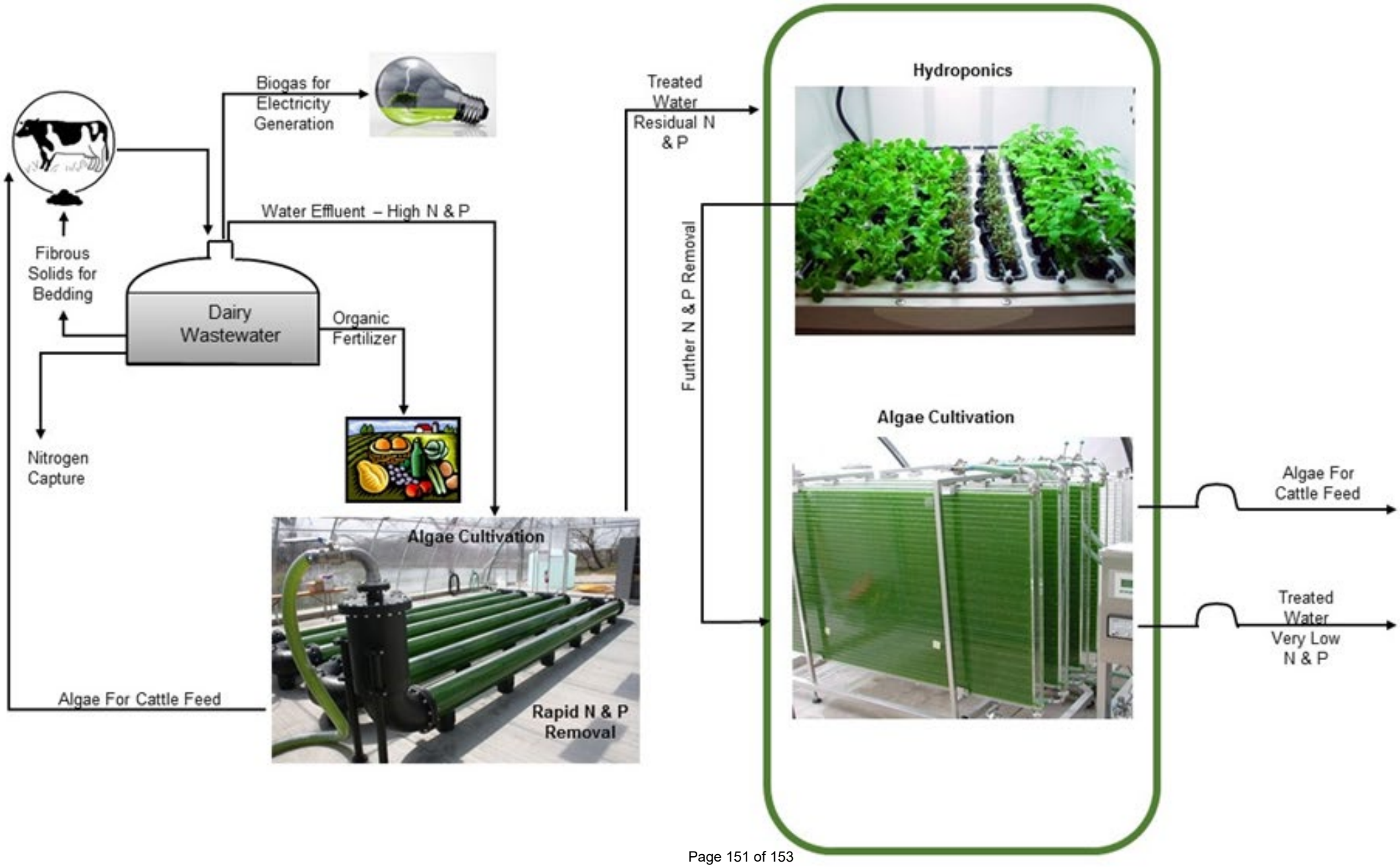
**X. RESEARCH ADDENDUM:** N/A

**XI. REPORTING REQUIREMENTS:**
**Periodic work plan status update reports will be submitted no later than January 1, 2017; July 1, 2017; January 1, 2018; July 1, 2018 and January 1, 2019. A final report and associated products will be submitted between June 30 and August 15, 2019.**

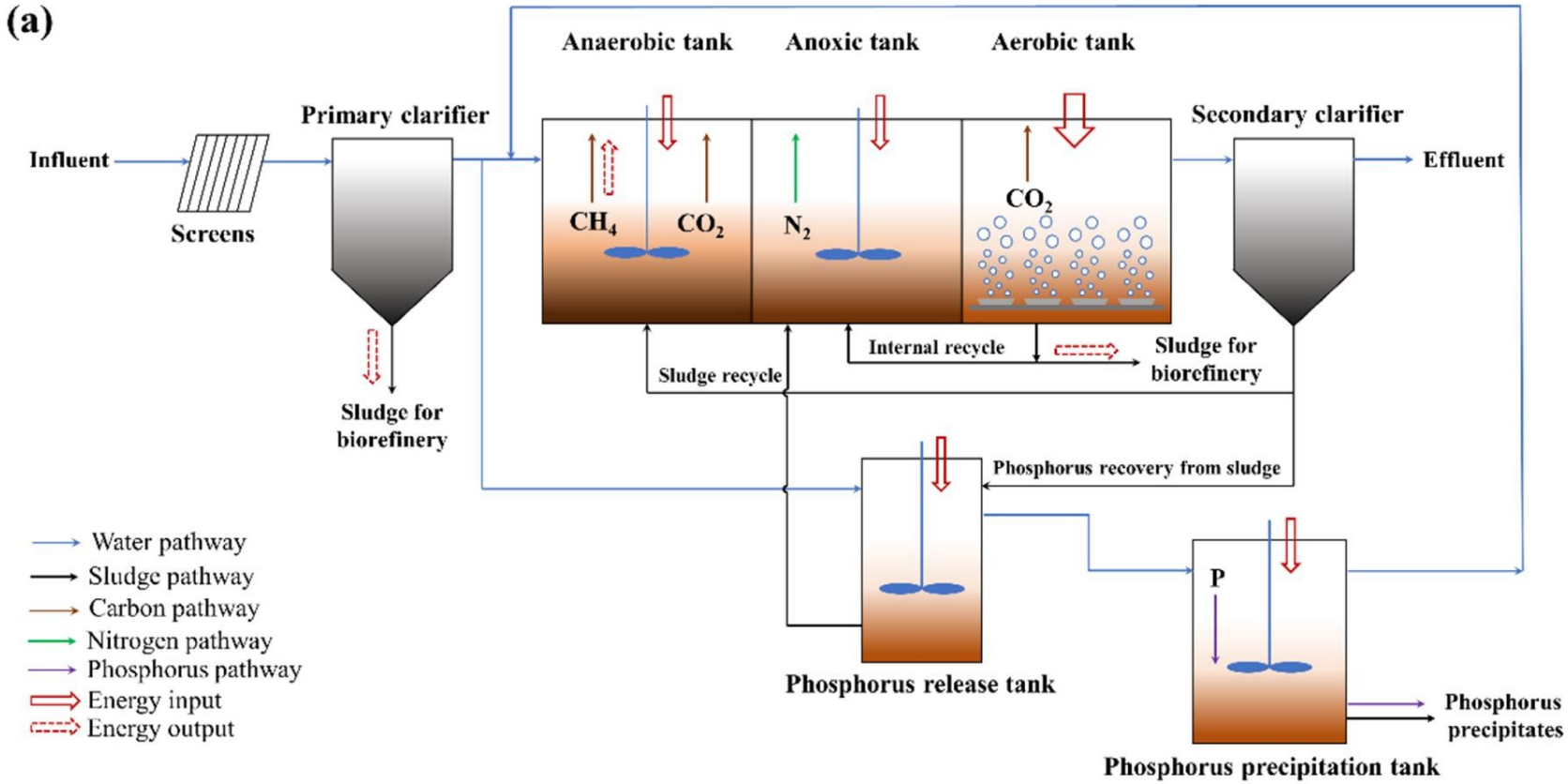
**Environment and Natural Resources Trust Fund**
**M.L. 2016 Project Final Budget**

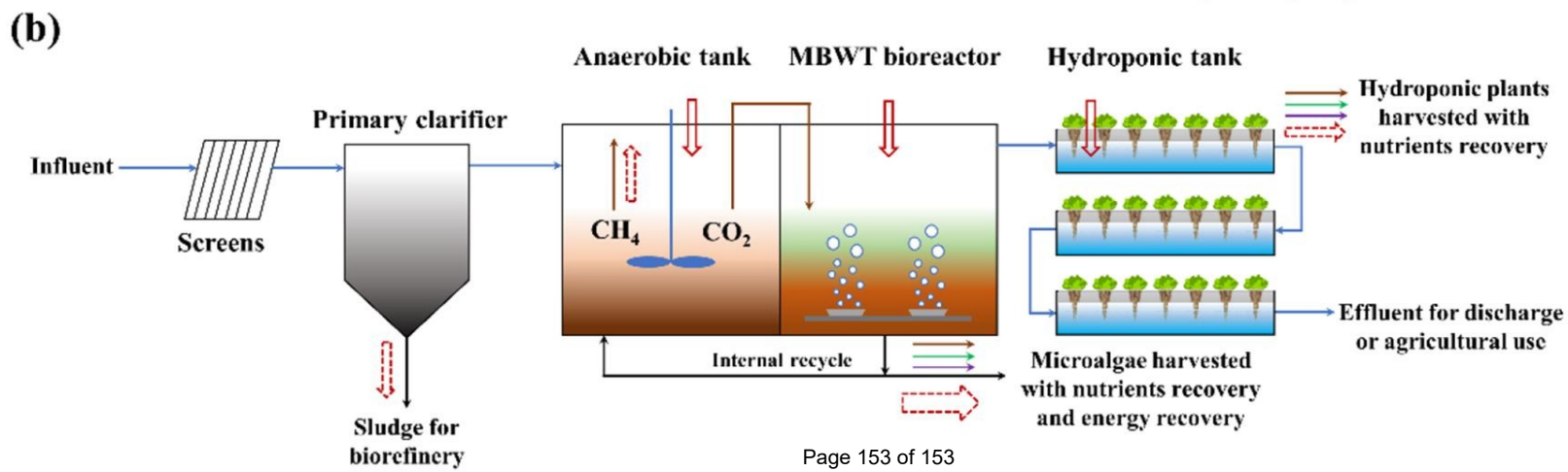
**Project Title:** *Utilization of Dairy Farm Wastewater for Sustainable Production*
**Legal Citation:** M.L. 2016, Chp. 186, Sec. 2, Subd. 07d
**Project Manager:** *Bradley Heins*
**Organization:** *University of Minnesota*
**M.L. 2016 ENRTF Appropriation: $475,000**
**Project Length and Completion Date:** *F: 3 Years, June 30, 2019*
**Date of Report:** August 16 2019

| ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET | Activity 1 Budget | Amount Spent | Activity 1 Balance | Activity 2 Budget | Amount Spent | Activity 2 Balance | Activity 3 Budget | Amount Spent | Activity 3 Balance | TOTAL BUDGET | TOTAL BALANCE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **BUDGET ITEM** | ***Develop and Test Integrated Dairy Wastewater System*** | | | ***Evaluate environmental impact and livestock algal feeding*** | | | ***Dissemination and Extension of Results*** | | | | |
| **Personnel (Wages and Benefits)** | $121,610 | $121,610 | $0 | $74,890 | $74,890 | $0 | $10,000 | $0 | $10,000 | $206,500 | $10,000 |
| *Paul Chen, 6% FTE in year 1, 2, and 3; 33.7% fringe rate; estimated $20, 720* | | | | | | | | | | | |
| *Pedro Urriola, 2.7% FTE in year 1, 2, and 3; 33.7% fringe rate; estimated $10,000* | | | | | | | | | | | |
| *Bioproducts and Biosystems Engineering Graduate research assistant for 2 years; 17.60% fringe, plus tuition remission during the academic year and 17.60% fringe summer; estimated $85,910* | | | | | | | | | | | |
| *Animal Science Graduate Research Assistant for 2 years; 17.60% fringe, plus tuition remission during the academic year and 17.60% fringe summer; estimated $79,890* | | | | | | | | | | | |
| *Food Science partial graduate research assistant; 17.60% fringe, plus tuition remission during the academic year and 17.60% fringe summer; estimated $9,980* | | | | | | | | | | | |
| **Equipment/Tools/Supplies** | $75,000 | $75,000 | $0 | $159,000 | $147,098 | $11,902 | $2,500 | $788 | $2,183 | $236,500 | $13,614 |
| *Column, reagents, HPLC vial, chemical standards, biochemical kits for Chi Chen laboratory in Food Science $5,000* | | | | | | | | | | $5,000 | |
| *Supplies for scoping parameters for the photobioreactor system for Roger Ruan laboratory in Bioproducts and BioSystems Engineering; Supplies include bags, tubing, chemicals, racks, pumps, lights; $25,000* | | | | | | | | | | $25,000 | |
| *Small research facility and vacuum ammonia stripping for both ammonia sulfate production and enhancement of the wastewater process; supplies include piping and mechanisms for data collection for ammonia sulfate production for testing algae strains; $45,000* | | | | | | | | | | $45,000 | |
| *Algal cultivation system, centrifuge to harvest algae, pumps for moving water and wastewater throughout system at the WCROC Dairy; supplies for the system include Bags, pvc piping, compressor, heat sealer, filters, centrifuge, chemicals, metal racks, pumps, lights, electrical wiring, pH monitoring and control, CO2 sparging equipment and storage. $159,000* | | | | | | | | | | $159,000 | |
| *Costs include Extension programming, workshops, field days, factsheets, and dissemination of information at the WCROC. $2,500* | | | | | | | | | | $2,500 | |
| **Capital Expenditures Over $5,000** | | | | $32,000 | $32,000 | $0 | | | | $32,000 | $32,000 |
| *Automatic calf feeder and mixer for mixing algae with calf feed for feeding algae as a probiotic to pre-weaned dairy calves. $32,000* | | | | | | | | | | | |
| **COLUMN TOTAL** | **$196,610** | **$196,610** | **$0** | **$265,890** | **$253,988** | **$11,902** | **$12,500** | **$788** | **$11,712** | $475,000 | **$23,614** |


Biogas for Electricity Generation
Water Effluent – High N & P
Fibrous Solids for Bedding
Dairy Wastewater
Organic Fertilizer
Nitrogen Capture
Algae For Cattle Feed
Algae Cultivation
Rapid N & P Removal
Treated Water Residual N & P
Further N & P Removal
Hydroponics
Algae Cultivation
Algae For Cattle Feed
Treated Water Very Low N & P

**Environmental and Natural Resources Trust Fund**
**2016 Visual Graphics**
**Project Title: Utilization of dairy farm wastewater for sustainable production**
Graphics 1. Schematic representation of the integrated photo-bioreactor, hydroponics, and aquaponic facility and outcomes.


(a)
Anaerobic tank
Anoxic tank
Aerobic tank
Primary clarifier
Secondary clarifier
Influent
Screens
Effluent
CH4
CO2
N2
CO2
Internal recycle
Sludge for biorefinery
Sludge recycle
Sludge for biorefinery
Phosphorus recovery from sludge
Water pathway
Sludge pathway
Carbon pathway
Nitrogen pathway
Phosphorus pathway
Energy input
Energy output
Phosphorus release tank
P
Phosphorus precipitates
Phosphorus precipitation tank


(b)
Anaerobic tank
MBWT bioreactor
Hydroponic tank
Primary clarifier
Influent
Screens
CH4
CO2
Hydroponic plants harvested with nutrients recovery
Internal recycle
Effluent for discharge or agricultural use
Microalgae harvested with nutrients recovery and energy recovery
Sludge for biorefinery