12-2018

Using Microalgae to Remediate Food and Bio-Digester Effluents from Western New York Agro Industries and Prospecting Harvested Algae Biomass for Biofuel Feedstocks

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USING MICROALGAE TO REMEDIATE FOOD AND BIO-DIGESTER EFFLUENTS FROM WESTERN NEW YORK AGRO INDUSTRIES AND PROSPECTING HARVESTED ALGAE BIOMASS FOR BIOFUEL FEEDSTOCKS

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ENVIRONMENTAL SCIENCE

AT

THE ROCHESTER INSTITUTE OF TECHNOLOGY- USA.

DEPARTMENT OF ENVIRONMENTAL SCIENCE

THOMAS GOSNELL SCHOOL OF LIFE SCIENCES

DECEMBER 2018

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ACKNOWLEDGEMENTS

This thesis is made possible through the guidance and support from the almighty God, family, friends, faculty, and organizations. I am forever grateful to my advisor Dr. Jeffrey Lodge for mentoring me throughout my research work. He gave me the liberty to independently explore and investigate concepts relating to this work. His unique advising approach, encouragement and timely feedback made this piece of work achievable.

I also owe an ocean of gratitude to my committee members, Dr. Karl Korfmacher and Dr. Ali Ogut for their unrelenting efforts to advise and see to the success of this work. Their constructive criticisms have helped to shape and prepare me to achieve greater heights in academia. I am not also forgetting, Dr. Robert Osgood and Dr. Susane Pagano for their encouragement through out my postgraduate journey.

I am also thankful to Ghana Education Trust Fund (GETFUND) for providing the needed financial resources to funding this research in its entirety especially the fund administrator, Mr. Baffoe, his deputies and the Government of Ghana (GoG).

I am also grateful to friends and lab members especially Katelyn Whitburn and Thulfiqar Ali Al-Graiti for their encouragement and show of united spirit in difficult moments. Finally, I would like to dedicate this thesis to my wife Alice Fiatsonu and kids Jeffery and Jadon.

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Abbreviations

ADE	Anaerobic Digester Effluents
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
EW	Egg wastewater
EW+det	Detergent containing egg wastewater
FFA	Free Fatty Acids
HRAP	High Rate Algae Pond
HRT	Hydraulic Residence Time
LED	light emitting Diode
MDL	Maximum Daily Loads
POTW	Publicly owned treatment works
RIT	Rochester Institute of Technology
RPM	Revolutions Per Minute
The USEPA	United States Environmental Protection Agency
TSA	Tryptic Soy Agar
TSS	Total suspended solids
WWTP	Wastewater Treatment plants

ABSTRACT

Agro-industries of Western NY contributes to the US economy in diverse ways. Among these are dairy, poultry, cheese, tofu and Greek Yogurt plants whose processes discharge effluents high in pollutants such as NH_3, PO_4, NO_3, and Fe which adversely affect aquatic systems and the watershed if discharged untreated. Waste hauling causes an economic burden to industries as WWTPs remain restrictive to these effluents, but Algae Remediation Technology provides a sustainable alternative to treating agricultural wastewaters onsite. This study sampled, assessed and treated effluents from selected production plants within NY State with various algae. The research applied free suspended Algae technology to treat food-based waste waters that have pollutant levels exceeding USEPA limits. While *Botyroccocus sp* and *Chlorella sp* reduced 99% of NO_3 from Synergy's dairy and bio digester effluents within 5 days residence time, all algae species removed 75% of phosphorus within 5 days Residence Time (RT). *Nostoc sp* removed 98% NO_3 from Kreher farm's Egg wash effluents but moderately removed PO_4 within 6 days RT while *Anabaena* and *Chlorella sp* impressively removed 90% PO_4 and over 90% NO_3 within an average of 3 and 12-days RT respectively. Tofu, cheese, and Greek yogurt whey all achieved bioremediation targets in less than 15 days RT. Post-treatment biomass harvested contained triglycerides and FFA fraction. Ultrasonication did not influence lipids, glucose and methane yields. *Chlorella sp* showed an avg 27g/L sugar yield compared to coffee and other algae biomass which yielded only avg 10g/L sugars. Lipid or lipid-sugar extractions from biomass increased Bio methane potential (BMP) by 1 and 5-fold respectively to 10ml meth/gVS and 25ml meth/g VS. Analysis and results indicate that algae are effective at reducing pollutants in agro-industrial effluents while producing high quality biomass for bioenergy purpose.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

1.1.1 WASTE HANDLING AND TREATMENT

When Abraham Maslow described the hierarchy of human needs, he prioritized food, water, air, and health as the fundamental and most critical for human survival (McLeod, 2007). However, these basic human needs cannot be possible if we sideline Environmental quality and Sustainability since these complement one another. It is, therefore, imperative that industry players pay critical attention to how they handle and treat their waste even as we strive to achieve food security. As technology advances, various methods have been utilized ranging from wastewater treatment plants to landfilling, composting and bio-digesters to handle and treat different waste streams. However, these methods produce substandard outputs and the cost of handling and treatments of secondary waste are economically unfeasible. The efficiencies of WWTPs have been questioned in recent years (Panepinto, Fiore, Zappone, Genon, & Meucci, 2016) while state and federal regulations remain firm on the type of waste allowed in POTWs. The cost of hauling waste to landfills and compost sites burdens most food processing industries and facility managers (Oliveira, Oliveira, Bezerra, Silva Pereira, & Battistelle, 2017). In some cases, facility owners pay surcharges if BOD and TSS limits exceed 300mg/l for discharged waste into POTWs (Trabold, Ramchandra, Haselkorn, & Williamson, 2011). Therefore, a more efficient and cost-effective onsite treatment method is needed.

1.1.2 ALGAL BIOREMEDIATION AS SUITABLE ALTERNATIVE

Bioremediation technologies using both macro and microalgae have in recent years gained momentum as suitable and more efficient means of liquid waste treatment onsite. Microalgae are autotrophic species capable of utilizing nutrients in various waste media for growth. The algae treatment technology was used on dairy farm effluents at Poisy in the French Alps which resulted in 82-96% removal of nutrient loads removal compared to 76% removal of nitrogen from WWTPs (G. Merlin & A. Gaillot, 2010). Similarly, both mono and polyculture algae applied onto carpet mill effluents from north-central Georgia in open pond treatment style achieved over 90% pollutants removal efficiency (Chinnasamy, Bhatnagar, Hunt, & Das, 2010). *Chlorella sp* and *Scenedesmus sp* have been determined to be highly effective bio-sorbents in heavy metal remediation compared to conventional activated carbons and Zeolite methods (Suresh Kumar, Dahms, Won, Lee, & Shin, 2015). Hence, their potential to be used in the treatment of high metallic mine effluents is justified, although certain physicochemical parameters need to be constantly checked.

Higher treatment efficiencies were achieved when municipal wastewater mixed with dairy wastewater was used as growth media for algae cultivation for biofuel. The treatment pond, when supplemented with CO_2, recorded a near 100% efficiency, but biomass lipid content varied with nutrient availability and aging algal cells (Woertz, Feffer, Lundquist, & Nelson, 2009). Given that CO_2 can be sequestered into algae pond treatment systems, there is a high probability that algal remediation systems could simultaneously reduce global climate change via CO_2 sequestrations into treatment ponds. Eibl et al. (2014) went to the extent of isolating pH tolerant acidophilic algae from a lignite mine in Ontario, Canada. The Lig 290 species could withstand a

pH as low as 4.0, a condition crucial to reducing the risk of contamination and also serve as a very robust species in extreme environments.

1.2 KNOWLEDGE GAP

The applications of algae in diverse wastewater sources have seen many successes ranging from manufacturing, municipal waste to mining effluents (Suresh Kumar et al., 2015; Wang et al., 2010; Woertz et al., 2009). However, fewer investigations have been done to simulate the concept of algal bioremediation to treat effluents from the food and agro-processing industries in the United States, especially Western New York. The following case studies open up potential opportunities to utilize algal remediation technology in the agro-industrial sector.

1.3 FOOD WASTE STREAMS IN WESTERN NY

1.3.1 CASE STUDY 1: DAIRY AND BIO-DIGESTER WASTE

Today the United States is home to some 51,000 dairy farms which provide milk and beef to local and international markets. Production is expected to grow by some 5.1% in first half of 2018 with a projected decline in meat prices due to high production and relatively low demands ("USDA ERS - Market Outlook Dec 18^{th,} 2017," n.d.). The downside to commercial dairy and livestock production is the magnitude of wastewater generated which can be detrimental to the environment and human health. Tunçsiper et al. (2015) documented a mass flow rate of 500 -10,000 l/day of dairy wastewater from the University of Vermont dairy farm with high nitrate and phosphate concentrations above USEPA Maximum Daily Loads (MDL). Also, an assessment of two major stall dairy farm sites in Wisconsin and Indiana have on-site stabilization lagoons holding 23,400 and 48,212 cubic meters respectively of liquid manure waste in waiting to be treated. This glut of waste exceeds acceptable standards to be channeled

into WWTPs but could serve as an unending source of growth media for cultivating microalgae for the biofuel industry while the recycled water could be harnessed for secondary purposes.

As of 2011, New York State hosted 5,300 dairy farms (fig 1.1) with Synergy farms being the largest in the State housing about 2000 dairy cattle and generating close to 425 tons of

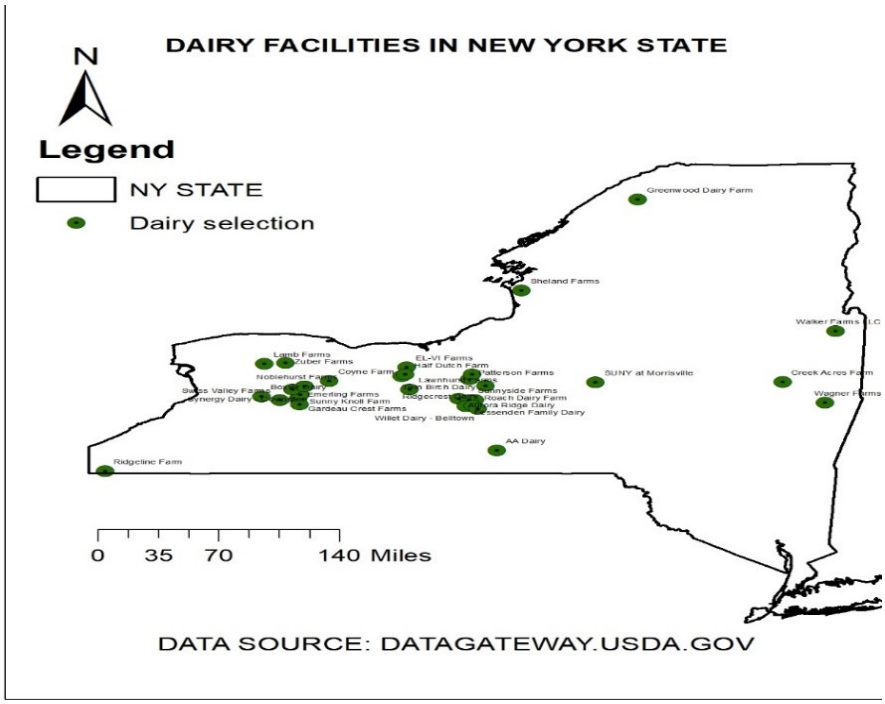


Figure 1.1 Dairy farms in New York State

dairy waste per Day ("New York's Largest On-Farm Biogas Power Project Generates Renewable Energy for Nearly 1,000 Homes," 2012). Although, Synergy farms based in Covington, NY partners with CH4-Biogas to co-digest most of its dairy waste in a 2.1M -gallon digester, digestate from the Anaerobic digester is confined in 3 onsite dug-out lagoons waiting to be treated. The digestate cannot be channeled into a local POTWs since pollutants far exceed acceptable limits and hence require some form of pre-treatment. Direct land application as fertilizer is not an option due to the risk of groundwater contamination from nitrate or phosphate.

Digestate confined in dug-out lagoons can also cause local air quality issues. The situation gets worse in the case of smaller farms which cannot finance exorbitant biodigester facilities. In the words of farm owner John Noble, "It is an expensive venture which our sister farms cannot afford. Thanks to our partners from Denmark and the NYSERDA providing $1M as an incentive" (personal communication, 2nd December 2017).

In the summer of 2016, Synergy – CH4 Biogas partnered with Dr. Jeffrey Lodge's lab at the Rochester Institute of Technology on a pilot project to use microalgae to treat wastewater from their digester (fig 1.2). Sample analysis of the digestate (ADE) shows phosphate and ammonia high above the State's permissible limits. Samples of ADE taken and tested in the winter and summer of 2016 showed around 1789ppm and 318ppm for ammonia and phosphate respectively while no nitrate was recorded. However, as a tiny fraction of ammonia volatilize, some ammonia will undergo undergo oxidation via ammonia and nitrogen oxidizing bacteria. The project could impact the environment positively by reducing the nutrient loads in wastewaters to avoid possible eutrophication in water systems. Additionally, an unending source of nutrient rich effluents could be used as growth media for algae cultivation for biofuels which could be a sustainable replacement for corn for ethanol in the United States while saving the company thousands of dollars in waste hauling. Equally large farms like the Willet and Sunnyside farms in the Cayuga County all generate tons of waste annually.

Figure 1.2: Synergy LLC site with a dug-out reservoir containing digestate and dairy waste

1.3.2 CASE STUDY 2: GREEK YOGURT, TOFU AND CHEESE WHEY

Two of the major leading brands of Greek yogurt, Chobani, and Fage are located in upstate New York. This type of yogurt has most of the water and whey strained out, hence the potential to generate high volumes of liquid waste to be treated (fig 1.3). By the year 2012, Alpina had increased its production of the Greek yogurt from 24 million pounds to 123 million pounds, and dairy farmers within New York State stand to benefit from the growing market (Neuman, 2012). The market boom stems from the Yogurt industry's reliance on milk from local dairy farmers for their production. As New York State officials hint of welcoming larger facilities for yogurt production, the most potentially sustainable way to handle and treat high volume whey is through remediation with microalgae. Whey contains a high amount of organic nitrogen due to its high protein content. At the time of this research, no concrete evidence was published on the fate of organic nitrogen (Org-N) in the wastewater although there is speculation that amino acids in the whey convert to hydrophilic compounds which could stimulate algal

growth (Huo et al., 2013). The research, thereofore, anticipates high Org-N and phosphate removal from cheese and yogurt whey by the microalgae.

Cheese Whey

Cheese whey (fig 1.3) is also another major issue as the whey from production is equally rich in lactose. It is estimated that every 1 kg of Feta cheese produced generates approximately 10 liters of wastewater also high in BOD and Chemical oxygen demand (COD) (Trabold et al., 2011) However, the whey containing lactose can be partly fed to *Kluyveromyces marxianus*, a unique Yeast capable of producing ethanol from lactose fermentation (Hegde, Lodge, & Trabold, 2018). Although high-quality whey from cheese can equally be processed into animal feed and food additive, most of the whey ends up as an environmental nuisance which could be treated using microalgae. In February 2015, the Yancy Fancy Cheese processing plant in Corfu, NY received a $100,000 grant from National Grid to expand its facilities for managing excess whey as a by-product from production ("Yancey's Fancy," n.d.). This research lab has conducted initial parameters testing from sampled Cheese and Yogurt whey from Yancy Fancy® and Lively Run® and found they contain high phosphate and nitrate above permissible levels of discharge. Cheese whey showed 90.9 ppm and 33.0 ppm for nitrates and phosphate respectively thereby far exceeding US EPA maximum allowable limits of 2.2ppm of Nitrates and 1.0ppm of phosphate for receiving waters. Also, low whey pH (≤4.6) makes land application inappropriate.

A) Cheese and Tofu whey B) Greek Yogurt Whey- Alpina

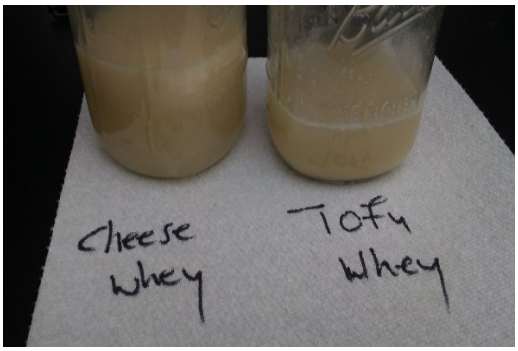


Figure 1.3 Sampled Cheese and Tofu whey from Yancy Fancy and Northern Soy respectively

1.3.3 CASE STUDY 3: EGGWASH WASTEWATER

The poultry industry is no exception to businesses generating high nutrient wastewater. Wastewater generated from eggwash (which contains detergent), meat processing, and cage cleaning contains high levels of PO_4, NO_3, and fecal coliforms which might wash into nearby surface waters. Kreher farms in Clarence NY is a conglomerate of poultry farms interspersed in the Empire State and has adopted a more sustainable approach to compost most of its poultry waste into fertilizers ("Kreher Family Farms - Compost & Fertilizer," n.d.). However, wastewater generated from egg processing facilities needs to be treated to reduce high nutrient levels. Two different eggwash wastewater samples are currently being analyzed in our lab:- 1) high detergent stream from the washing of eggs to remove coliforms 2) low detergent wastewater generated from equipment and cage cleaning.

1.3.4 CASE STUDY 4: TOFU WASTEWATER

Northern Soy, Rochester NY produces organic Tofu and other products sold in Wegmans and generates 40,000-45,000 gallons of wastewater weekly (see fig 1.3). Soybeans are fermented

and coagulated in tubs which are then passed through a belt press to squeeze the whey from the tofu and boiled at 90^0C. Tofu whey can have Biochemical oxygen demand (BOD) as high as 18,000 ppm. . The study assessed and found high P and N levels among other parameters of interest in sampled Tofu whey obtained from Northern Soy in Rochester, NY which results in a frequent surcharge due to high BOD and nitrogen exceeding 300ppm. Also, general food processing plants like tomato canneries, baked goods, milk, and oil processing exceed their MDLs of 300ppm of BOD. Hence, managers of these production plants constantly pay a surcharge to discharge into WWTPs.

1.4 SOME CURRENT TREATMENT TECHNOLOGIES AND LIMITATIONS

(a) POTWs (WWTPs): Various scientific disciplines have been working through various technologies to effectively treat wastewater and recover high-value products in an energy efficient manner. As mentioned earlier, POTWs currently remain our first point of call to remediate and handle most liquid waste. However, high energy demands and inefficiencies with their mechanical components (particularly aerators) have called this procedure into question. A re-assessment of Spain's WWTPs energy efficiencies after 18 years ranged from 5.3% to 16.1 % with efficiencies decreasing as facility ages (Hernández-Sancho, Molinos-Senante, & Sala-Garrido, 2011a). In some cases, desired nutrient removal efficiencies are not achieved, as effluents are partially treated and discharged, which could still be deleterious to receiving ecosystems (Kontas, Kucuksezgin, Altay, & Uluturhan, 2004). Pollutant removal efficiencies could be as low as 45% -66%, thereby having the potential to affect receiving waters through eutrophication (Kontas et al., 2004). Also, WWTPs are limited in their ability to receive high nutrient waste and in some instances industries have to pay the penalty for discharging over the pollution threshold.

(b) CONSTRUCTED WETLANDS (CW): Several scholarly works have explored the possible utilization of both natural and constructed wetlands in wastewater remediation efforts. Batavia and Red Creek WWTPs utilize constructed wetlands for nutrient reduction. The use of common reeds to remove pesticide residues like Boscalid have proven successesful (Papaevangelou, Gikas, Vryzas, & Tsihrintzis, 2017), as remediation of heavy metals from livestock wastewater yielded great results with *Phragmites australis* even in the presence of antibiotic residues from the farm. Although, constructed wetlands have the natural capacity to remediate high-level P and N effluents, the degree of nutrient levels and loading rates could defeat this purpose (White, 2007). There are instances where the USEPA has also raised red flags on that continued reliance on Natural Wetlands for wastewater remediation which could disrupt the ecosystem services provided by quality natural wetlands (Bastian, Shanaghan, & Thompson, 1989)

(c) CO-DIGESTION FOR BIOGAS: In the case of agriculturally related waste biomass, the US and highly industrialized nations have made significant efforts to co-digest dairy manure and food grade organic waste in anaerobic digestors for methane production while composting large portions of biomass for organic fertilizers. These technologies have helped to reduce waste while enhancing clean energy through methane production. However, digestates from these digesters are high in NH_3, PO_4, and NO_3 that they can neither be applied to land nor be channeled into public sewer systems and hence require some form of pre-treatment before discharge (Mendonça, Ometto, & Otenio, 2017). It is also necessary to carry out a detailed economic assessment to ensure constant and significant feedstock availability, and cost of operation as biodigesters are capital intensive to install and

operate. Installing Biodigesters for smaller farms seem to be economically conterproductive unless strategically sited to serve a cluster of farms.

(d) PHYSICO-CHEMICAL TREATMENT: This method has achieved some level of successes in recent years where certain chemical compounds are used to coagulate and flocculate pollutants in wastewaters. For instance, alum and ferric chloride could achieve about 90% removal and decolorization in dye wastewater. However, this method is highly affected by pH fluctuations, and the emergence of high metallic pollutants is seen (Teh, Budiman, Shak, & Wu, 2016). Biopolymers like chitosan and Moringa olifeira (drumstick tree) are non-toxic and recyclable, offering an environmentally friendly alternative to chemical reagents.

(e) BIOLOGICAL TREATMENTS: Agro-industrial waste is mostly treated either through aerobic or anaerobic means using microbial biofilms due to their high BODs, CODs and Total Suspended Solids (TSS). However, these methods, especially Upflow Anaerobic Sludge Blanket (UASB) and Anaerobic Filter (AF) which remove pollutants from wastewater using anaerobic microbes are not economical to small and medium scale farms due to their high energy demand and the requirement for high technical know-how (Labbé, Ramos-Suárez, Hernández-Pérez, Baeza, & Hansen, 2017). A more improved method, supplementing biological treatment with autotrophic algae, has proven to be successful with as high as 98% removal efficiencies while producing high-value products for the agricultural, pharmaceutical and the energy sector through through resultant biofuel feedstocks (Wang et al., 2010). However, it is imperative to know that treatment efficiencies differ among different algal species and lipid production is highly influenced by nutrient availability or deprivation (Park, Craggs, & Shilton, 2011).

1.5 PROBLEM STATEMENT AND OBJECTIVES

Western New York and the Fingerlakes contain about 280 dairy and food processing facilities (as of 2013) whose activities generate billions of dollars annually to the State. However, large volumes of wastewater with various degrees of pollutants are generated along the production chain which can neither go into POTWs nor be directly applied to land. Moreover, attempts to haul wastewater to specialized waste facilities increases the cost of production. It is, therefore, germane to have well functioning onsite treatment systems. This research strives to explore different algal strains for their efficiency in treating various food-based wastewaters and digester effluents in both laboratory based bioreactors and High Rate Algae Ponds (HRAP) in field trials. Bioreactor based treatments involve the use of closed vessels retofited with LED lights to treat wastewater under constant aerobic conditions where as HRAP utilizes an open pond system to treat wastewater. The outcome could be a trailblazer for sustainable agriculture through nutrient recovery and reapplication while serving as viable feedstock for the biofuel industry.

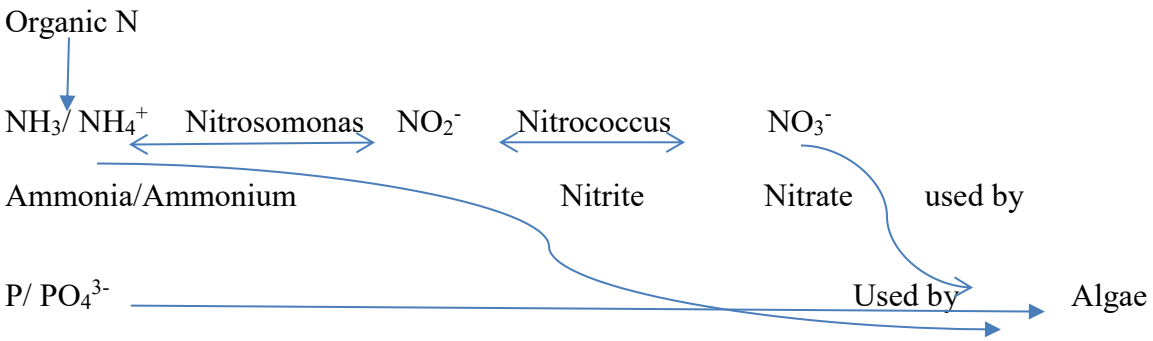
1.5.1 HYPOTHESIS

Various nutrient-rich synthetic media have been documented to promote microalgae cultivation effectively mainly due to the ability of microalgae to utilize the nutrients for growth among other environmental factors and stressors, but synthetic media at the large scale is expensive. Various types of wastewater can provide all the nutrients requirements for algae growth resulting in a nutrient reduction of these waste streams. Also, nutrient uptake by algae in a relatively shorter Hydraulic residence time (HRT) would conform to the periodic waste discharge schedules of partner industries to avoid a backlog of confined effluents.

1.5.2 SCIENTIFIC MECHANISM SUPPORTING THE RESEARCH

This research is underscored by the principle of the nitrogen cycle where ammonia and nitogen oxiding bacteria catalyze the aerobic conversion of organic ammonia into nitrate which is then readily utilized by primary producers (algae) for growth. The algae also can directly utilize ammonium (NH_4^+) as seen in fig 1.4 under aerobic conditions without undergoing complete nitrification.

Figure 1.4 Pathways from nutrient availability to transformation until algae intake



1.5.3 AIMS AND SPECIFIC OBJECTIVES

Aim: The primary aim of this research is to study the efficiencies of various algae to efficiently remediate various waste streams in shorter HRT and explore their potential to be used as biofuel feedstocks

Objectives:

- To test the nutrient reduction potential of various microalgae on different wastewaters (ADE, eggwash, tofu, cheese, and yogurt whey)
- To analyze and recommend specific algae for industries partnering with RIT.

- To harvest algae and extract lipids for thin layer chromatographic studies to profile harvested algae for biofuel potential.
- To establish carbohydrate content in various algae biomass as the potential for bio-ethanol
- To ascertain the potential of algae biomass for methane production through anaerobic digestion

1.5.4 SIGNIFICANCE AND BROADER IMPACTS OF THE RESEARCH

Algal wastewater treatment technology would not only ensure that less polluted effluents are released into streams, lakes or land but also contribute positively to sustainable agriculture, energy conservation, climate change, water conservation, to mention a few. For sustainable agricultural practices, biomass for algae treatment could be used as fertilizers to recylce N and P back to soils. Also, algal treatment has been documented to yield exceedingly high treatment efficiency compared to mechanical WWTPs at a rate of 96-99% as compared to the latter's 66-68% removal (Kontas et al., 2004). Since we can not take chances with or risk the integrity of aquatic ecosystems with quasi-treated wastewater, supplementing WWTPs with algal treatment as a tertiary level treatment would ensure environmental quality and sustain the health of receiving waters. Continuous research and development in this area could augment WWTPs with algal treatment and to mitigate the high energy demands from WWTPs (Hernández-Sancho, Molinos-Senante, & Sala-Garrido, 2011). For industries, algal treatment technology will not only lessen the burden of effluents hauling to WWTPs but will also save thousands of dollars through

significantly reducing pollutant levels to avoid fines and earn some federal tax credits for environmental compliance.

Treated water could always be used for irrigation onto crop fields or reuse in manufacturing industries for processes not requiring high water quality. The US is currently using Corn and Soybean as feedstocks for biofuels, this over-reliance on food crops frequently leads to prices hikes as well as food versus fuel competition in the market. The algae wastewater treatment could, therefore, provide a sustainable replacement for staple crops as feedstock in the biofuels industry. Climate change could be significantly minimized as cleaner fuels could be generated from lipid-rich algae biomass, and industrial generated CO_2 could be used for algae bioreactors for effluent treatment. Last but not least, resultant algae biomass can e used directly as nutrient-rich organic fertilizer for crop production thereby cutting down on the use of chemical fertilizers.

1.6 METHODOLOGY

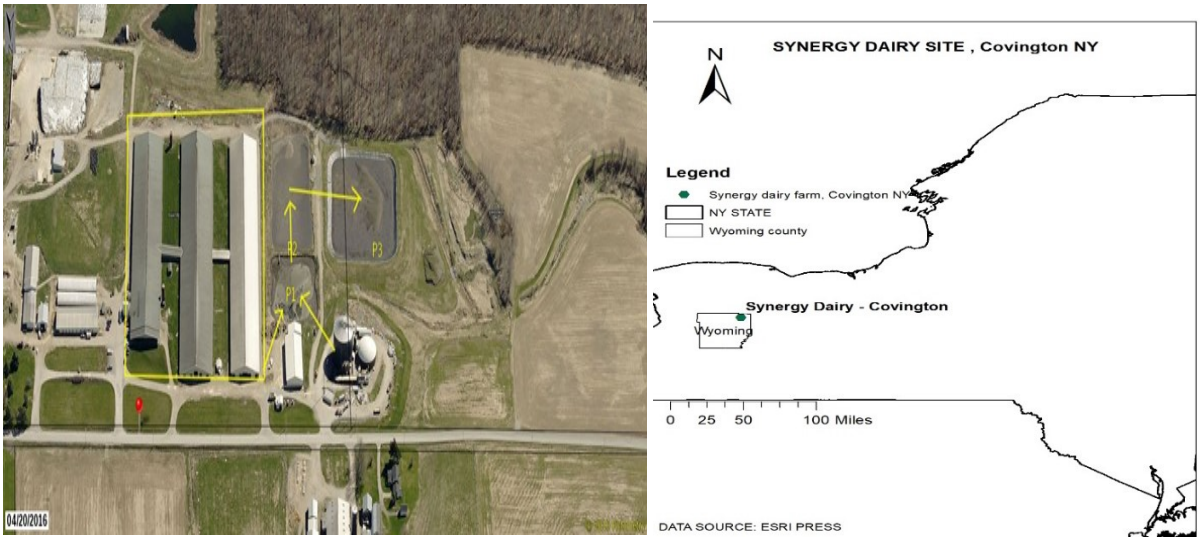


Figure 1.5 Field study area: Synergy Biogas Digester at Covington, NY

AlGAE STRAINS STUDIED: *Chlorella vulgaris., Nostoc sp., Scenedesmus sp Anabena sp* were obtained from Ward's Scientific®, *Boyteroccocus sp.*was obtained from the University of Texas algae culture collection, and Web3 algae were isolated from a primary clarifier of a WWTP in Webster, NY. The above strains have been tested in our lab in the past and exhibited higher longevity and robust in extreme wastewater environments.

1.6.1 ESTABLISHING BASELINE PARAMETERS

From fig 1.5 above, the aerial satelite imagery of Synergy-CH4 biogas facility shows effluents flow pathways into storage lagoons using arrows. Wastewater from both digester and dairy house are collected in Pond 1(P1) for gravity settling and later pumped into pond 2(P2) and pond 3 (P3) Samples of digestate were taken from stabilization pond 3, and initial baseline parameters were determined for Ammonia (NH_3), Phosphate (PO_4), Nitrate (NO_3), Nitrite (NO_2), Potassium (K), Salinity, pH, and Iron(Fe). Sampling was done at both peak (summer 2016) and off-peak times (spring 2016) of the year. Nutrient tests would be carried out using Hach Pocket Calorimeter II kit 58700-40 for NH3, Hach PC II 58700-02 for NO_3, Hach PC II 58700-06 for PO_4. Fe and NO_2 test were also determined with 0-1 mg/l IR-18A NJ1465-00 and 0-0.5 mg/l N1-15 21820-00 respectively. These Calorimeter kits can determine small to very high concentrations of nutrients with high sensitivity and with approval by the USEPA and the American Public Health Association (APHA). All pH measurements were made using an IQ Scientific Instrument probe IQ240 preceded by standard calibration.

1.6.2 ALGAE CULTURING AND GROWTH STUDIES

Strains of algae to be cultured for inoculation includes *Boytrococcus sp, Chlorella vulgaris,* Web 3 *sp, Scenedesmus sp., Anabena sp, and Nostoc Sp.* Stock cultures would be

obtained and cultured on synthetic Bristol salts prepared from stock solutions of ($NaNO_3$, KH_2PO_4, K_2HPO_4, $MgSO_4$. $2H_2O$, $CaCl_2.2H_2O$, NaCl, $FeCl_3$. $6H_2O$) diluted in distilled water for small scale experiments and pond water for larger scale experiements. Stock solutions were prepared by dissolving 10g $NaNO_3$, 7g KH_2PO_4, 3g K_2HPO_4, 3g $MgSO_4.2H_2O$, 1g $CaCl.2H_2O$, 1g NaCl in 400ml each of distilled water and 1g $FeCl.6H_2O$ in 100 ml distilled water. Portions of stock solution were added in a 1:100 ratio of pond water or distilled water and placed on Biomega® Magnetic stirrer for uniform mixing. Culturing was carried out in a 300ml Erlenmeyer flask and later scaled up to a 2000ml glass jar photobioreactors under White, Blue, and Red LED lights. Oxygen is constantly supplied to the system using 120V 60Hz 1.5 watts powered Whisper 10® aerators. In the process of culturing, growth rates of these species would be studied under White, Blue and Red LED lights at pH of 7.1- 7.4 at room temperature for 5- 7 days. Growth rates would be studied by taking 2ml of each set up at 2 –day interval and analyzing optical densities with the Clinical Diagnostics® Spectrophotometer 554142.

1.6.3 PHOTOBIOREACTOR GROWTH

A 10% inoculum of freshly cultured *Chlorella vulgaris* is inoculated into 1500 ml of 1:5 or 1:10 Digester effluent-pond water dilutions under a bioreactor set-up with an adjusted initial pH of 7.1-7.4. The reactor is then constantly aerated under a white LED powered 16:8 light-dark cycles while pH and other parameters of interest (NH_3-N, NO_3-N, NO_2, Fe, PO_4) were monitored in 2-3 day intervals over 30 days. Aeration for the bioreactor was carried out using 01G38 or 01B36 Whisper 10® airpump. Occasionally, 300ml of the initial dilution ratio of the Anaerobic digester effluents (ADE) may be added to revive the system's depleted nutrients. Nutrient top-up in the biorector is necessary to sustain algae cells in the bioreactor to mimic a

batch culture open treatment method. The entire cycle would be repeated with other algal strains, and other wastewater types like eggwash effluents, cheese, and yogurt whey. (Fig 1.6)

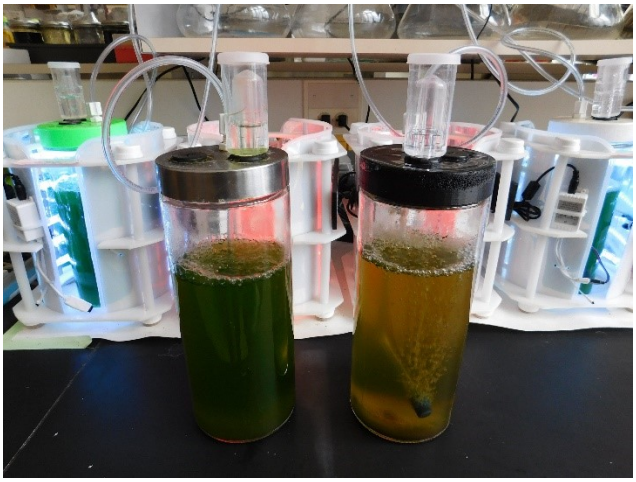


Figure 1.6 A Photo-bioreactor treatment set-up in the Lab

1.6.4 GREENHOUSE AND ON-SITE OPEN POND TREATMENT

Since laboratory conditions differ from ambient environmental conditions, it was necessary to simulate treatment design in RIT's greenhouse and in-the-field settings. A 2000ml culture of *Chlorella vulgaris* or any other algae strain was added to 10L wastewater in a 15L rectangular tank. It was necessary to dilute effluents to allow sufficient light penetration into the system. Constant aeration is carried out for 5-7 days, after which the process may be scaled up in the same ratio in a 50-gallon tank in the greenhouse. The necessary parameters were then monitored in the 3-day interval over 30 days.

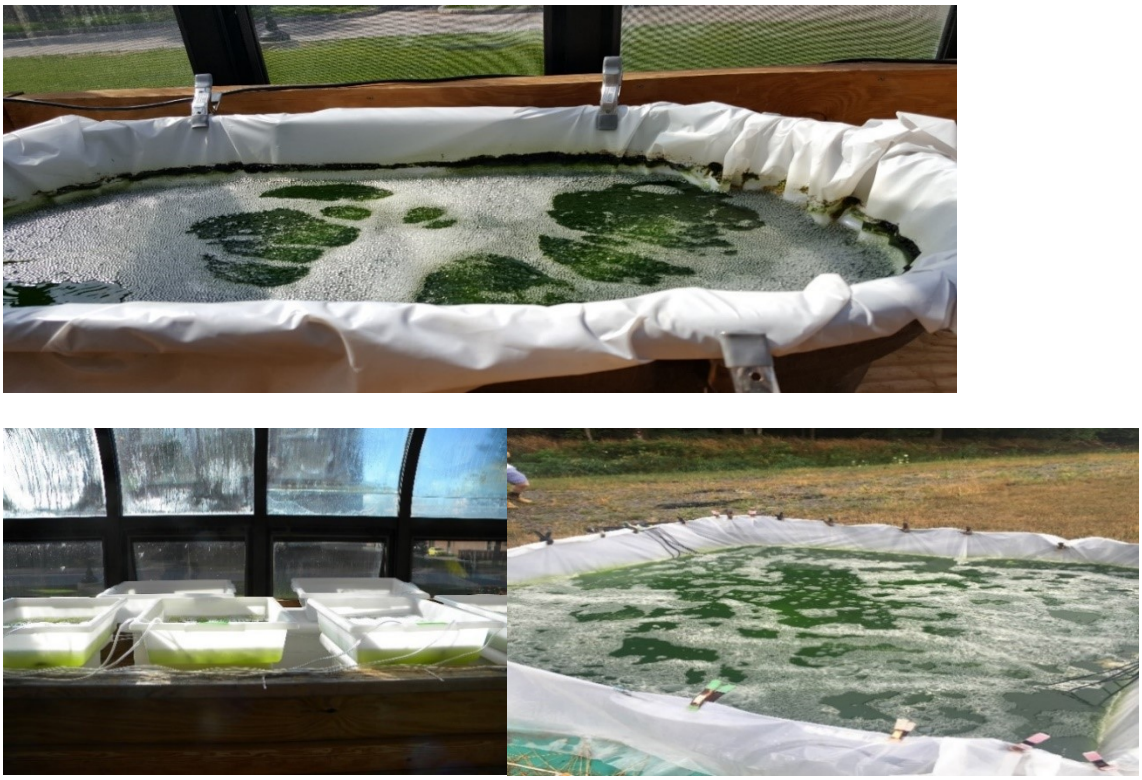


Figure 1.7 High Rate Algal Pond treatment at RIT's Green house and on-site. Treatment in a 50-gallon tank (Upper). Treatment in 1**5**L basins (Lower left) and treatment in 1220-gal tank onsite at Synergy LLC (Lower right)

For this research, a 1220 gallon tank was constructed onsite at Synergy Biogas in Wyoming County to serve as a study site. An 80 gallon volume of algae would be cultured in the Greenhouse and transported to site for use. To sustain a constant nutrient supply for algae growth, NutriCote 13:13:13 in pond water were used for culturing. Transported algae would then be inoculated into a 1220 gallon gallon tank with diluted ADE in batch culture with constant aeration at a rate of 440L/m. Environmental conditions such as light intensity, temperature, wind intensity, and humidity, and water depth would be monitored together with other parameters of interest at 2-day interval over 30 days.

1.6.5 ALGAE BIOMASS HARVEST AND LIPID EXTRACTION

Centrifugation was used as the main harvesting technique for fully grown algae from the treatment bioreactors. In open ponds and large-scale facilities, biomass may be harvested through gravity settling induced by pH. Biomass from photobioreactors is centrifuged at 25^0C at 2500 RPM for 5 minutes with Allegra™ 6R ALR 03b254 Centrifuge from Beckman Coulter (fig 1.8). Resultant pellets are collected and oven dried with VWR International® oven at 80^0C-90^0C for 72 hours in glass bowls for the lipid extractions stage. Treated water is then discharged.

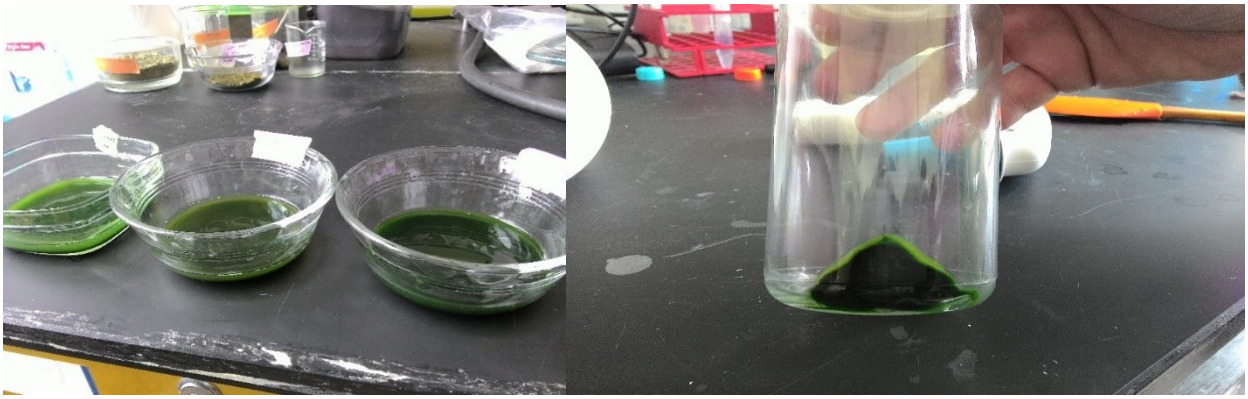


Figure 1.8 Harvested algae after centrifugation

Lipid extraction from algae biomass is performed by crushing dried algae in a mortar and pestle and the biomass is dissolved in a 3:2 hexane -isopropanol solvent. The mixture containing 20g of algae in 150 ml of hexane:isopropanol is shaken vigorously at 150rpm in an auto shaker Max Q 4000 instrument from Barnstead Labline® for 24hours and filtered. The filtrate is added to a separatory funnel, 40ml of hexane and 40ml distilled water is added. The addition of hexane-distilled water is to form a two-phase system with the more denser isopropanol-water layer at the bottom while a lipid-hexane layer is at the top (figure 1.9). Dissolved isopropanol in water is collected and discarded while dissolved lipid in hexane is collected into a beaker and

placed in an evaporator. The isopropanol-water mixture was tested for triglycerides before discarding to ensure no quantity of triglycerides was lost. Volatile hexane evaporates leaving lipids in the beaker. In a commercial production situation, hexane may be reclaimed through distillation apparatus for reuse.

1.6.6 ULTRASONICATION OF ALGAE CELLWALLS

Algae biomass disruption was tested using ultrasonication techniques to disrupt cell walls with the aim of maximizing lipid yield from algae cell walls. Algae were exposed to different magnitudes (0, 180, 280, 380, and 480 secs) of sonic waves in an attempt to break open cell walls before lipid extractions. A second experiment treated biomass longer sonication times (480, 960, 1440 secs) using a 160W Aucma ultrasonic device SU-767 from Intertek® and their effects on lipid yields compare (Fig 1.9)

A) **Sonicated Biomass** B) **Hexane-isopropanol extraction of lipids**

Figure 1.9: Lipid extraction from sonicated algae biomass using organic solvents
A. Sonicated biomass B. Lipid extraction using hexane-isopropanol

1.6.7 THIN LAYER CHROMATOGRAPHY (TLC)

Thin Layer chromatography was used to assess the quality of lipids extracted from the algae biomass for biodiesel. TLC analysis is used to separate non-volatile lipids into its constituents using a TLC Silica gel F_{254} (5 X 20cm) plates obtained from VWR Wheaton. The goal of this step is to assay the composition of algae lipids especially its triglyceride to FFA content. Lipids may be composed of free fatty acids (FFA), monoglycerides, diglycerides and triglycerides which may influence the quantity and quality of ester formation into biodiesel. For TLC analysis, the solvent was 85:15:2 (hexane: ethyl ether: glacial acetic). Lipids were then dissolved in small amount of solvent. An origin is made at 2cm length on the plate, and a 2µL-8µL of the sample is spotted at the origin and allowed to dry. The silica plates were placed in the developing tank containing 100ml of the solvent after 20 minutes of equalization and allowed to run for as long as 1 hour such that the solvent travels ¾ of the distance of the TLC plates. The plate is then air dried and sprayed with a detection spray made of 10g of $CuSO_4$ dissolved in 10% phosphoric acid. After drying, the plate is then placed a Napco® Vacuum Oven 952 at a pressure of 18-20 Hg for 10 minutes at a temperature of 90-100^{0}C to char the lipids.

1.6.8 CARBOHYDRATE EXTRACTION- QUALITATIVE AND QUANTITATIVE

Algae biomass does not only contain lipids but is also rich in carbohydrates which can be extracted and fed to yeast to produce Bio-ethanol. 20g of lipid extracted algae is added to the 250ml flask and 100ml of 2% sulfuric acid is added. The mixture is then placed in an oven at 105 ^{0}C for 4-8 hours after which it is allowed to cool. Residual algae are separated from the carbohydrate-rich supernatant by filtration, and the solution's pH is adjusted to 6.0-6.5. The

filtrate collected is then quantified as described below and the remaining algae biomass can be composted or added to a biodigester to boost methane production.

QUANTIFICATION OF SUGARS FROM ALGAE BIOMASS

A 0.4 ml of a 1:1000 dilution of the Carbohydrate extract is placed in a test tube and mixed with 0.4ml of 5% phenol, 2.0 ml of concentrated H_2SO_4 is then added to the mixture which results in orange coloration. The mixture is read at 490nm wavelength upon cooling using Clinical Diagnostics Spectrophotometer 554142, with 0.4ml distilled water instead of carbohydrate sample to serve as a blank. The value obtained is then compared to a standard curve to obtain the concentration of the carbohydrate. The standard curve was made using pure glucose at 2%, 4%, 6%,8% and 10% which were taken through the same procedure and their respective absorbence recorded at 490nm. Table 1.1 shows some selected algae and qualitative analysis of their cell wall constituents.

TABLE 1.1 Representative algae and their cell wall composition

MICROALGAE	CELLWALL COMPOSITION	REFERENES
Boytryococcus braunii	Cellulose, hemicellulose, glucans	Uno et al.; Wiess et al., 2012
Chlorella sp.	Glucosamine, galactose, Rhamnose, Arabinose, Uronic acids,	Taked et al.; Baudelet et al., 2017

	rhamnopyrnose, glycopyranurosyl	
Scenedesmus spp.	Hydrocarbons,unsaturated fatty acids, glycoproteins, carboxyl groups, neutrl sugars, uronic acids	Allard et al, 2002 Voigtet et el 2015
Nostoc sp.	Rhamnose, mannose, galactose , glucose A	Delattre et al 2016

1.6.9 FERMENTATION OF ISOLATED ALGAE CARBOHYDRATE BY *SACCHAROMYCES* AND *KLUVEROMYCES*

To test whether algae sugars can be fermented by yeast to produce bioethanol, extracted sugars were fed to *Kluveromyces marxianus* and *Saccharomyces cereviciae,* ethanol producing yeast strains. Seed cultures were made by adding 50ml of SAB into a sterilized 250ml flask and adding a loopful of yeast cells and then shaken for 1-2 days in MaxQ 4000 auto shaker at room temperature. 5.0ml each of culture is centrifuged and suspended in 5.0ml of sterile saline (0.9%NaCl). 5ml of *Saccharomyces* and *Kluveromyces is* cells are added to 45ml of extracted sugar in a 250 ml flask, and the fermentation is run at room temperature and shaken at 130 rpm. To test whether the additional protein is required for the yeast fermentation, 0.5g of peptone is added to the 50ml culture. The pH of the Yeast fermentation was between 5.5-6.0.(table 1.2)

TABLE 1.2 YEAST FERMEMNTATION PROCESSES

FLASK	CELLS	Amount of cells/ ml	Amount of carbos/ml	Peptone in grams
A	*Kluveromyces*	5.0	50.0	0.5
B	Saccharomyces	5.0	50.0	0.5
C	Kluveromyces	5.0	50.0	0
D	Saccharomyces	5.0	50.0	0
E	No yeast (control)	0	50	0

Each culture is shaken at room temperature and 1.5ml of each culture including the control, removed roughly 3-4hr intervals for 33 hours. Each sample is centrifuged in microfuge tubes for 5 minutes at 13000 rpm using the VWR® Galaxy 16 C0170. 1 ml of supernatant of each tube is removed and diluted with 1000 parts of distilled water. Carbohydrate concentration was determined using already descried process above. The value is then compared to a pre-existing standard curve to determine the concentration of carbohydrates. A graph for carbohydrates concentration versus time is plotted to show carbos utilization by each yeast.

1.6.10. BIOCHEMICAL METHANE POTENTIAL

The Biochemical methane potential (BMP) is a standard method for testing substrates in small scale anaerobic digesters. Algae biomass was co-digested with dairy manure. Microcrystalline cellulose with 20-µm (Sigmacell type 20) used as a positive controls sample for system testing. The inoculum was obtained from a full-scale anaerobic digester operated at mesophilic temperatures that co-digested dairy manure with food related waste. The inoculum was pre-incubated at 37^0 C for 5 days to minimize gas production from undigested biomass. Samples were prepared to achieve an inoculum-to- substrate ratio (ISR) of 1:2 (gVS inoculum: gVS substrate added) to prevent biomass limiting kinetics. The total solids content of all samples was less than 3% in prepared samples, and the dairy manure inoculum provided nutrient requirements for anaerobic microorganisms. No additional external nutrients or elements were added, and the average pH range for the entire process was between 6.9 for the initial feedstock to 7.9 after the process run to completion. Feedstocks were flushed with N_2 to create an anaerobic environment and incubated at 37^0C with mixing rate at 10 sm^{-1}. The 500ml BMP vessels were used with working volumes ranging between 300-400ml. Biomethane production from substrates, blanks, and controls were all operated under standard temperature and pressure of 0^0C and 1 atm respectively. The BMP assay was conducted for 33 days after which biomethane production climaxed. All samples and blanks (only inoculum) were run in triplicate, and actual Bio-methane production was obtained after subtracting values of the blank the experiments from that from substrates.

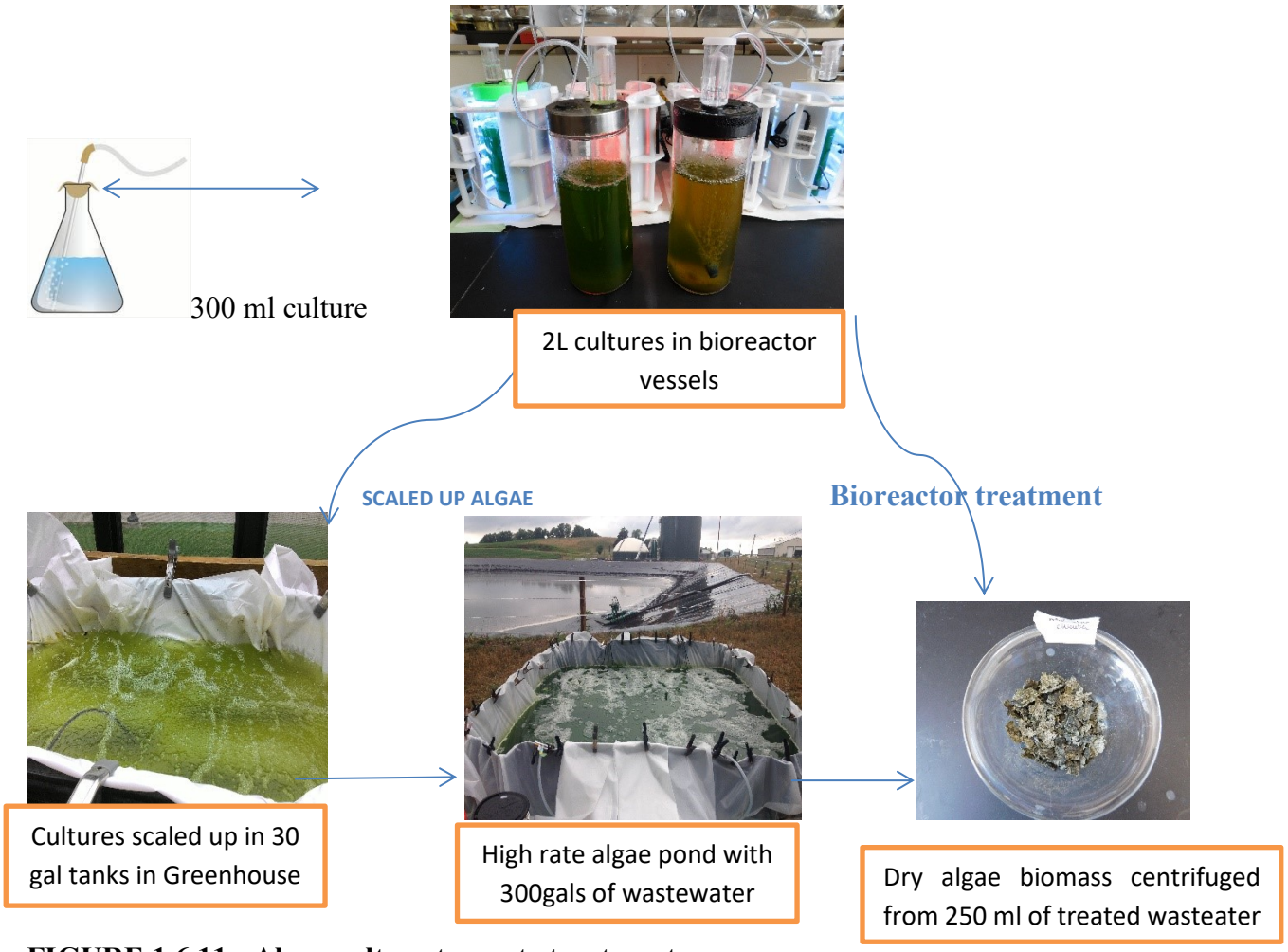


FIGURE 1.6.11.: Algae culture to waste treatment

From fig 1.10, startup cultures were initiated in 500ml vessels or conical flasks which is later scaled up to 1.5L in 2L vessels using Bristol salts as growth medium. Matured algae cultures were added to wastewaters in a 1:10 ratio (algae: waste). For field trials, cultures were scaled up in 60 gal tanks in the greenhouse which is later transported to the site to be applied onto agricultural wastewaters. Biomass from treatment tanks was harvested and brought to the lab for further analysis.

CHAPTER TWO

ANALYSIS OF THE DIFFERENT WASTE STREAMS

2.1 BASELINE CHARACTERIZATION OF THE DIFFERENT WASTEWATERS

It was imperative to ascertain the various pollutant levels in the AD digestate and other wastestreams before treatment. Initial site visits, sampling, and analysis of ADEs from all three stabilization lagoons located on-site at Synergy CH4-biogas were carried out. Concentrations of ammonia, nitrate, nitrite, Iron, and pH were determined using the Hach test kit if the needed samples were diluted with distilled water to be in the proper range of test kits. The pH probe was calibrated daily within ranges of 7.0- 10.5 before pH readings for quality assurance.

Table 2.1 Concentrations of various parameters of digester effluents from Synergy's three storage lagoons

	ANAEROBIC DIGESTER EFFLUENTS				
	NH_3 ppm	NO_3 ppm	NO_2 ppm	PO_4 ppm	Fe ppm
storage lagoon 1	2145	0	0	330	553
storage lagoon 2	1922	0	0	330	393
Storage lagoon 3	1789	0	0	318	190

Table 2.1 shows, high ammonia levels between 1700 ppm-2000 ppm were recorded as compared to phosphate and Iron which shows concentrations of 300 – 400 ppm. However, all three concentrations are higher than USEPA, and NY State standards especially 0.075 ppm and

0.3 ppm for phosphorus and Iron respectively which justifies the need for urgent remediation. ("USGS Fact Sheet 2010–3078: Nutrients in the Nation's Streams and Groundwater: National Findings and Implications," n.d.). Although the USEPA has not estabiliahed clear cut-off concentrations for these nutrients, it has recommended limiting nitrate in discharged effluents to 10ppm and a near zero tolerance for phosphorus as these nutrients are triggers for harmful algal blooms. From the Table 2.1, there is absence of Nitrates (NO_3) and Nitrites(NO_2), but high ammonia (NH_3) is a cause for concern as ammonia can be nitrified to nitrate to cause eutrophication in waters. In addtion, successive gravity settling from lagoon 1 to lagoon (3) has little to no impact on nutrients concentrations but rather on Total Suspended Solids (TSS). This research, therefore, focused on treating lagoon three effluents due to its reduced TSS, less murky which otherwise, could cause photo-inhibition during treatment processes.(Fig 2.1)

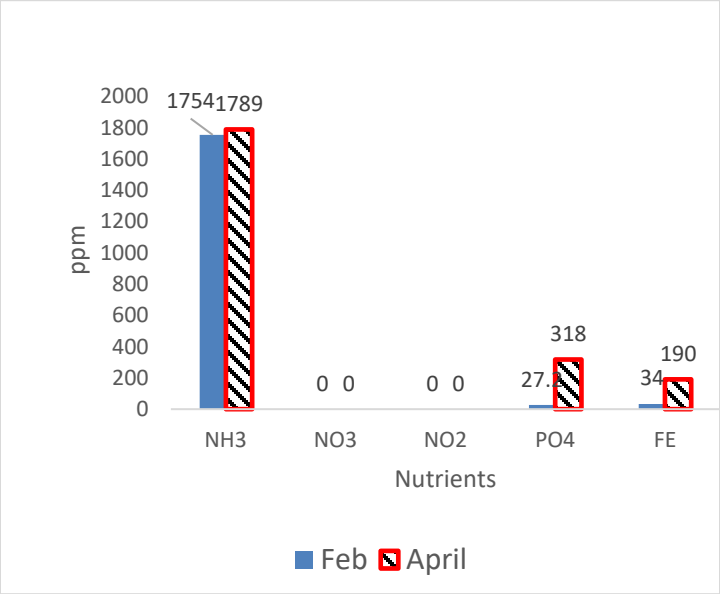


Figure 2.1 Nutrient variations in lagoon 3 between February and April of 2016

Figure 2.1 goes a step further to access how nutrients vary over time between February to April 2016 within Synergy's lagoon 3 ADE. While there was no significant difference in NH3 concentration, PO4, and Fe concentrations were higher in April compared to February.

TABLE 2.2 CHARACTERIZATION FOR VARIOUS WASTEWATER TYPES SAMPLED FROM PARTNER INDUSTRIES

EFFLUENT / PARAMETER	USEPA STANDARDS	A.DE	FETA CHEESE WHEY	TOFU WHEY	EGGWASH + DET	EGG WASH WASTEWATER	GREEK YOGURT WHEY
AMMONIA (NH3) in ppm	1.9 mg TAN/L (EPA 2015)	1789	49.8	60.5	335	1037	28.47
NITRATES ($NO3^-$) in ppm	10 ppm (EPA 2018)	0	82.9	33.8	308	792	30.8
PHOSPHATES ($PO4^{-2}$) in ppm	< 0.1 ppm	318	33	15.3	23	350	13.2
Nitrite ($NO2^-$) in ppm	1ppm (EPA 2018)	0	0	0	0	0	0
Iron (Fe)	0.4ppm (USEPA 2015)	190	7.2	2.6	0.3	45	0.73
pH	6.0-90	9.29	4.65	4.3	8.47	6.45	4.16
Hardness (Caco3)	<60ppm	4000	-	-	-	-	-
Salinity in ppt	<1200 ppt in drinking water and <3000ppt in fresh water (epa.gov 2018)	30	-	-	-	-	-

From Table 2.2, higher than acceptable concentrations of nitrate nitrogen (NO_3-N) in both eggwash wastewater types were recorded. 300ppm and 780ppm were recorded in detergent and detergent free egg wastewater respectively. Tofu, Yogurt, and Feta cheese whey all recorded some level of nitrate between 30-80 ppm, but ADE showed no nitrates. Phosphate was highest in egg wastewater and ADE with both having concentrations between 300-400 ppm, but

other wastewater types recorded relatively lower phosphate levels with a range between 13-35 ppm. Nitrate and phosphate are major plant nutrients. Hence, exceedingly higher concentrations in discharged effluents could cause eutrophication and uncontrolled algal growth in receiving waters. Significant Iron levels were observed in egg wastewater without detergent and ADE as other wastewater types showed negligible amounts. Although there is no definite toxicity from Iron in the environment, concentrations betweeen 5- 200 mg/l aqeuous Fe concentration may be fatal to plant communities ("Iron (Fe) and water," n.d.).

2.2 MICROBIAL DIVERSITY IN ADE WASTEWATER

Pretreatment analysis of ADE effluents was extensive to include assesssment of microbial community on the wasewater. Microbes play a critical role in nitrification and break down of organic matter during treatment under both aerobic and anaerobic conditions. In one study, microbial granules were used to treat abattoir wastewater which caused a reduction in phophorus by 97% (Lemaire, Webb, & Yuan, 2008). Tryptic Soy Agar is commonly used to enumerate heterotrophic populations in soils, water, and wastewater.

2.3 DISCUSSION OF RESULTS

2.3.1 SYNERGY'S A.D.E

Assessment of pollutant levels in digestate from Synergy's three storage lagoons all exceeded the permissible limits for any wastewater treatment plant in New York state. Ammonia ranged between 2145 ppm in storage pond 1 to 1789 ppm in the lagoon (3) which meant discharge through WWTPs for treatment was not possible. High ammonia resulted from excreta discharging directly from the dairy farm into lagoon 1. According to the farm owner and manager John Nobles, only 20% of dairy waste is co-digested in the Anaerobic digester to boost methane

production (personal communication, 2nd December 2017). This revelation implies that the bulk of the dairy waste is directly discharged and stabilized in storage lagoon 1. Although total ammonia concentration is dependent on conditions like pH, temperature and moisture, treating manure waste in Anaerobic digester had been documented to significantly increase ammonia concentrations and volatilization (Evans et al., 2018). High ammonia levels disrupt local air quality and can be toxic to aqautic life through acidification and eutrophication in waters, whereas low concentrations in soil and water could also be beneficial for nutrient cycling. Synergy Biogas receives different foodgrade waste from Foodlink, surrounding restaurants and other food industries in Western New York which could contribute to the presence of high phosphorus and Iron in the digestate. Also, digester conditions like pH variations, moisture, and temperature, could all result in the High PO_4 (318-330ppm) and Fe (190- 553 ppm) levels. No nitrate nitrogen (NO_3-N) and nitrite (NO_2-N) were recorded in the digester effluents.

Intermittent qualitative and quantitative analysis of the digestate carried out over the Winter, Spring, Summer, and Fall of 2016 continued to reveal higher than normal levels of NH_3, Fe, and PO_4 but no nitrates which are characteristic of the type of waste constantly fed to the digester. Although seasonal variations for NH_3 was not signifiant as shown in Fig 2.1, variations in PO_4 and Fe were significant. These variations could result from the diverse biodegradable waste fed to the digester in addition to the roller coaster operational behaviour of the digester during peak and off-peak times. Similarly, high Iron (Fe) content in digestate could stem from the possible use of FeO or $Fe(OH)_2$ coated woodchips as solid scavengers for hydrogen sulphide removal H_2S during the biogas upgrading and cleaning process (Greer, 2010).

Gravity sedimentation across the three storage ponds caused slight reductions in NH_3, and PO_4 levels but signifiantly reduced Fe levels by 65%. The Fe could have precipitated and be sequestered into solids removed through gravity settling. Stabilizing effluents in storage ponds is a primary treatment technique used to reduce BODs and TSS drastically. The pH of the digestate ranged from 8.5- 9.2 which favors microbial communities expediting the nitrification process during treatment. Ammonia nitrogen was the predominant nitrogen type in the ADE before treatment representing total kejeldahl nitrogen (TKN) of 1789 ppm which was high enough to intoxicate and cause eutrophication in receiving waters bodies if untreated.

2.3.2 FETA CHEESE, TOFU, AND GREEK YOGURT WHEY

Whey from cheese, yogurt, and tofu are generated in high volumes along the production chain of which a fraction is channelled to Synergy's Anaerobic digester to boost methane production. Other conversions to animal feed and bioethanol as a clean fuel source are also currently being explored (Hegde et al., 2018), but a large fraction of whey from these processes remains an environmental issue. Also, transport of whey to digester facilities incurred additional production cost. The study carefully analyzed the different whey seperately to ascertain their potential to serve as growth media for algae cultivation and subsequent remediation. Nitrate-nitrogen (NO_3-N) was detected to be 82.9 ppm in cheese whey and relatively lower in Greek yogurt, and tofu whey is showing approximately 31 ppm and 34 ppm respectively. Nitrate in the different Whey all exceeded US EPA levels for discharge into waters, and their low pH (4.1-4.7) rendered them unsuitable to be channelled into POTWs. Wastewater treatment plants operate within a stringent pH range of 6.0- 9.0 for which the whey could cause pH imbalances and hence disrupt treatment efficiencies. Total Kejeldahl nitrogen (TKN) across the three different whey was 50mg/l for cheese, 61 mg/l for tofu and 29mg/l for greek yogurt, so total nitrogen (Total-N) was

found to be 133mg/l for cheese, 60mg/l for greek yogurt and 95mg/l for Tofu. Total nitrogen in the different whey can be used as growth media for algae to reduce nitrates levels to permissible levels for discharge. Phosphate levels in the three different wheys were approximately 33ppm, 16ppm, and 14ppm for cheese, tofu, and greek yogurt. These figures far exceed allowable limits for discharging wastewater which needs to be remediated. The acceptable limits for total phosphate-phosphorus (Total PO_4-P) for any fresh water systems are at 0.1mg/L above which accelerated eutrophication would be inevitable ("Lead, Plumbosolvency, and Phosphates in the Environment," 2016). Microalage are capable of utilizing the phosphorus in the whey to levels which can then be applied to soils for irrigation and fertilizer without risking the health of surface and ground waters.

2.3.3 EGGWASH WASTEWATER

Both eggwash with (EW+det) and without detergent (EW) were sampled from Kreher Farms and characterized which showed high levels of kejeldahl nitrogen (TKN) of 335ppm and 1037 ppm respectively. Also, Nitrate-nitrogen for EW+det and EW were determined to be 308 ppm, and 792 ppm respectively bring total nitrogen in both wastewater types to 643ppm and 1829 ppm respectively. The total nitrogen in both wastewater types makes them sustainable media for microalgae growth while remediating the eggwash wastewater. Moreover, phosphate phosphurus (PO_4-P) recorded for EW+det was 23 ppm as compared to 350ppm for EW, therfore, rendering both effluent types inappropriate for direct discharge. Nutrient levels in EW were significantly higher than that of EW+det due to the former resulting from the first phase of washing and cleaning of farm equipment. Daily routine cleaning in the pens and coop which contains debris from the feed, broken eggshells and fecal matter are contributory factors to high total kejeldahl

nitrogen (TKN) and phosphate in EW than EW+det. However, detergent usage for second phase cleaning of egg processing facilities resulted in high pH (8.47) for EW+det over that of EW (6.45) although both effluent types had pH values within regulatory limits of 6.0-9.0.

Fe recorded in EW was excessively high (45mg/l) and exceeded USEPA limits of 2.0mg/L as compared to 0.3 mg/l for EW+det. While the cause of high iron (Fe) content in the former remains unknown, wear-out of older machinery parts with other management pratices could contribute to the high Iron content. Over 75 % of egg processing facilities in the US utilize about 4.4gals/min of fresh water in eggwashing, and most farms and egg processing facilities regularly suffer closure by the USEPA due to their inability to meet regulatory discharge requirements (Northcutt, Musgrove, & Jones, 2005). Hence, The implementation of the algal treatment technology could be a relief to the poultry industry through pollution reduction and water recycling back to processing plants.

2.3.4 MICROBIAL DIVERSITY

The ADE effluents sampled from Synergy-CH_4Biogas was qualitatively and quantitatively assayed for their microbial diversity. TSA plates which are non restrictive and non selective contain an array of nutrients wide enough to enable a diversity of bacterial populations to grow. Higher TSA counts are indications of this diversity and associated role in wastewater treatment. Relatively high counts for TSA show the presence of heterotrophic bacteria population which play a critical role in nutrient transformation and CO_2 production for the algae (Table 2.3). Microbial diversity is good for nutrients transformation and BOD reduction through various microbiological activies. For instance, lithotrophic bacteria play a critical role in NH_3-NO_3 conversion under aerobic condiions.

Table 2.3 Bacterial culture counts in ADE

	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
D0 TSA*	2	9	87	TNTC*	TNTC
D3 TSA	1	12	191	TNTC	TNTC
D7 TSA	-	-	TNTC	TNTC	TNTC

TSA = Tryptone Soya Agar *TNTC = Too numerous to count

2.4 CONCLUSIONS

From the above analyses, it can be deduced that all the effluents types analyzed have shown concentrations extrememly above that which can be directly discharged hence the need for remediation efforts. Digester effleunts and egg wastewater contained high ammonia and Fe levels where as the different whey all exhibited high N and P with low pH. Also, microbial diversity in the biodigester effleunts is generally helpful for biological treatments of the wastewater.

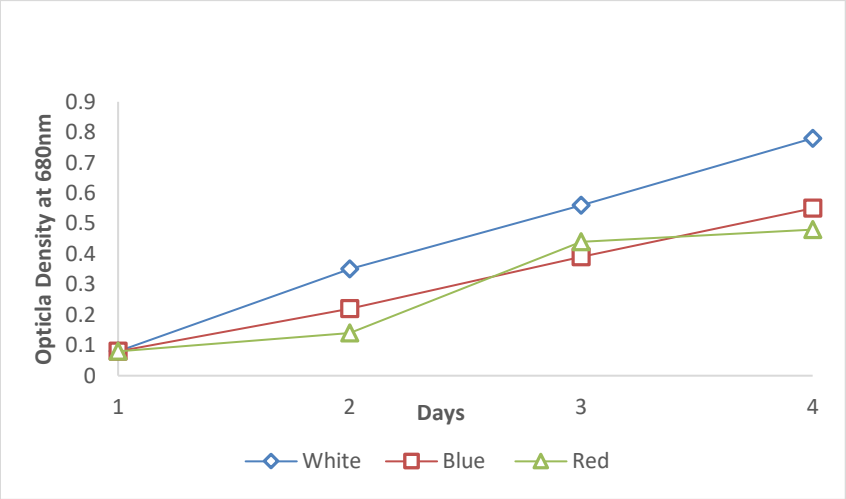
CHAPTER THREE

3.1 PRELIMNINARY ALGAE GROWTH STUDIES ON WASTEWATER

Intitial growth studies were carried out, and cell densities were measured at 680nm. Fig 3.1A shows Web3 microalgae growing under different light spectra on synthetic bristol salts as medium. Full spectrum white light maximized growth compared to blue and red lights individually. Fig 3.1b below further investigated the potential of egg wash wastewater to be used as growth media for algae cultivation and treatment purposes. Significant growth densities were recorded for four different algae with *Botyroccocus* sp exhibiting the highest growth and longevity over time. *Chlorella sp* and *Anbaena sp* showed steady growth over time, but

Scenedesmus sp exhibited stress and settled after just seven days of cultivation. Optical densities were translated into biomass per litre of wastewater as shown in table 3.1.

A.



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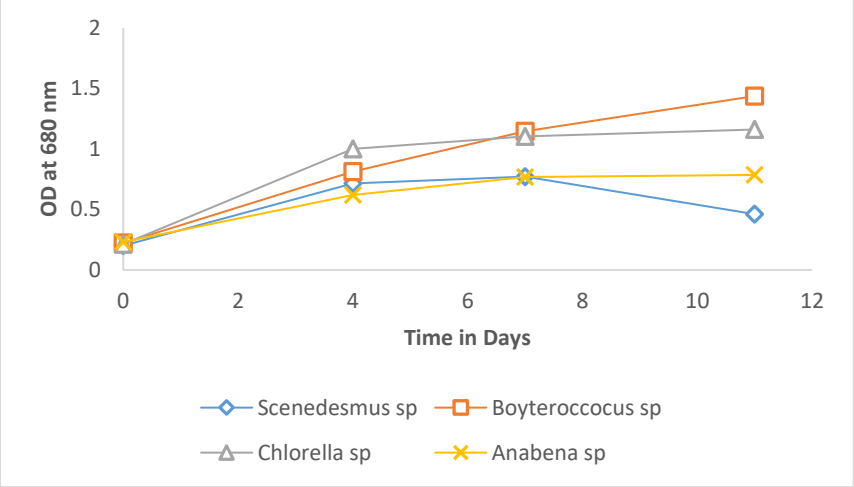


FIGURE 3.1 Growth studies of algae under various media

A)Web3 growing under different light spectra

B) The growth of various algae on eggwash

Table 3.1 Biomass **yield from various microalgae after growing on egg wasetwater.**

1 unit Optical density = 0.39 ±0.01 g/dry cell/L (Yoon, Shin, & Park, 2008)

	D0 g/dry cell/ L)	D4 g/L	D7 g/L	D11 g/L
Scenedesmusp	0.08	0.28	0.3	0.12
Botyroccocus sp	0.09	0.32	0.45	0.56
Chlorella sp	0.08	0.39	0.43	0.45
Anaebena sp	0.09	0.24	0.3	0.31

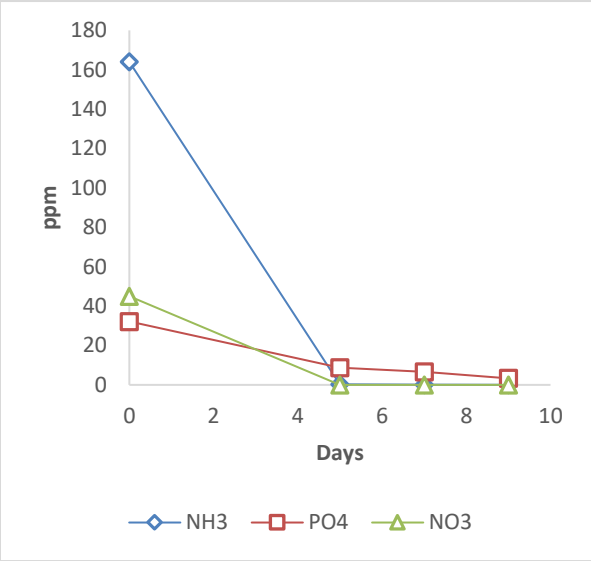
3.2 ADE TREATMENT WITH MICROALGAE

3.2.1 SMALL SCALE TREATMENTS IN BIOREACTORS

This research investigated how various microalgae can reduce nutrient levels. *Botyrococcus sp, Chlorella sp., Scenedesmus sp,* and Web3 were grown on diluted lagoon 3 ADE, and the nutrient reduction was monitored over time. Treatments were carried out in 2L bioreactor vessels at an initial pH of 7.2-7.4 under 16:8 light -dark daily cycle. *Botyrococcus sp* and *Chlorella sp* reduced PO_4 (70%), NO3 (99%), and NH_3(98.9%) below 10 mg/l in a relatively short Residence Time (RT) of 5 days. For *Scenedesmus sp*, NH_3 was reduced at below 10 mg/l at RT of 5 days while NO_3 and PO_4 achieved the target reductions at HRT of 7- 9 days. Nutrient reduction rate for Web3 on ADE was relatively low with a very low nitrate uptake rate of 57% and 40% for PO_4. Nutrients uptake by individual algae is considered efficient if levels are reduced below the 10-ppm threshold to enable secondary uses of the treated water such as irrigation without much ecological risk. Although the NH_3 conversion was equally efficient, nitrates uptake by web3 was slow as compared to the other algae. (Fig 3.3.D)

There were rises in pH identified with all the species from 7.5- 9.0. Rising pH is indicative of algal growth and activity occurring in the process of treatment. Also, the algae use carbonates as CO_2 source and release OH^- in the process which all contributes to the rise in pH.

A)



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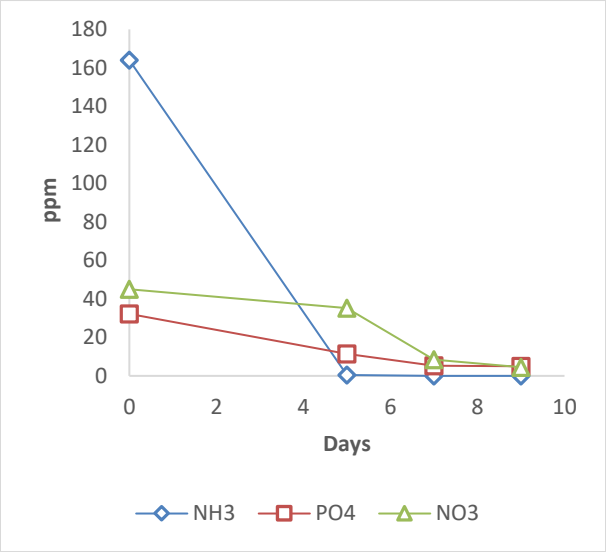

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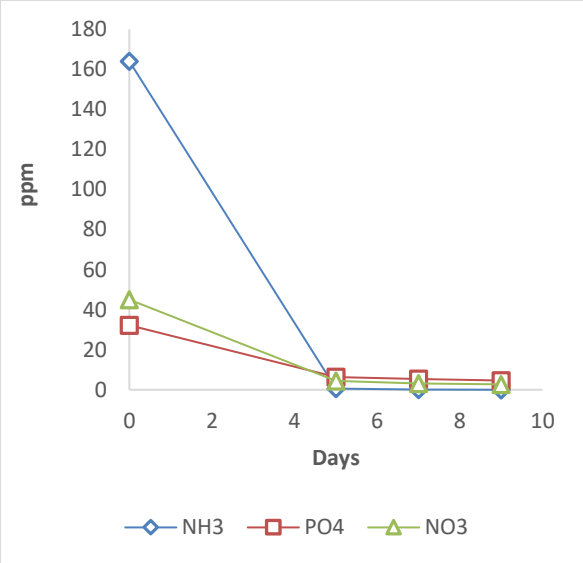

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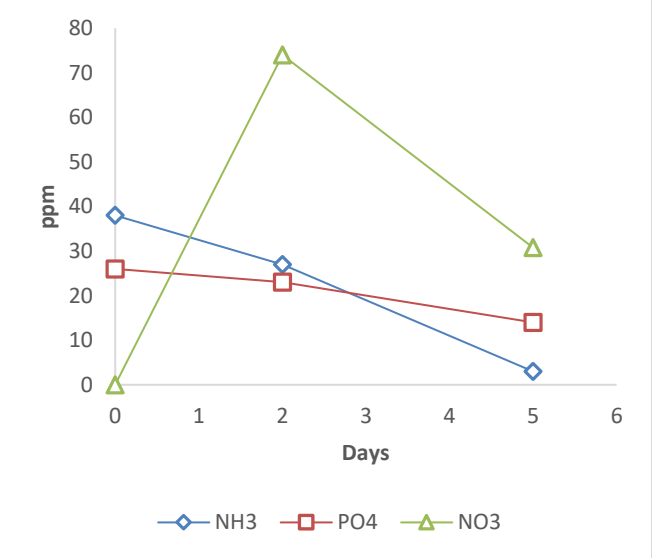

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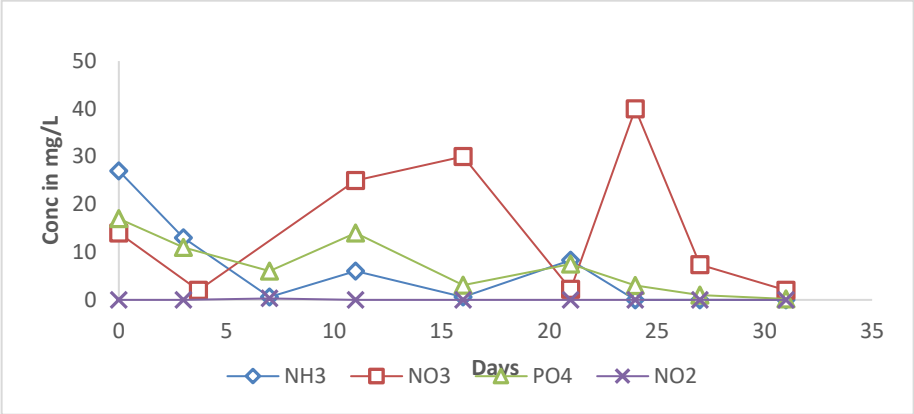

Figure 3.2 Nutrients reduction by various algae on ADE

**top up with 300 ml of fresh ADE on day 11 and day 21

A) *Botyroccocus sp* on ADE C) *Chlorella sp* on ADE E) *Nostoc* sp on ADE
B) *Scenedesmus sp* on ADE D) Web 3 on ADE

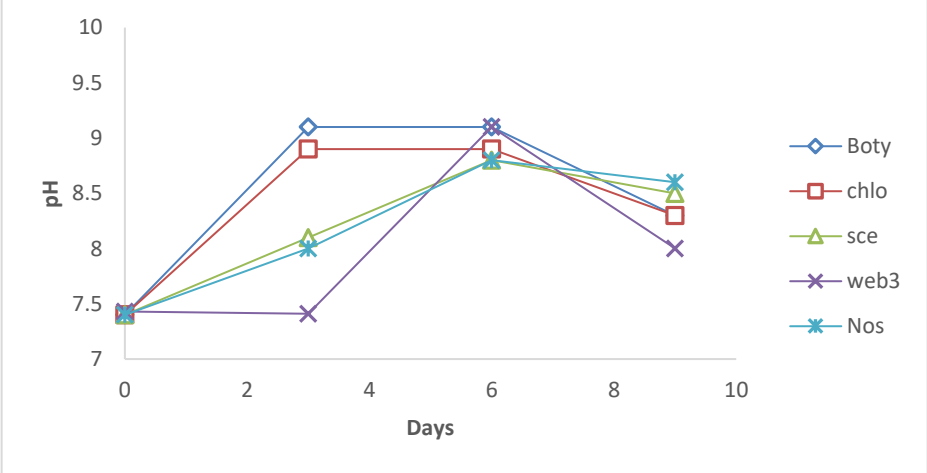

Figure 3.3 Changes in pH during mutrient reductions of ADE by various microalgae

Filamentous *Nostoc sp* was tested for a nutrient reduction on ADE in a 30-day cycle in a 1:100 ratio of ADE- pond water bioreactor system. Nitrate (NO3) and ammonia (NH3) were drastically reduced at day 3 and day seven (7) respectively. Occasional top -up with 300ml fresh

ADE was necessary to sustain algal growth in the medium after nutrient depletion. Top-ups resulted in spikes in nutrient levels as evident on day 11 and day 21. Phosphate reduction by Nostoc below threshold was achieved at day 7 RT and significantly reduced at day 16 RT.

3.2.2 EXTENDED TREATMENTS IN THE GREENHOUSE

Three algal strains -*Anabaena sp, Scenedesmus sp, and Chlorella sp* were all tested for ADE treatment for extended periods in the Greenhouse in 15L tanks. *Anabaena* sp and *Scenedesmus* sp were grown on 1:5 ratio of ADE-to-pond water dilution with 10% by volume of algae inoculated onto each system. Chlorella sp, however, was the only exception which was grown on a blend of ADE, Feta Cheese Whey, and Yoghurt Whey in a 70:15:15 ratio. An initial working pH of 7.2 under ambient sunlight. Bioreactors were run over 30 days as sampling and testing were carried out at 3-4-day intervals. Results and analysis are shown below in the graphs and tables.

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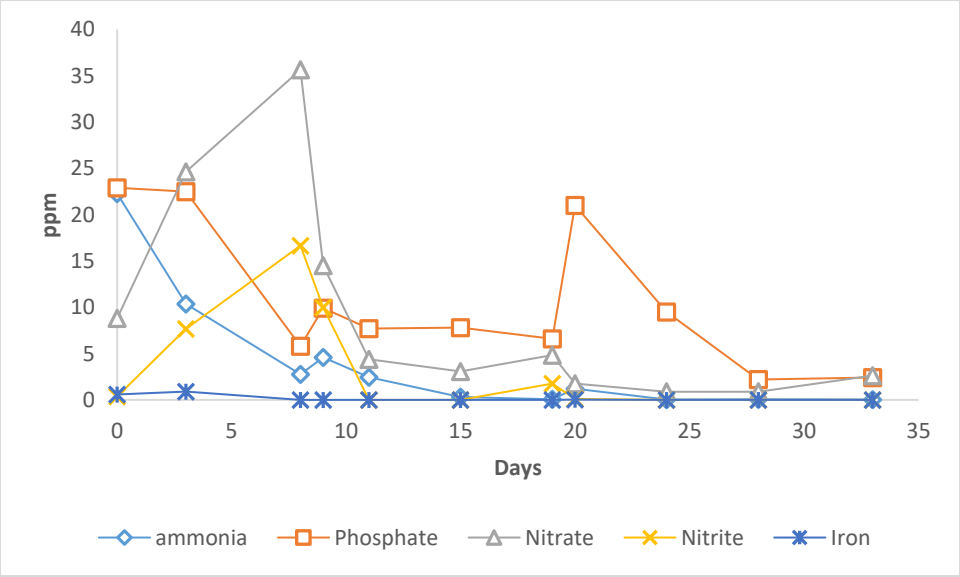

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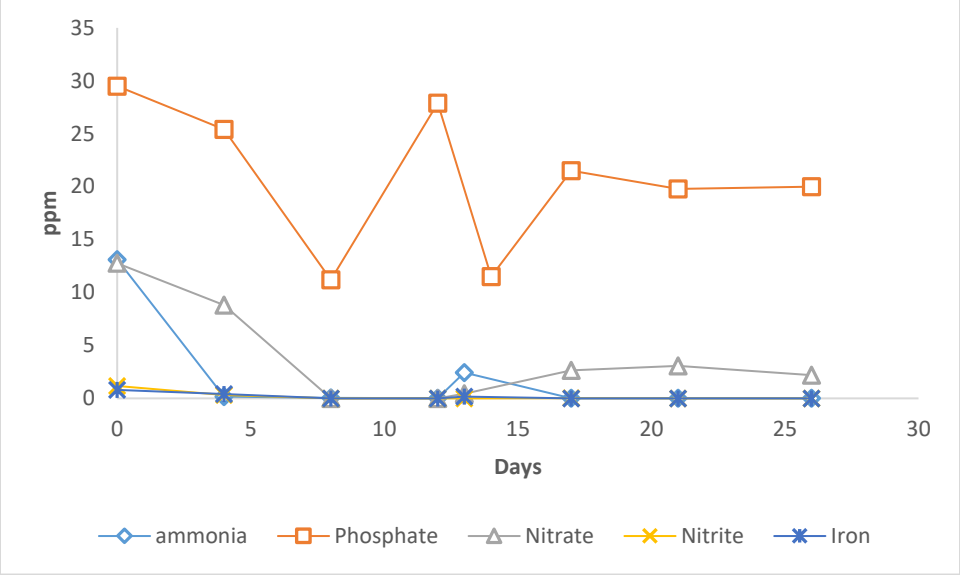

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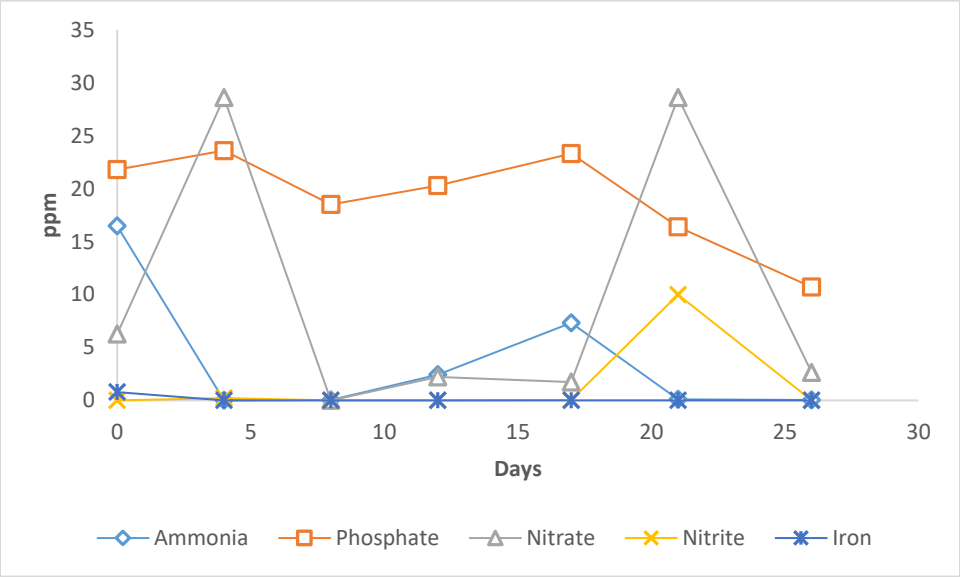


FIGURE 3.4 Prolonged treatment of ADE by various microalgae

A) *Scenedesmus sp* on 1:5 diluted ADE
B) *Chlorella sp* on 70:15:15 ADE: Feta cheese whey: yogurt whey
C) *Anabaena sp* on 1:5 diluted ADE

In all the above set-ups, Fe was swiftly removed from wastewater within an average of 5 days. *Chlorella sp* and *Anabaena sp* removed 99% NO_3 within eight (8) days residence time. NO_3 reduction by *Scenedesmus sp* was steady and slow over time achieving above 90% reduction at 24 days RT. However, total nitrogen reduction by *Scenedesmus sp* below the target concentration of 10mg/l was achieved on day 10 of residence time. Phosphate reduction was 50% at day 26 RT for Anabaena sp, as 70% and 60% reduction was achieved for *Scenedesmus sp* and *Chlorella sp* respectively at day 8 RT. Nitrite (NO_2^-) is a transient compound whose formation and conversion to Nitrate by nitrifying bacteria occurs within a very shorter period. All bioreactors recorded a sharp rise in pH values to 9.0 within four days due to the absorption of CO_2 from bicarbonates in wastewaters, hence causing a rise in pH values (data not shown).

3.2.3 TREATMENT ONSITE AND EFFECT OF PRE-TREATMENT DILUTIONS ON TREATMENT OUTCOMES

Treatment of ADE in HRAP onsite at Synergy LLC in the summer of 2016 achieved a remarkable outcome by reducing Odor, Nitrate, Phosphate, and Iron as shown below. Before field treatment, *Chlorella sp* was tested on ADE in bioreactors under different dilution ratios to optimize light penetration for effective treatment on site. *Chlorella sp* when grown on 1:25, 1:50 and 1: 100 of ADE to pond water ratio recorded the following results over 7 days as shown in table 3.3. Set ups with relatively high dilution factors exhibited higher treatment efficiencies compared to the least diluted ones. A further assessment investigated and compared algal remediation efficinecy of diluted and undiluted ADE with HRAP in different runs at Synergy's site.

The figure 3.5 and Table 3.2 below is an overview of general summer site and greenhouse conditions during treatment of effluents in the summer of 2016. Since these physical factors could influence treatment outcome, continuous measurement of water depth, pH, light intensity, and temperature were carried out.

A) B)

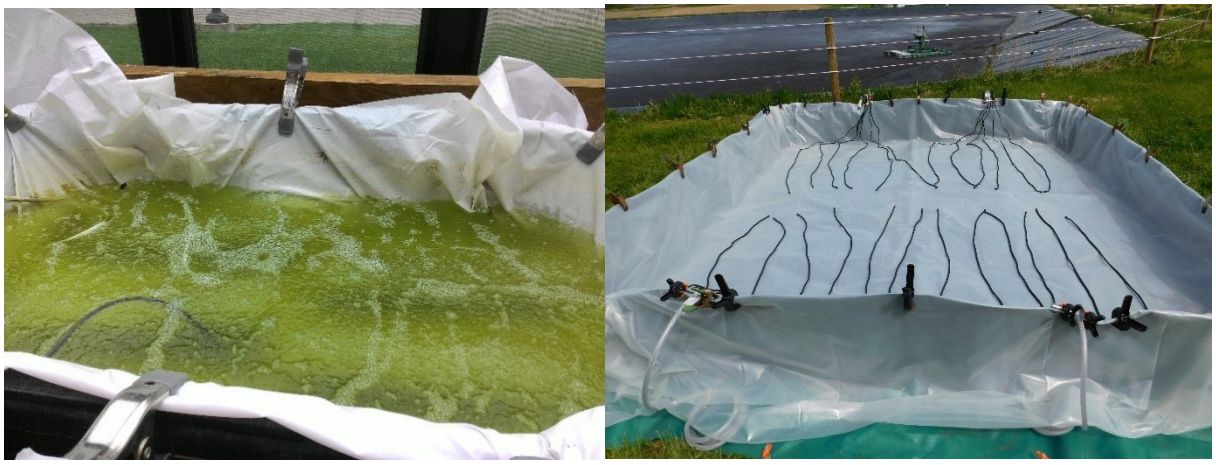


Figure 3.5 HRAPs of Chlorella sp growing on ADE

A) 50-gal greenhouse tank
B) 1220gal onsite tank

TABLE 3.2: Comparing Green house and Site conditions durng treatment

PARAMETER	RIT GREEN HOUSE	SYNERGY'S SITE
Light Intensity	217.4 – 252.2 w/m^2	452.2 w/m^2 – 521.7w/m^2
Water depth	-	5.0 inch (initial)- 2.2 inch(fin)
pH	7.3- 10.23	8.89 – 9.42
Water temperature	-	21.1^{0}C – 29^{0}C
Ambient Temperature		Cloudy with 22.2 -26.1^0 F
Observations	Water levels decline frequently	1. Foul smell reduces within four (4) days RT
		2. Water level declines rapidly from evaporation

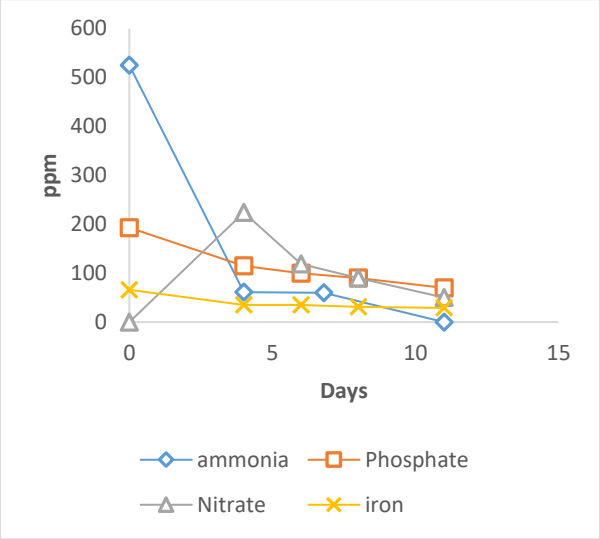
Footcandle(FC) * 0.2/ 4.6= watts/m

Dilutions influenced nutrient uptake by reducing pollutant levels in shorter residence time in diluted effluents than undiluted samples as shown (fig 3.6)

TABLE 3.3: EFFECT OF DILUTION ON TREATMENT EFFICIENCY WITH *CHLORELLA Sp* GROWING ON ADE

DILUTION	NH_3 (ppm)		PO_4 (ppm)		NO_3 (ppm)		pH	
	D0	D7	D0	D7	D0	D7	D0	D7
1:25	45	10	92	30	0	40	7.4	8.7
1:50	26	1	37	20	0.01	0.01	7.4	8.9
1:100	21	0	22	12	0.01	0.01	7.4	9.2

A)



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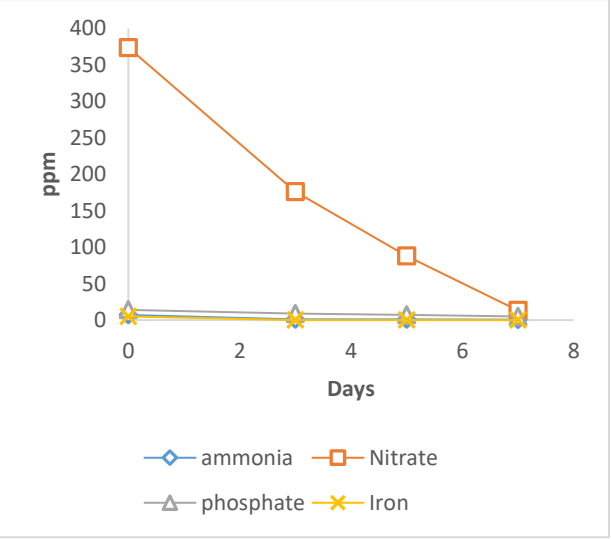

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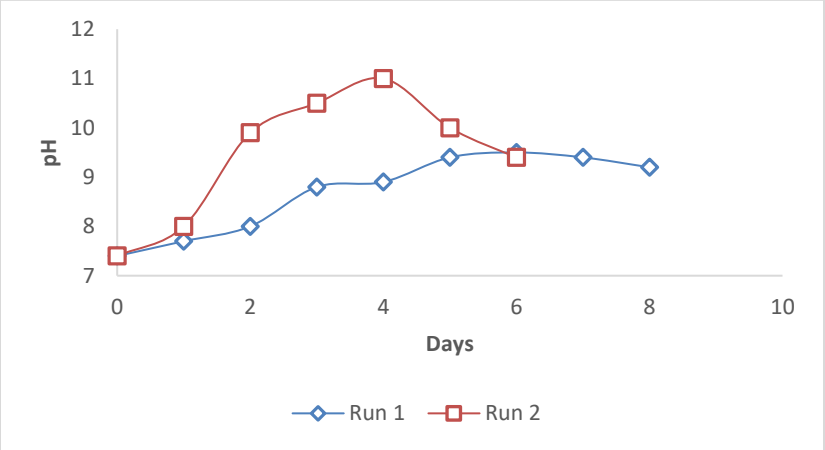


FIGURE 3.6: Nutrient reduction trends in diluted versus undiluted effluents in HRAP

A) Undiluted ADE with Chlorella sp
B) 1:10 dilution with Chlorella sp
C) pH changes

3.3 ISOLATION OF ADE ALGAE

A blank treatment of 1.0 L of 1:50 dilution of ADE was carried out in a bioreactor under 16:8 light-dark cycle for 10 to 14 days without any algae inoculation. The blank treatment turned green which was an indication of algae present in the biodigester effluents. The algae were harvested by centrifugation at 5000xg for 10 minutes. Extracted algae were then placed in Algae culture broth (ACB) for 7 days for further growth and storage. Growth rates for ADE algae under ACB, Bristol salts, and ADE were studied for 7 days. Results from growth rate studies are shown in (Fig 3.7.)

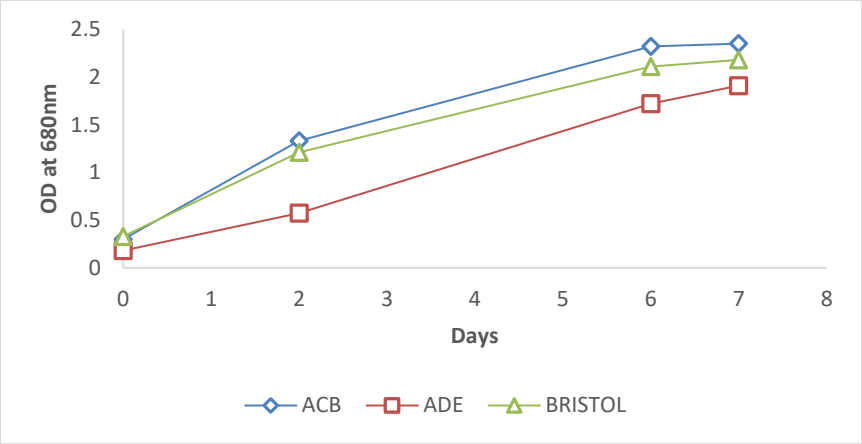
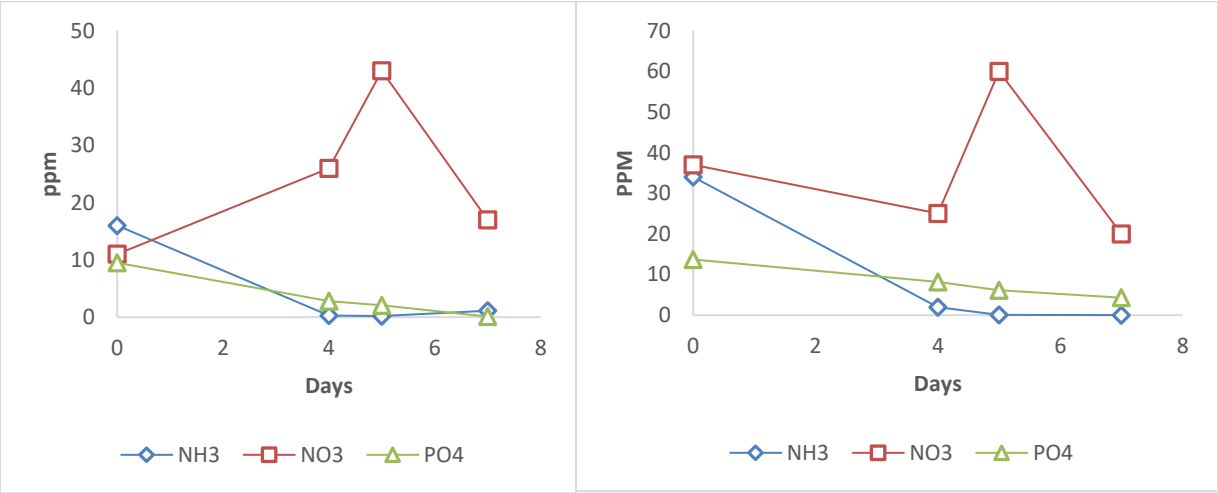


FIGURE 3.7: Growth rates for ADE isolated algae on various media

From the growth rates assessment, ADE algae have the potential to utilize both synthetic nutrients and ADE for growth. There was a steady rise in growth with ADE. The ADE might have taken some time to undergo NH_3 oxidation and nitrification by nutrifying bacteria to make nutrients available to the algae and hence the steady. The ADE algae were, therefore, grown on eggwash and ADE to examine nutrient removal capabilitities as shown in (Fig 3.8)

A) B)



FIRGURE 3.8: Nutrient reduction by isolated ADE algae on ADE and Egg wash wastewater.

A) Egg wash Wastewater Treatment B) ADE treatment

From the Fig 3.8, it can be seen that the ADE algae have the potential to reduce nutrients in waste water within a relatively short residence time. Nitrates were reduced by 50% at day 5 RT for both wastewater types while PO_4 removal for eggwash was 70% at day 4 as compared to ADE. In comparism to the previous experiments, NO3 uptake was not as efficient in both wastewater types which could indicate that ADE algae prioritized PO_4 uptake over NO_3 in the treatment set-ups.

3.4 DISCUSSIONS

3.4.1 ALGAE GROWTH STUDIES

Web 3, a locally isolated polyculture micro-algae from a Wastewater treatment plant in Webster NY was grown on Bristol salts (synthetic nutrients) under different light intensities and growth rate was determined by OD at a 680nm wavelength as shown in fig 3.1(a). The growth rate was the highest under white light compared to the blue and red light spectra. In the case of red light, intermittent lag phase in growth was observed. Microalgae are photosynthetic organism whose growth does not depend solely on nutrients availability in wastewater but also the presence of light and temperature among other physicochemical factors. Excessive illumination during algal treatments could result in oxidative stress while the optimum ligh-dark stage cycles allow for re-oxidation, thereby, preventing radiation stress and impeded photosythetic activity (Sforza, Simionato, Giacometti, Bertucco, & Morosinotto, 2012).

Moreover, *Anabena sp*, *Chlorella vulgaris*, *Scenedesmus sp,* and *Botyroccocus braunii* were cultivated on eggwash wastewater over 11 days under white light spectrum, and growth rates were measured at 680nm wavelenth as shown in fig 3.1(b). *Botyroccocus sp* and *Chlorella sp* exhibited relatively high growth as compared to *Scenedesmus sp* and *Anabena sp* which showed slower and steadier growth. Different algae react to light and nutrients differentlty although it was obvious that all algae utilized nutrients in the eggwash wastewater for growth.

Anabena sp, for instance, has been found to effectively grow at the optimal light intensity of above 110 µmol/s/$m^{2,}$ and 7.0 PH of which any extreme from these conditions might cause radiation stress and growth inhibition (Yoon et al., 2008). From the growth rate studies carried out in fig 3.1b, it is evidential that microalgae could utilize nutrients in wastewater for photosynthetic productivity.

TREATMENT OF DIGESTATE WITH MICROALAGE

3.4.2 SMALL SCALE LAB EXPERIMENTS

Different strains were grown on ADE in 4 separate 2.0 litre LED powered photo-bioreactor vessels with simultanious monitoring of nutrients reduction over a period as shown in figure 3.3. *Botyroccocus sp, Scenedesmus sp, Chlorella vulgaris, and Web3* were the four main algae utilized in the treatment process at 16:8 light-dark cycle. During the treatment process, the rates of absorption and trend of major nutrients by the individual algae species were of significant interest. The conversion of total kejeldahl nitrogen (TKN) to NO_3-N for use by the algae was efficient (>98%) for all four bioreactor systems occuring within five days RT which is a function of autotrophic nitrifying bacteria in the wastewater. Microbial communities in ADE coupled with constant aeration played a significant role in NH_3-to-NO_3 conversions rather than volatilization which is found to contribute only 2% of ammonia reduction in any high rate algae pond (Zimmo, van der Steen, & Gijzen, 2003).

Nitrate (NO_3) uptake in ADE was most efficient (99%) with *Botyrococcus sp* and *Chlorella vulgaris* which reduced nitrate below 10mg/l within 5 days of residence time compared to *Scenedesmus sp* which took 7-9 days RT to achieve target reduction. Nitrate uptake by Web3, on the contrary, was the lowest recording 30ppm as at day 5 RT representing only about 57% reduction. Many scholarly works over the years have explored the molecular bases

and factors triggering nitrates uptake by various algae. Although outside the scope of this study, factors such as the presence of transcriptional proteins, NO_3-NH_4-PO_4 gradients, CO_2 concentrations, and trigger hormones have been documented to contribute to algal nutrient uptake behaviour (Sanz-Luque, Chamizo-Ampudia, Llamas, Galvan, & Fernandez, 2015). For instance, *Chlorella vulgaris* will prioritize ammonim (NH_4^+) uptake over Nitrates (NO_3) in times of equal concentrations for both nutrients in the system, which implies that NH_3 reduction in treatment systems is not dependent solely on nitrification. The implication is that the nutrient uptake behaviour varies from species to species which can be more innate with individual algae as opposed to environmental existing conditions.

Similarly, Phosphate (PO_4) uptake followed the same trend with all the algae utilizing phosphorus and reduction below 10 ppm within 5 days of residence time. However, phosphate removal by Web3 algae was very slow only 40% removal at day 5 as compared to above 75% of the other three algae species. The P uptake in wastewater is impacted by factors such as pH, algae settling, algae decay and N-P concentration ratio. The pH in the bioreactors rose significantly during treatment from an initial adjusted pH of 7.2 to above (>10) at certain times due to CO_2 – O_2 dynamics. Higher pH may result in phosphorus precipitation making them inaccessible to the algae for uptake, therefore resulting in “deceptive” phosphurus removal which can easily be reversed when pH begins to decline. Another study posits that there is a cycling effect between algae biomass and wastewater where dead algae biomass settles and release phosphorus back into the wastewater (Grifiths, 2010). From figure 3.3 (e), there was a sharp rise in pH during treatment by all algae except the Web *3* which exhibited a lag phase for 3 days before a significant change in pH. The rising pH, while positive for algal treatment systems tend to be inhibitory for the algae at above a value of 10. At extremely higher pH, algae physiology

and microbial community are affected, and hence nutrient uptake by algae may be retarded. Hence a working pH range of 7.2- 9.0 is always recommended by monitoring and regulating pH in treatment tanks. Another study proposes that removal of phosphate from wastewater might not be a function of the algae itself but rather the nitrogen to phosphate ratio of the wastewater (Kube, Jefferson, Fan, & Roddick, 2018). The relatively higher concentration of one pollutant over the other in the N:P ratio could trigger a scenario known as "luxury uptake" where there is a propensity for algae to prioritize the uptake of the more concentrated compound. This scenario could account for Web *3* opting for phosphate reduction until a point where NH_3-to-NO_3 conversion results in high NO_3 in the system that triggered NO_3 removal as shown in figure 3.3d.

3.4.3 EXTENDED TREATMENT PERIODS IN THE GREENHOUSE

The ultimate aim is to remediate wastewaters to minimum concentrations within as short as 7 days hydrualic residence time (HRT) to cut down energy consumption and cost. However, erratic industrial discharge behaviour coupled with unpredicted environemntal conditions might shorten or prolong treatment times. It is, therefore, imperative to investigate algae treatment behaviour over extended periods to check for nutrient dynamics as well as algae longivity and resilience. The filamentous *Nostoc sp* was grown on ADE and monitored over 30 days with a periodic top-up of the digestate day 11 and day 21 as in figure 3.3f. Target reduction for phosphate was achieved on day 7 of treatment to 5.0 mg/L representing 71% removal while the NO3 reduction achieved above 90% at a relatively shorter residence time of 3 days. Rising pH for the Nostoc based treatment as in figure 3.3e was an indication that both biological and algal activity in the treatment system proceeded efficiently. Periodic NO_3 spikes from the graph showed rapid NH_3-NO_3 coversion which consequently increases total nitrate in the system. Nitrification from NH_3/NH_4^+ to final nitrate might have occurred less than 3 hours because

nutrient monitoring was carried out at atleast 4hrs after top -up. By this time enough time would have elapsed for equalization to occur within the system. *Nostoc sp* showed high resilient and longivity for nutrient uptake below targeted concentrations up to day 30. However, subsequent nitrogen removal efficiency over the 30 days declined steadily from day 21 to day 30. The decline in uptake efficiency may be due to equilibrium established between accumulated nitrogen concentrations in algae biomass and surrounding wastewater which impedes the active transport across algae tissues. At this point, there is a propensity to switch to increased phosphate uptake as proposed by Kube et al. (2018). Also, algae naturally tend to stress out over time for which reason their ability to continuosly reduce nutrient loads in wastewater at high rates may be impeded.

The extended treatment period was carried out in the summer of 2017 with *Chlorella vulgaris*, *Scendesmus sp,* and *Anabena sp.* Elevated pH from 7.3 to above 9.0 was recorded in all bioreactors within 4 days of treatment due to the speedy algal uptake of CO_2 and bicarbonates (HCO_3) which, hitherto, could have caused a more basic medium. Treatments with *Chlorella vulgaris* and *Anabena sp.* resulted in a pH rise which might have contributed to phosphorus reductions in these systems. As earlier discussed, pH dynamics impact the availability of nutrients for algal uptake and above 9.0 pH could create a nonoptimal environment for the algae. While all algae showed rapid nitrate uptake to above 90% with 8 days of residence time, phosphate reduction rate was slow among all algae with some occationally released back to the wastewater. Algae take up phosphurus within initial treatment until a time where cellular equilibrium and pH changes begin to impact P intake negatively. Also, allowing treatment to run over extended time means longer algae retention time which causes some algae to die out and settle, therefore, releasing P back into the wastewater. It is, therefore, advisable that any algal

treatement system hoping to reduce phosphurous loads should harvest matured algae and replace with fresh cultures regularly to prevent the back release of phosphorus.

3.4.4 ONSITE TREATMENTS IN HRAP AND IMPACT OF DILUTION

Treatment at Synergy, Covington NY was run twice in a batch mode with and without prior dilutions. For the first run, *Chlorella sp* was innoculated onto raw ADE without any dilutions and allowed to run for 11 days. The start-up concentrations of all pollutants recorded on day 0 were relatively high, and levels in treated effluents were still higher even after day 11 as shown in the graphs above. From Table 3.3, it can be deduced, that dilutions before treatment are capable of lowering concetration of pollutants by more than 50%. A 1:50 and 1:100 dilution of the ADE reduces turbidity and allow maximum penetration of light to reach autotrophic algae. Also, nitrate (NO_3) reduction were almost 100% for a 1:50 and a 1:100 dilutions compared to 1:25 ratio.

Furthermore, the outcomes from the onsite HRAP as shown in Fig 3.6 A-B confirmed the crucial need for dilution before treatment. The *Chlorella sp* on undiluted ADE (run 1) could not take up much of the nutrients when left to run for 11 days. It was also observed that algae died out quickly and turned brownish within the first four days of treatment. Undiluted ADE is dark and muddy and hence blocked sunlight from reaching the algae for nutrient uptake and growth. Athough, complete nitrification occurred on day 8, the NO_3 reduction was poor hence leading to nitrogen build -up in the treatment pond. Also, PO_4 and Fe remained significatly higher after treatment and could not be directly applied to crops. A second run (run 2) with a more diluted ADE yielded a greater reduction due to its pellucid nature and hence allowed much

light penetration. Moreover, dilution significantly reduced nutrient concentration and above 97% of NO_3 was removed on day 7.

3.5 CONCLUSIONS.

Microalgae showed the ability to utilize light for growth with full spectrum white light being the most preferred and efficient. Also, all the selected strains of microalgae exhibited the ability to grow using nutrients from the egg wastewater. All microalgae applied to ADE reduced nutrients in less than 10 days RT except Web3 which exhibited stress and poor P removal. Removal efficiencies ranged from 70%- 95% for NO_3, PO_4, and Fe as ammonia nitrification mostly occur within 6 days RT. Although there was no significant difference in nutrient removal efficiency between small scale, greenhouse and field based experiments, dilutions of muddy wastewater with pond water proved to be helpful for algae performance. Also, extended treatment times would require continues feeding of the system with wastewater as nutrients deplete rapidly. It is also advisable to replace stressed algae cells with fresh cultures when running a batch mode treatment project.

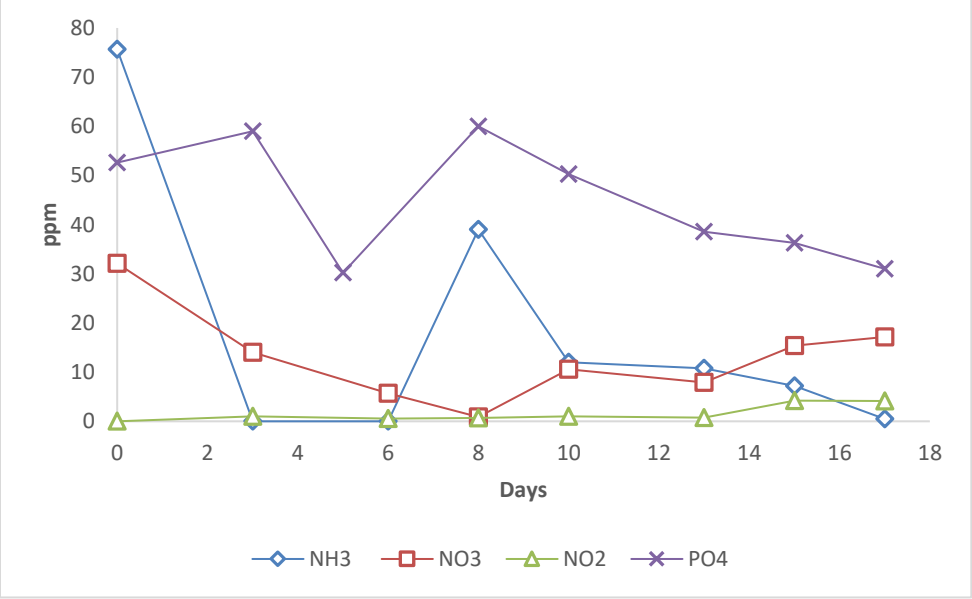
CHAPTER FOUR

EGG WASTEWATER NUTRIENT REDUCTION BY VARIOUS ALGAE

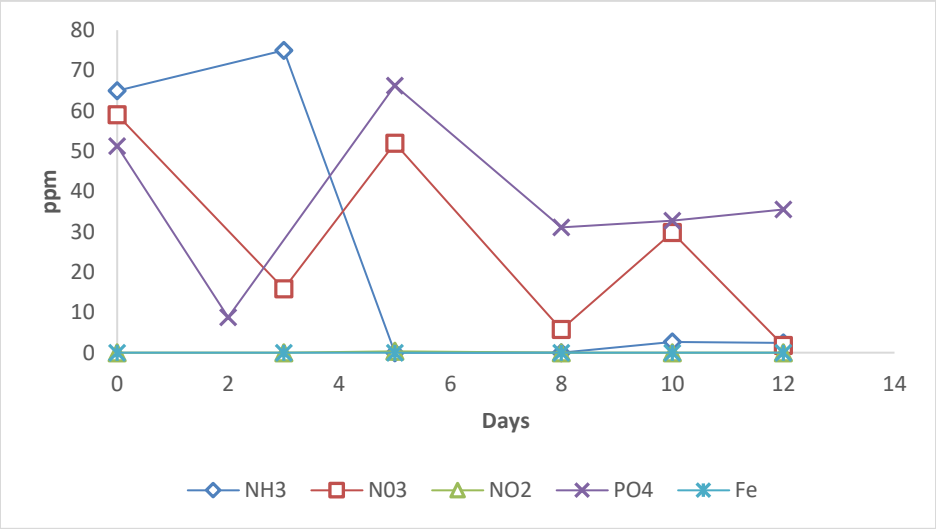
4.1 INTRODUCTION:

Egg wash wastewater sampled from Kreher farms, New York State is high in phosphorus for which reason the direct discharge of the egg wash is not permissible under federal and state regulations. The egg processing wastewater stems from the initial washing of eggs whereas the detergent containing wastewater is as a result of cleaning of equipment and cages quarterly with detergent. The egg wastewater contained high phosphates and debris (EW) as compared to the follow up washing with detergent which contained low phosphate levels(EW + det). Selected algal strains were grown on both detergent-free and detergent based egg wash in bioreactors and nutrient reductions constantly monitored. The results are depicted in Fig 4.1.

(A)



(B)



*EW+ DET = Egg wastewater with detergent

Figure 4.1: Egg wastewater (with detergent) nutrient reduction by various algae

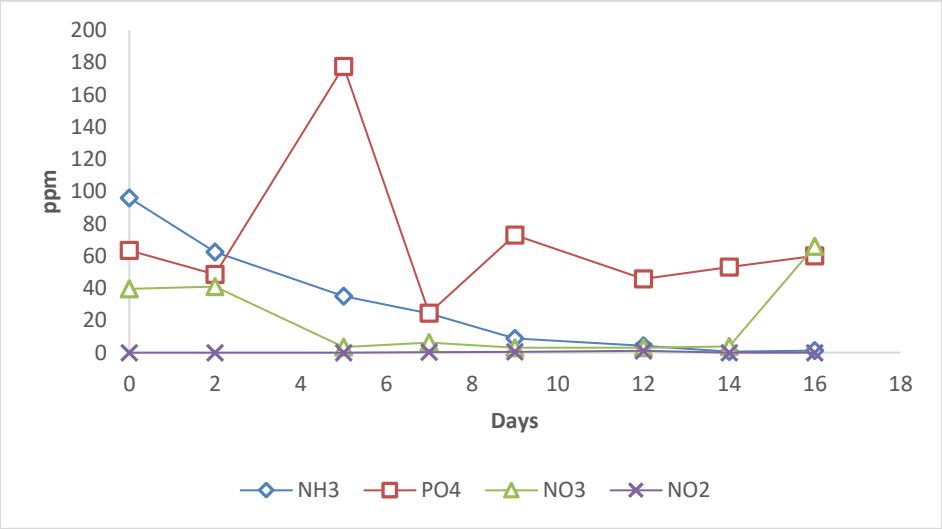
(A) *Nostoc sp* on egg wastewater

(B) *Anabaena sp* egg wastewater

While both strains reduced nitrates below the desired level in a short RT (≤ 8 days), Phosphate reduction by Nostoc sp was not as efficient as that of the Anabena sp which achieved a sharp reduction of both nutrients within 3 days RT. However, phosphate removal rates were relatively slow after day 5 for both strains of algae.

Nutrient reductions for Web3 and Chlorella sp. were also investigated on Egg wastewater with detergent for 16 days. The treatment set-up comprised algae inoculated onto 1:10 parts of egg wastewater to pond water and an initial pH of 7.2-7.4. Treatment was allowed to run for 16 days under a 16:8 light-dark cycle. While 98% nitrate (NO_3) was reduced within 5 days RT, phosphate (PO_4) reduction was more apparent with Chlorella *sp*. at 12 days RT. The pH varied significantly in the system for all algae strains from the initial 7.5 to 9.5 to 10 which is a good indicator of efficient biological activity and nutrient transformations during treatment.

A)


200
180
160
140
120
100
80
60
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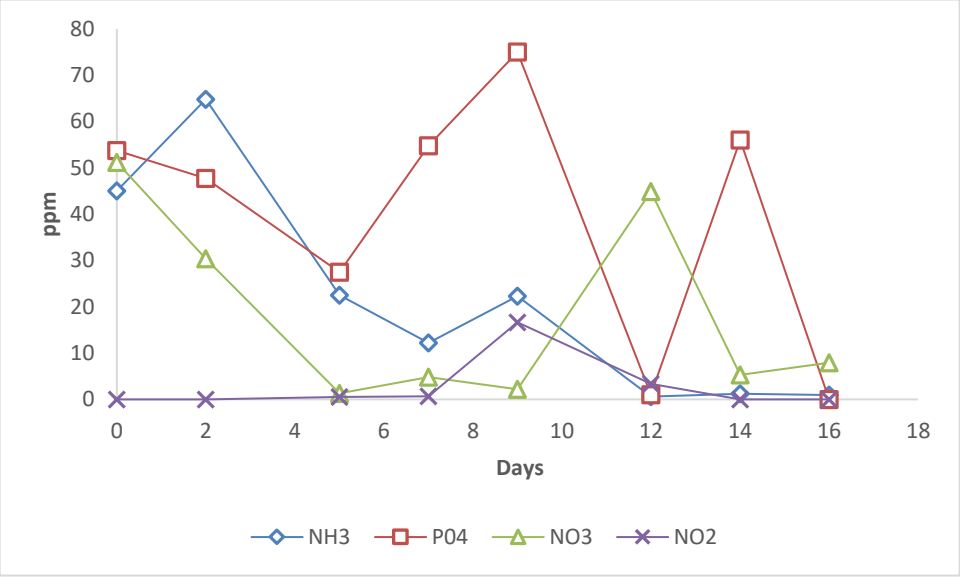
Days
NH3
PO4
NO3
NO2

B)


80
70
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ppm
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2
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16
18
Days
NH3
P04
NO3
NO2

C)

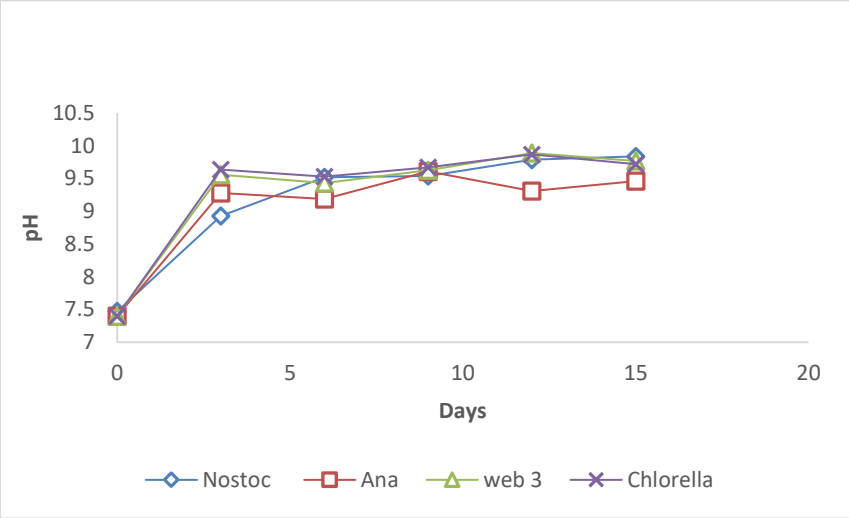


FIGURE 4.2: Nutrient reduction studies for *Web3* and *Chlorella sp* on egg wastewater

A) Web3 on egg wastewater with detergent
B) Chlorella sp on egg wastewater with detergent
C) pH changes

4.3 NUTRIENTS REMOVAL FROM EGG WASTEWATER WITH NO DETERGENT

The study also investigated nutrient reduction in detergent-free egg wastewater in a bioreactor under lab conditions. Egg wastewater was diluted with pond water in a 1:5 ratio at initial pH 7.2-7.4. A top-up was made at day 9 when the system's nutrients level was not enough to sustain algal growth. Monitoring was done on an average 16-day period, and results are presented in Fig 4.3. In Fig 4.3a, *Scenedesmus sp* reduced phosphate levels to desired levels (>90%) within 4 days RT as nitrate reductions were achieved at day 8. Ammonia conversions in all bioreactors were efficient within first 4 days except with Anabaena which showed slower NH_3 conversions. Chlorella sp, as shown in Fig 4.3(c), achieved maximum (~95%) reductions for all nutrients within day 8 but microalgae began to stress out at day 13 to release nutrients

back into the system. It was observed that prior dilutions of the various wastewater with pond water reduced nutrient levels significantly.

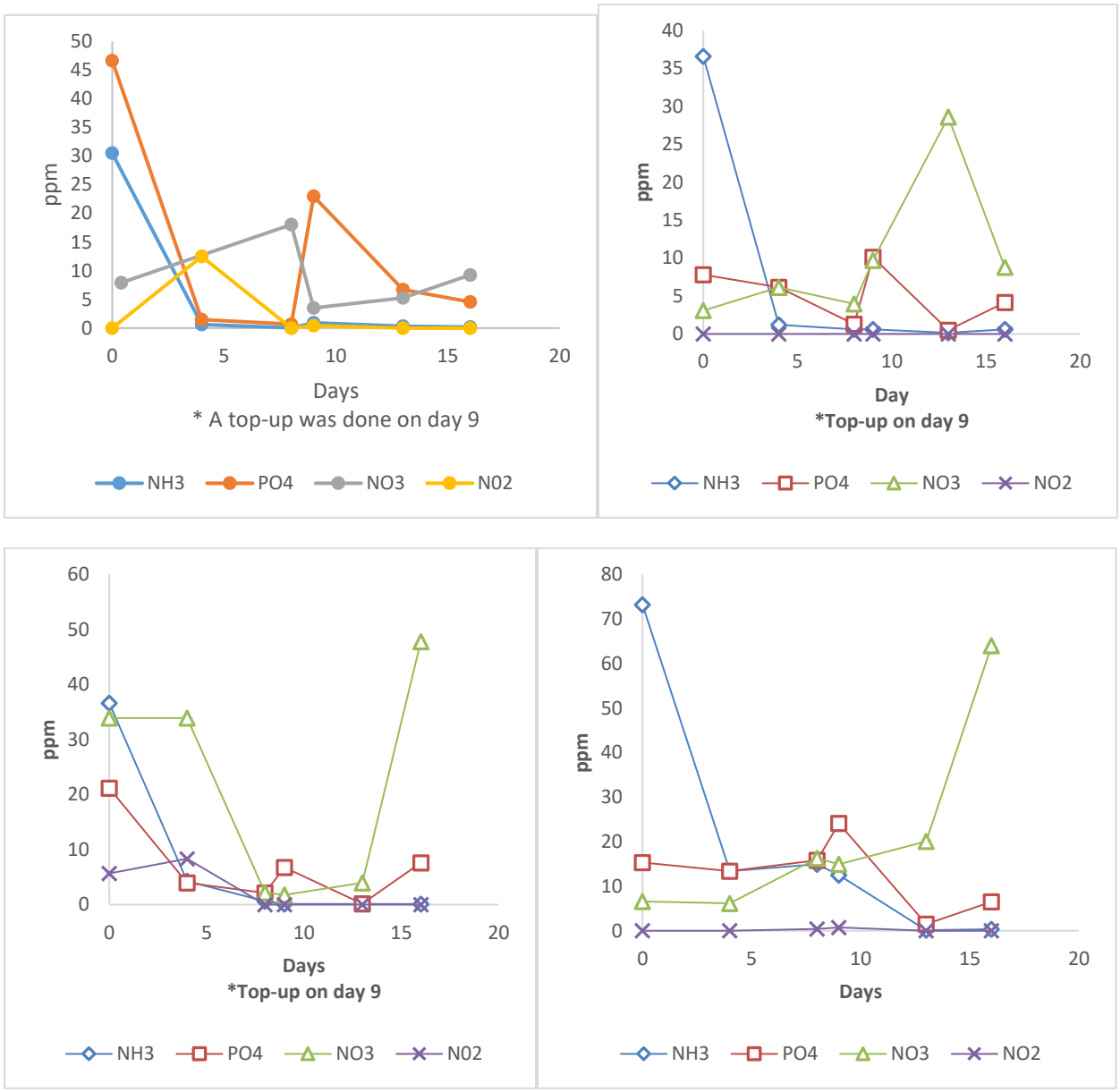


FIGURE 4.3: Nutrients reduction by various algae grew on egg wastewater.

A) *Scenedesmus sp* on Egg wastewater
B) *Botyroccocus sp* on Egg wastewater
C) *Chlorella sp* on egg wastewater
D) *Anabaena sp* egg wastewater.

4.4 EGG WASTEWATER TREATMENT IN THE GREENHOUSE

The study on Egg wash nutrient reduction was further scaled up to 10L in the greenhouse using *Botyroccocus sp*. Egg wash was diluted with pond water in a 1:4 ratio and treatment started in a 10L white basins with initial pH set to 7.3. Treatment to desired levels for PO_4 and NO_3 were achieved 7-9 days after inoculation with microalgae as ammonia (NH_3) conversion nitrate was swift. Also, cell longevity was observed with *Botyroccocus sp* when occasional top-up at day 12 became necessary, and nutrients were retaken at a faster rate. Moreover, pretreatment dilutions also contribute to lowering concentrations of various nutrients in the system. Centrifuge harvested biomass was oven dried at 87^0C and weighed.

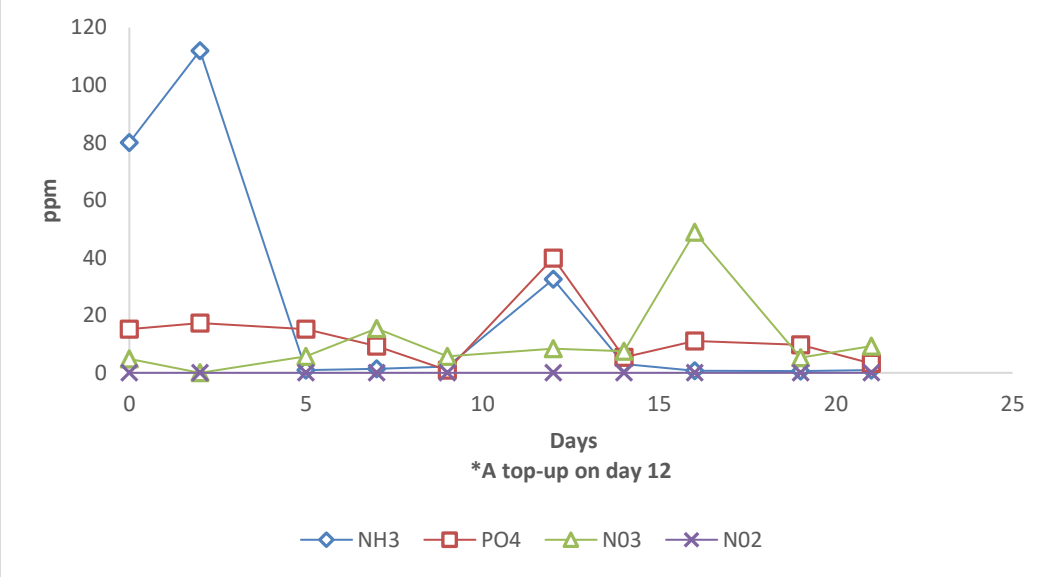


FIGURE 4.4: Nutrient reduction in egg wastewater with *Botyroccocus sp* grown in Greenhouse

4.5 EXPERIMENTAL CONTROLS

Also, eggwash treatments under certain controlled conditions were carried out to investigate the contributory effects of aeration and algae on treatment processes.

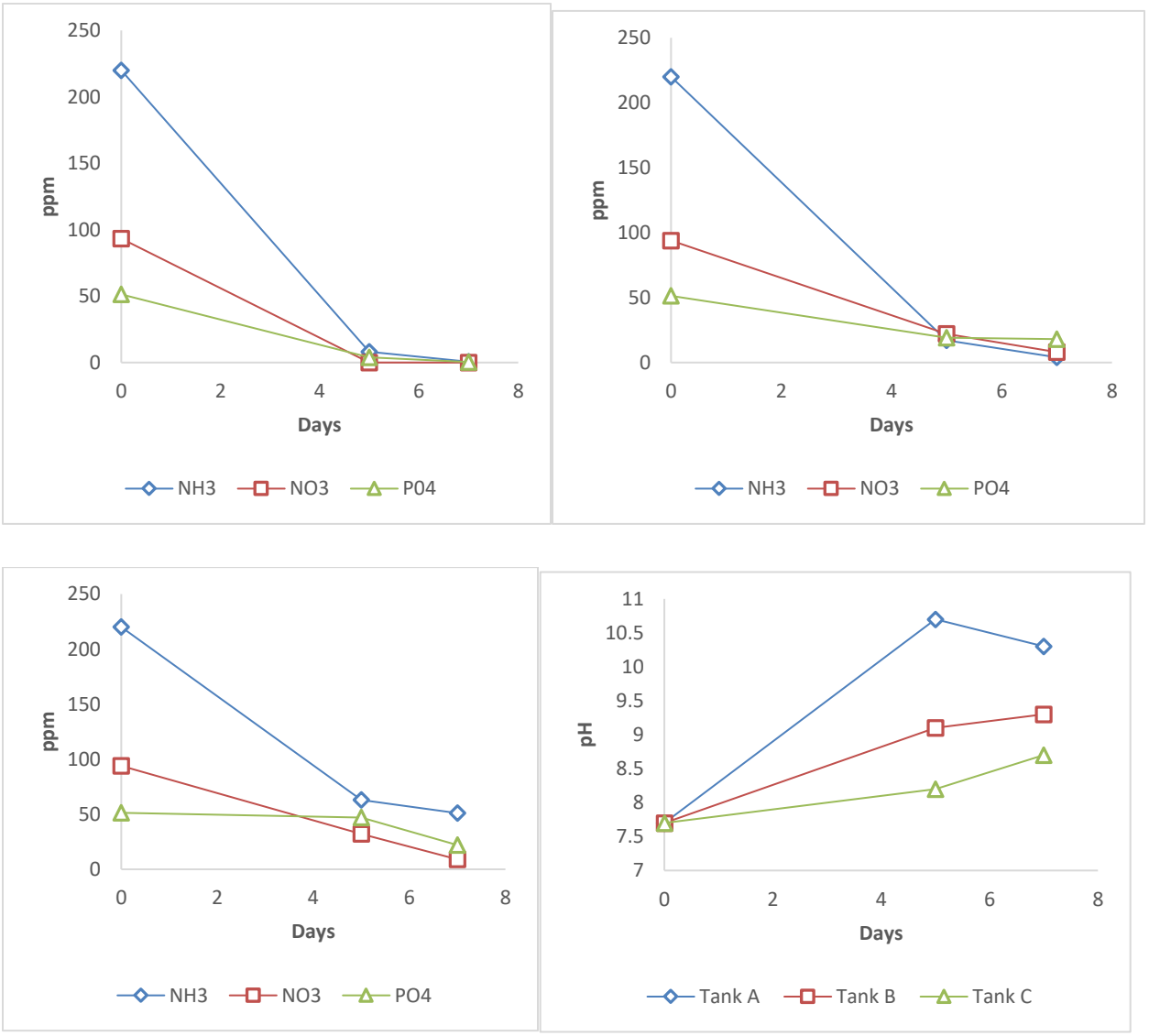


FIGURE 4.5: Egg wash treatment under controlled condition's at RIT's Greenhouse

A) Botyroccocus on egg wastewater
B) Eggwash under aeration only
C) No algae-No aeration set up
D) pH Changes

Treatment under controlled conditions was aimed at investigating the effect of the presence or absence of certain factors on treatment outcomes. In set up A, all conditions were present while in B, Algae inoculation was absent. In set up C, both algae, and aeration were absent, and parameters were monitored over 7 days. Diluted egg wash in three different 10 litre capacity white basins was run at the RIT Greenhouse. Set up (a) composed of *Botyroccocus sp* grown on Egg wastewater under constant aeration. In Set-up (B), Egg wash was aerated without added algae while in set up C, neither algae nor aeration was provided. These experimental set-ups sought to identify the main factors contributing to nutrients reduction in algal remediation systems. In respect to the graphs below, set-up A achieved above 98% of nutrients reduction for all nutrients present. In set-up B, ammonia (NH_3) conversion and nitrate uptake were relatively higher despite a lack of algae in the system. Phosphate was also significantly reduced but not to desired levels (<10ppm). Set-up (C) also showed high ammonia conversion (>75%) as phosphate and nitrates reduced by 50% and 90% even the absence of algae and adequate aeration.

4.6 DISCUSSIONS

EGGWASH NUTRIENT REDUCTION BY MICROALGAE

Kreher farms is a family owned farm operating withing the Erie, Genesse, and Wayne Counties in New York to produce fresh and quality eggs for market. Since egg production and processing generated high volume wastewater containing high NO3 and PO4, the study investigated the suitability of algae to use the high nutrients in the effluents for growth and remediation. *Nostoc sp, Anabena sp, Web3, Botyroccocus sp* among other species experimented on the Eggwash wastewater.

4.6.1 **Reduction by *Nostoc and Anabena sp***

Treatment of detergent containing egg wastewater with *Nostoc and Anabena sp* showed NH3 conversions were highly effective in both systems achieving complete nitrification within 3 and 5 days respectively. *Nostoc sp* removed over 98% of NO_3 within 6 days RT where as Anabaena utilized only 67% of the nitrate within 3 days RT and over 90% within 8 days RT.

There was high phosphate reduction (90%) by *Anabaena sp* within 3 days RT while 50% reduction by *Nostoc sp* was seen at day 6 RT. Although initial PO4 concentration was relatively higher than NO3 for *Nostoc sp*, there was a preference for NO_3 over PO_4 contrary to findings by Kube et al. (2018) that increasing phosphate concentration will shift selective absorption behavior of the algae to favor phosphate. Trends from previous treatment bioreactors support the assumption by Whitton et al. (2015) that there is a possibility that P uptake in algal treatment systems is enhanced by external factors like pH, light, and temperature and rather than the algae under study. Also, prolonged treatment periods were found to have negatively impacted nutrient uptake by both *Nostoc sp* and *Anabena sp* as they stressed hence harvesting, and replacement with fresh culture should be done to avoid the reverse release of nutrients.

4.6.2 Nutrient uptake by Web3 and Chlorella sp

Detergent containing eggwash wastewater was used as growth media for Web3 and Chlorella over 16 days, and nutrients reductions were monitored over time as shown in fig 4.2 NO_3 removal was 98% within 5 days RT by both algae while complete NH_3 conversions were achieved within 12 days RT. The PO_4 reduction was 67 % within day 7 RT for Web3 and 99% removal within 12 days RT for *Chlorella sp*. High nitrate removal rates exhibited by both algae may be due to the high initial concentration of the nitrate which creates a high concentration

gradient between algae cells and the wastewater. Hence luxury uptake could be the mechanism driving nitrate reductions. Previous treatment systems also have further confirmed the phenomenon of luxury uptake where relatively lower initial NO_3 contrations caused a lag phase until enough nitrates build up in the bioreactor to triger nitrogen removal. For instance, from fig 4.3 (a) where initial NO_3 concentrations at Day 0 were below 10 ppm, there was a surge in NO_3 until high concentration gradients were established. Phosphate reduction, on the other hand, could be a function of pH among other factors contributing to P removal. Both algae began stressing after day 12 and released N and P back into the wastewater.

4.6.3 No Detergent Eggwash

From fig 4.3, detergent free Eggwash was also remediated in different bioreactors using *Chlorella sp, Scenedesmus sp*, *Anabena sp,* and *Botyroccocus sp* in 16 days. For *Scenedesmus sp*, NH_3 and PO_4 have reduced by 99% in 4 days RT, and 80% of NO_3 has removed in 8 days RT. Occassional top-up of nutrients on day 9 became necessary to keep the system running. The *Botyroccocus sp*, although removed 99% of NH_3 within 4 days RT, PO_4 reduction above 95% was attained on day 8. However, NO_3 uptake by Botyroccocus was not impressive until day 13. From fig 4.3b, initial startup cocentration for NO_3 was relatively lower (<5mg/l), hence NO3 uptake was delayed until high concentration gradient was established between algae and surrounding wastewater at day 13 which then triggered nitrogen removal.

For *Chlorella sp,* all nutrients in the eggwash wastewater recorded a decline more 95% within 8 days RT. Hence nutrient top-up became necessary on day 9. On the contrary, poor nutrient uptake performance was recorded for *Anabena sp* with delayed NH_3 conversion until day 13 when 99% conversion was achieved. Since Ammonia (NH_3) conversion is a function of

nitrifying bacteria, there could be a lag phase after 80% NH_3 conversion occurred on day 4. However, there was poor uptake of nitrate by *Anabena sp* which resulted in continuous accumulation in the system over time. Contrary to nitrogen removal, slow but steady phosphorus removal was observed with Anabena which could be mainly due to precipitation as pH soar in the system (data not shown). Phosphorus removal is highly efficient under low pH where it exists in solution and can easily be taken through ative tranport but at higher pH, they precipitate and hence removal become difficult.

Table 4.1: Algae Biomass yield from 1.5L photobioreactors

	Anabaena sp	***Nostoc sp***
Egg wastewater +Det Biomass g/L	1.22	0.89
Eggwastewater (Biomass in g/L)	0.67	1.09

Algae biomass from bioreactor based treatments was harvested through centrifugation and oven dried at 80^0C. Dry weight in grams per litre of wastewater was determined for both *Anabena sp* and *Nostoc sp* after growth on eggwash with and without detergent. Productivity was relatively high in the high detergent eggwash compared to the ordinary eggwash Table 4.1. The ability of various microalgae to yield high biomass is a characteristic innate to the algae under study and varies from algae to algae which may come with their own trade-offs the depending on the goal of the algae cultivation.

4.6.4 GREEN HOUSE AND FIELD BASED HIGH RATE ALGAL POND (HRAP) TREATMENTS

The high cost of construction and resource demand in setting up bioreactors makes the option for open pond (HRAP) treatment as a more economical and suitable alternative. Moreover, high energy demands through LED powered lightening and limited loading capacity found with bioreactors are absent with HRAPs. Shallow HRAPs retrofited with paddle-wheels for constant mixing has been found to increase algae biomass growth to about 30 tonnes/haYr, effectively reducing nutrients in wastewaters and makes algae harvest convenient through bioflocculation (Craggs, Heubeck, Lundquist, & Benemann, 2011). Also, industries like Synergy LLC, Northern Soy, Lively Run, and Kreher farms whose operations generate high volume mass flow of wastewater would most likely prefer HRAPs to bioreactors as a more economical alternative.

Both Egg wastewater and ADE were treated in HRAP style on a small scale in the RIT Green house and on a larger scale onsite at Synergy LLC respectively. A 1220 gal capacity tank was constructed onsite at Synergy LLC in the summer of 2016 while a 15L and 50 gal capacity tanks were mostly used for Greenhouse based treatment. Tanks were lined with white plastic to enhance light penetration to the bottom of the tank. A 120V and 1.5 watts powered whisper10® aerators were used to provide constant oxygen from a bottom-up direction. Treatments were carried out under conditions depicted in Table 3.2.

4.6.5 GREENHOUSE BASED TREATMENT OF EGGWASH

From figure 4.4, *Botyroccocus sp* was grown onto Eggwash in a 50 gal HRAP in the Greenhouse showed total NH_3 conversion occurring within 5 days of RT which shows efficient nitrification in the system. Also, prior dilutions before treatment significantly lowered

concentrations of the various pollutants. Dilutions before treatments also enhance light penetration to maximize nutrient reduction. While there was no significant difference in Phosphorus reduction rate when compared to its counterpart bioreactor setup, Nitrate (NO_3) removal in the Greenhouse open tank treatment was more efficient than that of bioreactor setting due to the former being exposed to adequate sunlight and good aeration systems. Both NO_3 and PO_4 were reduced below target concentrations of 10ppm and required nutrient top-up on day 12. However, NO_3 removal by *Botyroccocus sp* was not as efficient under bioreactor based treatment compared to that of the Greenhouse which demonstrates that open pond treatments provide the enabling environment, better lighting and natural conditions to enhance treatment.

4.6.6 **EXPERIMENTAL CONTROLS**

A further run of eggwash treatment was carried out at the Greenhouse while conditions such as oxygen and presence and absence of algae were controlled during the treatment process. In tank A, Botyroccocus sp was added to Egg wastewater in a 10L tank and aerated. Additionally, set -ups in other two 10L tanks were simultaniously started to control algae and oxygen in tank B and C respectivly. *Botyroccocus sp* under aeration in tank 'A' swiftly reduced all nutrients (>99%) to the minimum in shorter residence times than those under controlled conditions.

In tank 'B', NO_3 and PO_4 were reduced by 90% and 65% respectivly in addition to a near 100% conversion for NH_3. Although no algae were present in this tank, the aeration rate of 20 Lm^{-1} could have aided heterotrophic bacteria to degrade and utilize NO_3 using organic carbon as an energy source. Research has found that under aerobic condition *Enterobacter cloacae* grew significatly and exhibited heterotrophic nitrification and aerobic denitrification properties

(Padhi, Tripathy, Mohanty, & Maiti, 2017). Padhi et al. (2017) further proposd a nitrogen preference in the order NO_3-N>NO_2-N > NH_4^+-N for E. claocae signifying that the bacteria could utilize nitrates in wastewater under aerobic conditions.

For tank 'C,' a no-aeration-no alage still exhibited some reductions in levels of NH_3, NO_3, and PO_4 but not as drastic. Anaerobic Ammonia oxidation (anamox) reactions could be possible for some fraction of the ammonia reduction in the absence of oxygen. Since enhanced aeration was absent in this tank, it could be that the little water surface-air interaction was enough to trigger the oxidation of ammonia by anamox and hence the denitrification to nitrogen gas. Nancharaiah, Venkata Mohan, & Lens, (2016), in their review, stated that Anamox process utilizes only 40% of a typical nitrification aeration requirement hence could be a sustainable energy saver process for wastewater remediation.

4.7 CONCLUSIONS

Both *Anabaena sp* and *Nostoc sp* removed about 95% of NO_3 within 7 days RT while PO_4 was reduced over 90% within 3 days by *Anabaena sp.* Similarly, high NO_3 removal was observed among *Chlorella* and Web3 sp but *Chlorella sp* removed over 90% of PO_4 compared to 67% removal by web3. All strains showed satisfactory to excellent NO_3 removal capacity while P removal from Egg wastewater was impressive with detergent free wastewater than detergent containing wastewater. Luxury uptake and active transport mechanisms play an active role in NO_3 removals where as pH among other physico-chemical factors influence P removal. Greenhouse based open systems and open systems presented better treatment outcomes compared to bioreactors. Control experiments showed that other microbial entities all play vital roles in nutrient transformation and removals.

CHAPTER FIVE

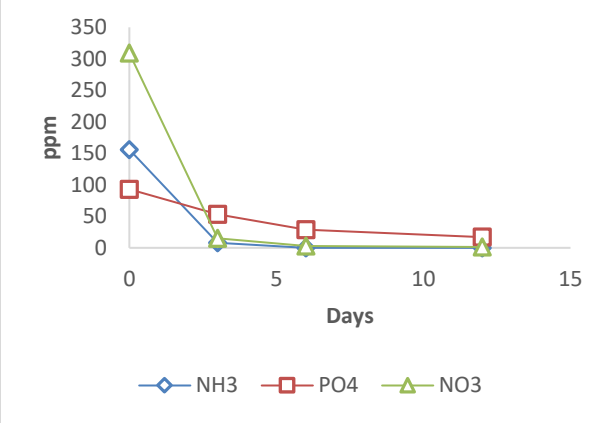
5.0 NUTRIENTS REDUCTION IN FETA CHEESE AND TOFU WHEY

The whey from Feta cheese productions was used as growth media for *Scenedesmus sp, Chlorella sp*. and *Botyroccocus sp*. while simultaneously reducing nutrients to desired levels. All wastewater types required some level of dilutions before treatment and pH was adjusted accordingly. Nutrients were monitored over 12 days. While nitrate (NO3) reduction was achieved within a shorter residence time of 6 days, Phosphorus concentrations remained high above the 10 ppm target in both set-ups as at day 12 although reductions were continuous and steady. Ammonia-to-nitrate conversions in both systems were highly efficient. Simultaniously, there was a rise in pH during treatment from 7.0- 9.5. A 7-day treatment of Tofu whey with *Scenedesmus sp* and *Botyroccocus sp* was carried out as seen in Table 5.1

TABLE 5.1: TOFU WHEY TREATMENT WITH MICROALGAE

Parameter	Tufu whey nutrient reduction by *Scenedesmus sp*		Tofu whey nutrient reduction by *Botyroccocus sp*	
	Day 0	Day 7	Day 0	Day 7
NO_3 (ppm)	44.8	7.6	44.8	9.9
NH_3 (ppm)	5.8	0	5.8	0.27
PO_3 (ppm)	20.6	6.0	20.6	4.2
pH	7.3	9.8	7.3	9.7

A)



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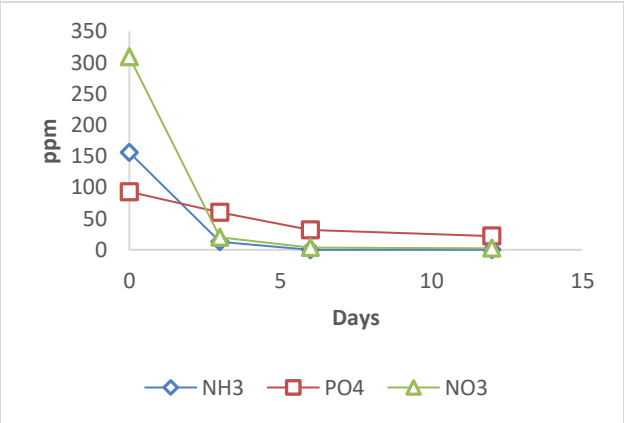

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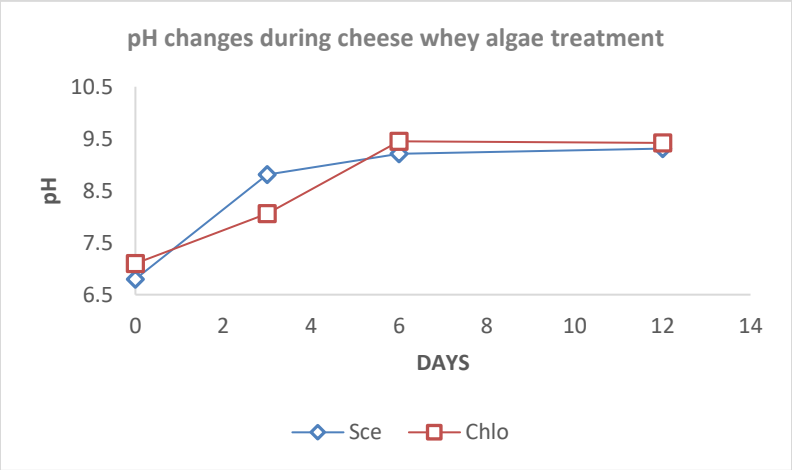


FIGURE 5.1: Cheese whey nutrient reduction by various algae and pH changes

A) ***Scenedesmus* on cheese whey**
B) ***Chlorella sp* in Cheese whey**
C) **pH changes with cheese whey treatment**

5.1 TREATMENT OF COMBINED A.D.E AND WHEY EFFLUENTS

Bio digesters are designed to receive and convert degradable agricultural waste from eclectic sources. In some instances, co-digestion with dairy manure and whey significantly increase methane production to above 70% methane production and reducing COD by 98% (Hublin, Zokić, & Zelić, 2012; Yan, Liao, & Lo, 1988). Various co-digestion strategies to enhance methane production could alter pollutants composition and dynamics in a given digestate at any time. Given this recent development, the study tested algal nutrient reduction in mixed ADE-Whey effluents in a ratio of 70:30 and 1:10 dilution a using the *Chlorella vulgaris* in a bioreactor set-up (fig 5.2)

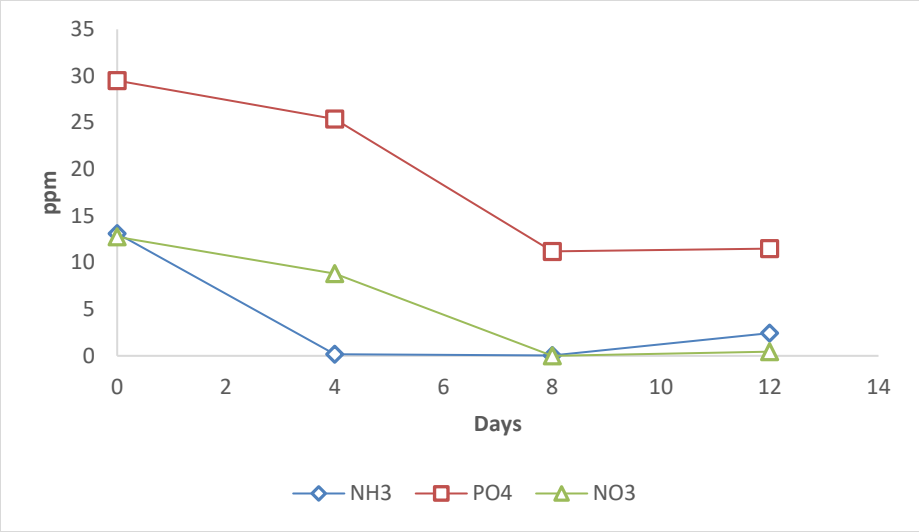


FIGURE 5.2: Mixed ADE-whey wastewater treatment by Chlorella sp

5.2 DISCUSSIONS

NUTRIENT REDUCTION IN THE CHEESE, YOGURT AND TOFU WHEY

Whey from tofu, Greek yogurt, and feta cheese production was high in NO_3 and showed significant levels of phosphorus. Iron levels also exceeded permissible limits (0.2mg/l) especially in Feta Cheese and Tofu whey. From fig 5.1, cheese whey was used as growth media to cultivate *Scenedesmus sp* and *C. vulgaris* over 12 days in bioreactors. Complete nitrification occured within three 3days of RT, and while both algae reduced nitrate by 99% in 3 days, there was no significant difference in NO_3 uptake between the two algae. Similarly, 80% of PO_4 has reduced in both systems at 12 days RT as pH rose in both tanks from 7.0-9.5. Although both algae species have proven to be very vibrant in remediation systems, higher initial NO_3 concentrations (300ppm) might have played a significant role in establishing concentration gradients for NO_3 uptake. Releatively higher initial concentrations above prelimnary established concentrations could stem from transferred nitrate from initial cultures during innoculation. Also, whey sampled at different times at the source may show varying concentrations of pollutants due

to different management practices and choices of raw material and cleaning detergents made by manufacturing industries.

Nutrients in tofu and yogurt whey also significantly declined when used as growth media for *Scenedesmus sp* and *Botyroccocus sp* respectively as shown in Table 5.1 The 7 -day period of treatment using *Scenedesmus sp* recorded an 82% and 70% reduction in NO_3 and PO_4 respectively while 100% NH_3 oxidation and nitrification were achieved for Tofu whey. For Yogurt whey, 75% and 80% reductions were achieved for NO3 and PO4 respectively when treated with *Botyroccocus sp.* (data not shown). Treatment of mixed ADE-whey effluents presented no adeverse effect, and as NO_3 over 98% NO_3 was removed although P remained significantly high. From all indicaions, it is evident that algae can utilize nutrients from Cheese, Yogurt and Tofu whey hence a very good alternative waste remediation effort to offset the cost of waste hauling and surcharge from WWTPs.

5.3 CONCLUSIONS:

The nutrients in the various whey types supported algae growth and removed within relatively shorter (<5days) RT. There was a sharp decline to about 95% of nitrogen and P in whey when treated alone with microalgae. However, treatment of mixed ADE – whey recorded a steady decline of P as complete N removal was achieved at a prolonged period.

5.4 SUMMARY AND RECOMMENDATIONS TO RIT PATNER INDUSTRIES

This study aims not only to remediate agricultural wastewaters with the algal treatment systems but also through technology transfer, recommend and implement specific strains to industry's that partners RIT in this research. Since the different strains perform differently on different wastewaters, it is necessary to customize algae performance to industrial requirements factoring time and resource constrians. For instance, Synergy Biogas has a 30 day discharge schedule, hence a batch mode treatment of any kind of algae may not exceed 25 days RT to avoid untreated waste storage backlog. From Table 5.2, residence times (RT) for N and P implies days taken to reduce each nutrient to below 10mg/L (target concentration). At this level, the land application can be utilized without risking ground water contamination.

For Synergy's ADE, performance in the order *Botyroccocus sp> Chlorella sp, > Nostoc sp> Scenedesmus sp* was exhibited as all reduced ADE nutrients to target concentrations within 10 days RT, hence highly recommended for any onsite treatment project. However, prior dilutions of ADE effluents and periodic replacement of stressed algae with fresh cultures are recommended to ensure efficient treatment process. Also, light intensity to HRAP should be regulated and maintained within threshhold to avoid photo-inhibition. Other strains may be tested for research and development (R&D) purposes over time. Adding whey to ADE in the ADE-Whey mixture situation did not pose any adverse effect on treatment times but rather positvely reduced residence time for *Chlorella sp.*

For Kreher farms' eggwash, *Scenedesmus sp* and *Chlorella sp* rapidly reduced nutrients below target concentrations with 10 days RT compared to the other strains which might take >15 days to achieve the same treatment standards. While *Chlorella sp and Scenedesmus sp* seem to

reduce Nitrogen in Lively Run's Cheese whey rapidly (<10 days RT), P reduction is always slow and might span over 15 days RT. Future research may be necessary to ascertain the potential for other algae strains to reduce the P level. The *Botyroccocus sp* and *Scenedesmus sp* utilized nutrients from Northern Soy's tofu whey bringing N and P below target concentrations within 7 days RT. Onsite treatment of these different effluents with algae would reduce production cost and earn industries some federal tax credits. Also, pressure on WWTPs would be reduced while water can be recycled and recirculated back to the manufacturing plant or irrigation on farms.

TABLE 5.2: NUTRIENT REDUCTIONS BY VARIOUS MICROALGAE

Algae strain tested	AD effluents treatemt		Egg wash treatment		Treatment of ADE-Whey mixture		Treatment of Cheese Whey		Tofu whey treatment	
	Avg RT (days) – N	Avg RT-P	Avg RT-N	Avg RT-P	Avg RT-N	Avg RT-P	Avg RT-N	Avg RT-P	Avg RT-N	Avg RT-p
Chlorella sp	6	9	7	8	4	8	6	>12	n/a	n/a
Botyroccocus sp	5	5	10	7	-	-	-	-	7	7
Scenedesmus sp	8	8	8	4	-	-	6	>12	7	7
web3	>16	8	5	>16	-	-	-	-	-	-
Nostoc sp	3	7	6	>17	-	-	-	-	-	-
Anabaena	6	26	12	8	-	-	-	-	-	-
ADE algae	>7	4	>7	4	n/a	n/a	n/a	n/a	n/a	n/a

***RT = Retention time**

CHAPTER SIX

6.0 INTRODUCTION

LIPID EXTRATION AND THIN LAYER CHROMATOGRAPHY

After treatment, algae biomass was harvested by centrifugation and oven dried at 80-90^{0}C. Biomass from field experiments was harvested after draining treated water and drying sludge in open sun for 24 hours. Microalgae contain both carbohydrates and lipids which can be extracted for secondary products. Lipids are extracted from biomass to serve as feedstock for biodiesel and other bi-products. Sugars are also extracted for bioethanol purpose as left over biomass from the algae can still be digested into biomethane. For this research, harvested algae were first taken through lipid extraction using hexane: isopropanol solvent at a 3:2 ratio. TLC analysis assessed components of lipid extracts to determine quality and suitability as a biodiesel feedstock.

6.1 TLC ANALYSIS

TLC glass plates support coated with silica gel as sorbents were used for the analysis which showed bands of different lipids as seen in fig 6.1. The separation of algae oil into its component lipids was achieved with a mixture of organic solvents. The study qualitatively investigated triglycerides content in various algae lipids through the use of mixed solvents from hexane: ethyl ether: glacial acetic acid, in an 85:15:2 ratio. Some experiments added sonication to the lipid extraction with the purpose of breaking up the cell wall of the algae to maximize lipid extraction and to see if sonication may increase lipid extraction. For biodiesel purposes, triglycerides are highly desirable since they can undergo catalyzed transeterification to yield biodiesel from fatty acids methyl esters (FAMEs). From figure 6.1, TLC profile from 2µL and 4µL spots showed a

significant amount of Triglycerides, free fatty acids (FFA), diglycerides and monoglycerides (Top to bottom). Excessive amounts of free fatty acids could disrupt the transesterification process and may lead to undesirable soap formation (Saponification) and increased biodiesel production cost (Leung, Wu, & Leung, 2010).

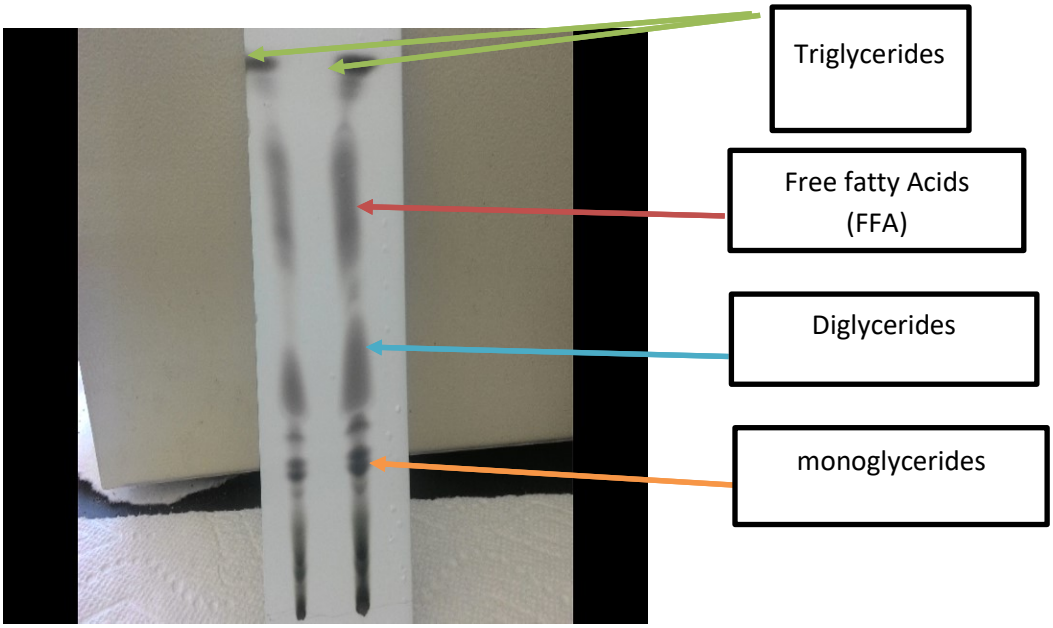


Figure 6.1: (a) Lipid profile extracted from Chlorella sp used to treat wastewater

Figure 6.2 (b) takes the algae TLC analysis, a step further by comparing to a TLC made from spent coffee grounds and heating oil canola. While coffee grounds and canola oil showed triglycerides, they also contained some amount of FFA which affects their suitability for biodiesel and raise production cost. In comparison to the algae biomass, FFA content in the algae biomass is relatively less as shown in figure 6.1 (a) and (b) and fig 6.2.

T= Triglycerides F= Free fatty acids D= Diglycerides M= Monoglycerides

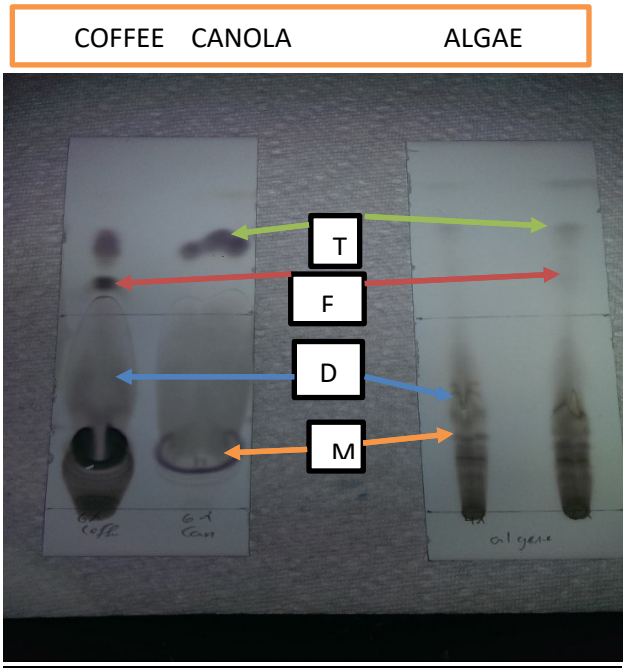


Figure 6.2 Comparison of Coffee, Canola and Algae oil via TLC

6.2 SONICATION EFFECT ON LIPID YELD

TLC analysis was carried out with algae biomass after applying a magnitude of sonication to investigate the effect of sonication on lipids extractions from algae. 20g of Algae biomass was added to 150 ml 3:2 hexane: isopropanol and sonicated for 180 secs, 280 secs, 380 secs and 480 secs of sonication at 160 watts before lipid extraction. A control experiment with no sonication effect was also carried out, and TLC chromatograms from sonicated algae biomass are shown in fig 6.3

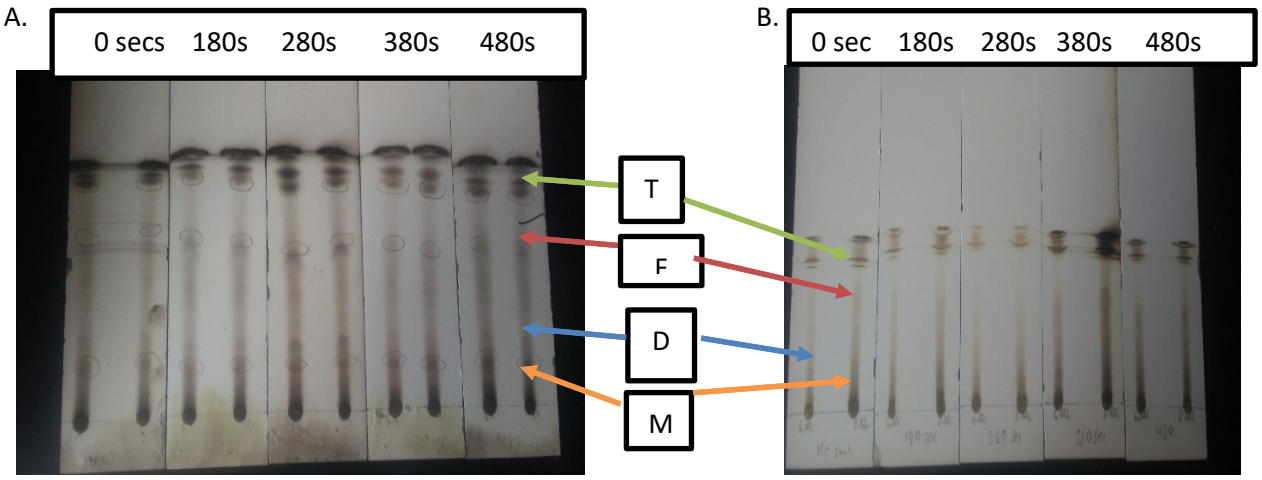


Figure 6.3 (A) TLC Chromatograms of sonicated biomass run 1

(B) TLC chromatograms of sonicated biomass run 2

To further investigate the relationship between sonication effect and high lipids yield, algae biomass harvested from ADE treatment was taken through yet another high and extended period of sonication time for 480sec, 960 sec, 1440 sec, 1920 sec and 0 sec as a control experiment. Chromatogram is seen in figure 6.4. Although the individual bands appear conspicuous, there seems to be no established correlation between the magnitude of sonication and band surface area

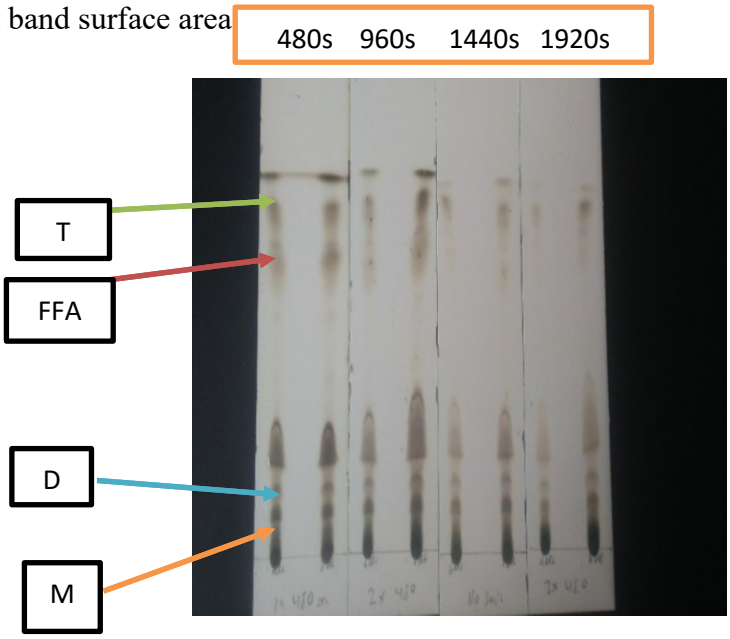


Figure 6.4 Thin layer chromatogram from increased sonication times

6.3 CARBOHYDRATE EXTRACTION AND QUANTIFICATION

Algae biomass also contains carbohydrates in the cell wall or storage granules which can be extracted and fed to yeast to yield bioethanol. Lipid extracted algae (20g) were allowed to dry and 100ml of 2% sulphuric acid (H_2SO_4) was added in a 250ml conical flask. Each flask was placed in the oven at 105^0C for 4-8 hours. Samples were brought out and allowed to cool, and the carbohydrate rich supernatant was separated from the residual algae biomass by filtration. Each carbohydrate extract was diluted to 1:1000 with distilled water and 0.4ml of each sample was assayed by adding 0.4 ml of 5% phenol and 2ml of concentrated H_2SO_4. Each sample was allowed to cool and read at 490nm wavelength in the spectrophotometer. Samples of 2%, 4%, 6%, 8% and 10% glucose were taken through the same procedure and also read at 490nm to generate a standard curve to determine the concentration of glucose in each algae biomass sample.

TABLE 6.1. Optical densities of standard glucose concentration (1:1000 dil)

absorbance at 490nm	% Concentrations g/L
0	0
0.212	2
0.415	4
0.583	6
0.684	8
0.987	10

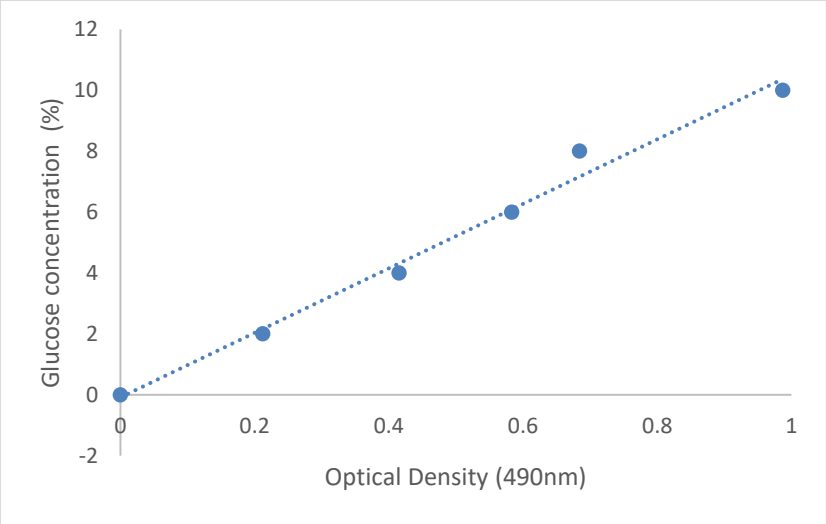


Figure 6.5 Standard curve for sugar concentration

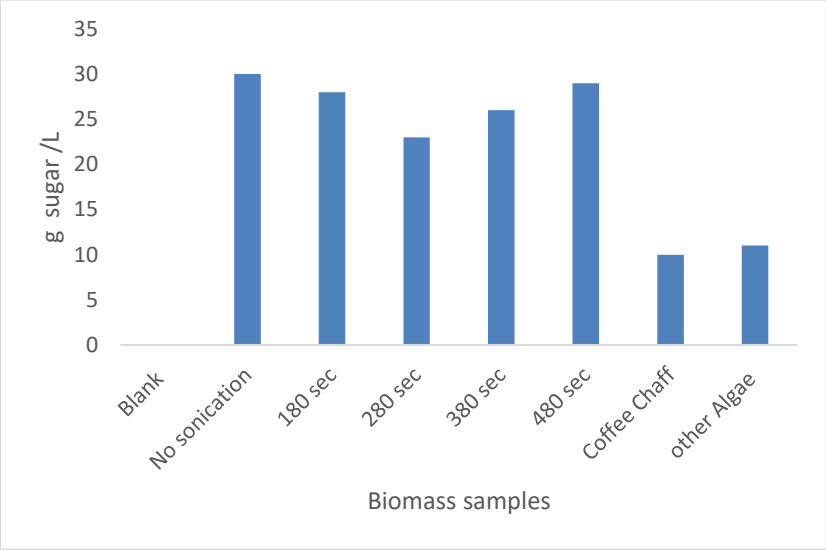


Figure 6.6 Sugar concentrations from dry algae biomass and coffee chaff.

6.4 EFFECT OF SEQUENTIAL EXTRACTIONS ON YIELD

Further investigations were carried out to ascertain whether sequential extractions of lipids and sugars can have any significant effect on yield. This time sugar extraction from biomass was preceeded by lipid extraction and compared to that from a nonlipid extracted algae all taken through sulphuric acid based sugar extraction. Supernantant were taken through a 1:1000 dilution of sugar solution were made and read at 490 nm. Absorbence from

spectrophotometry were compared to standard curve to obtain carbohydrates concntrations in the algae. From the below data, it can be infered that preceeding sugar extraction by lipid extraction significantly increased sugar yield although sonication, even at a higher magnitude of sonication did not contribute significantly to sugar yield.

A) B)

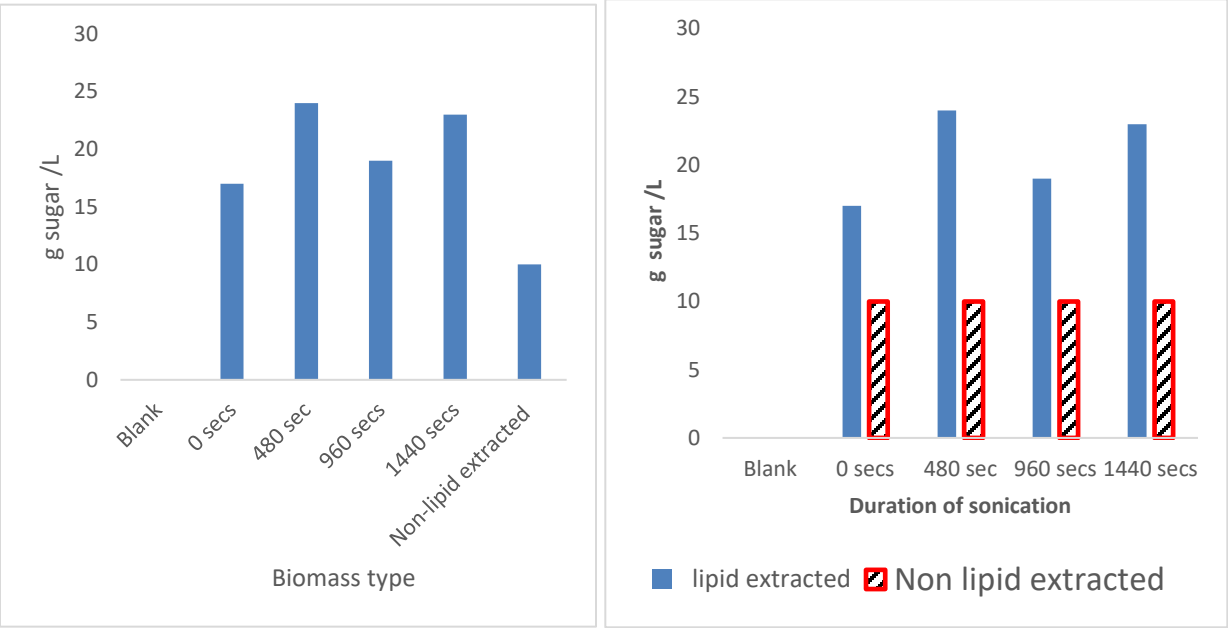


Figure 6.7: Sugar yields from algae.

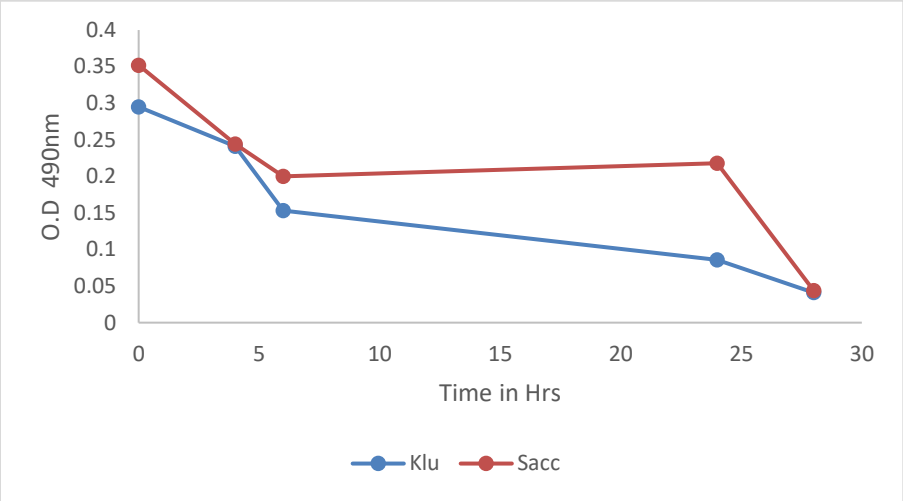
a) Lipid extracted followed by sonication b) Lipid extracted versus non lipid extracted

6.5 YEAST FERMENTATION ON ALGAE SUGARS

K. maxianus and *S. cereveciae* were fed extracted sugars from algae biomass and fermentation were observed over 28-30-hour period. From fig 6.8, *Kluveromyces* sp utilized 95% of Algae biomass sugar into bioethanol within 28 hours compared to Saccharomyces utilizing 88% within the same time frame. The process was repeated, and both Saccharomyces and Kluveromyces sp were supplemented with 0.5g of peptone. The addition of peptone is to serve as

additional nitrogen for the fungi. The peptone boosted fermentation recorded a 63% carbohydrate utilized by Saccharomyces sp and 54% sugar utilized by Kluveromyces sp`.

(A)



(B)

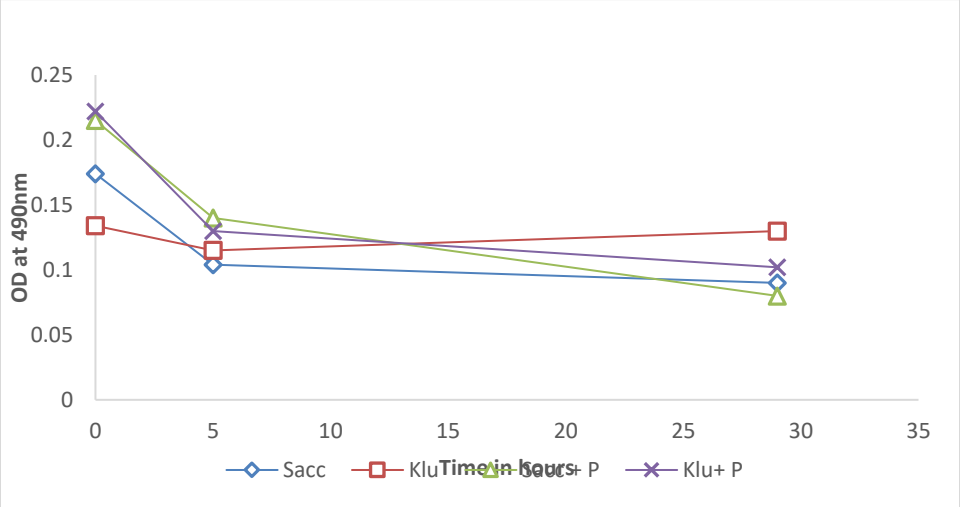


Figure 6.8: Fermentation of algae sugars by Kluveromyces and Saccharomyces sp

A) Fermentation by *Kluveromyces sp* and *Saccharomyces sp*. B) Comparing fermentation with or without peptone.

6.6 UTILIZING ALGAE BIOMASS FOR BIOMETHANE

The concept of using algae to boost methane production has been experimented with in numerous literature and yielded positive outcomes. For instance, methane production from both single and two-stage fermentative co-productions of hydrogen and methane was studied and found that energy output efficiency in the latter was higher compared to the former (Ding et al., 2018). However, as the organic loading rate exceeded 6.0 g VS/L/d and 2.0g VS/L/d for Hydrogen and methane production respectively, diminishing returns on corresponding yields were recorded. Some works highlight the need to co-digest algae biomass with other feedstocks to improve the C/N ratio and hence boost methane production. For instance, the co-digestion of blue algae extracted for the Taihu Lake in China with corn straw substrate in a 20: 1 ratio increased methane yield by 64% and compared to single substrate digestion (Vo Hoang Nhat et al., 2018). Co-digesting algae with Feta cheese whey increased methane yield by 25% compared to co-digesting Feta cheese whey alone (J. Lodge, personal communication). The biomass-to - methane process is a 4 stage process comprising hydrolysis, acidogenesis, acetogenesis and methanogenesis. Hydrolysis ensures that complex chemical polymers are broken down by hydrolytic enzymes to trigger subsequent reactions until methane is produced and cleaned. Although all these processes work in a coordinated manner to enhace biohydrogen and biomethane production, the biochemical composition and quantity of the biomass in co-digestion can affect yield. For instance, rigid cell walls preventing accessibilty to enzymes and mixotrophic behaviour allows *Scenedesmus sp* to survive anaerobic digestion for six months(Chia et al., 2017). In such situations, pretreatment of the biomass through several cell disruption techniques to break up recalcitrant cellwalls for reactions becomes a necessity.

This study, in collaboration with the Golisano Institute of Sustainability, investigated and compared methane yield from 3 different feedstock types; 1). Algae (no extraction), 2) lipid extracted algae and 3) lipid-carbo extracted algae biomass with dairy manure. It was anticipated that the exposure of algae biomass to thermochemical disruptions during lipids and carbohydrate extraction could be substituted for conventional pretreatments and boost methane yield as shown the the data below.

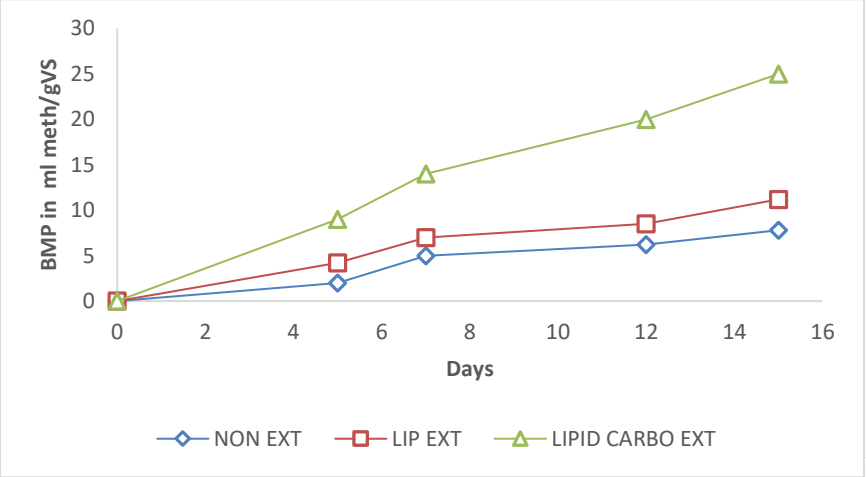


Figure 6.9 Comparing methane yield from extracted and non- extracted algae and dairy manure.

6.7 DISCUSSIONS

BIOMASS -TO-ENERGY

This study, in addtion to using algae to treat the different wastewater, further harvested biomass for other sustainable use and co-products. Algae biomass has valuable uses in the biofuel, agricultural and pharmaceutical industries. Previous years witnessed a surge in corn use for ethanol and soybean use for biodiesel. The practice has caused unnecessary hikes in food prices and competition for arable lands. It is theoretically estimated that a total land area of 1-3 million acres of land area would be required to cultivate algae to satiate entire US biodiesel

demands. This value is less than the total land area of the State of Connecticut and represents only 2-4% of US arable farm lands. Algae can be cultivated using unwanted high nutrient wastewater as an alternative to synthetic nutrients, therefore, reducing pollution and cultivating high quality biomass for different purposes at low or no cost. Microalgae grow faster and require less land area to cultivate compared to traditional biofuel feedstock like corn and soyabean. This study explored biomass from harvested algae after treatment and analyzed for their lipids, sugars and methane booster properties. Triglycerides component can be transesterified to biodiesel while sugars were fed to yeast for bioethanol.

6.7.1 LIPID EXTRACTION AND TLC

Hexane:isopropanol extraction was employed to extract lipids from dry biomass algae that were harvested from the various treated wastewater types. On a large scale production, solvent extraction may not be feasible, but thermochemical conversion methods like pyrolysis, liquefaction, and gasification methods could be utilized (Beal, Smith, Webber, Ruoff, & Hebner, 2011). Lipid content varies from algae to algae, and as *Botyroccocus sp* can have between 20-80% lipids, *Anabena sp* has been studied and found to contain a very scanty amount of lipid between 4-7% (Ghasemi et al., 2012). Lipids are comprised of phospholipids, triglycerides, free fatty acids, diglycerides, and monoglycerides present in typical algae cell. From the figs 6.1-6.4, a TLC run with hexane-ethylether-acetic acid over 2µl and 4µl spots from *Chlorella sp* showed visible triglycerides among bands of Free fatty acids, Di, and monoglycerides. Triglycerides are the most useful lipids found in algae which can be transesterified into fatty acid methyl esters (FAME), a major chemical component of biodiesel. Transesterification process with methanol is catalyzed by NaOH or KOH producing glycerol as bi-product as shown in fig 6.10. From the

TLC plates above, conspicuous triglyceride bands on the plates is an indication of *Chlorella sp* as a good biodiesel feedstock.

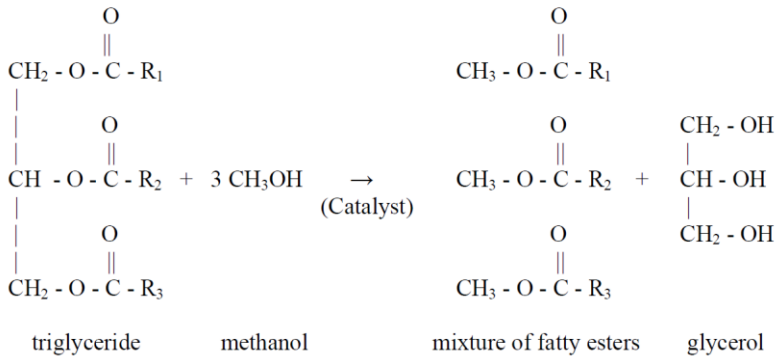


Figure 6.10: TRANSESTERIFICATION REACTION TO BIODIESEL

6.7.2 AlGAE OIL VERSUS FOOD OIL FOR BIODIESEL

TLC analysis from algae was compared to that from coffee and canola oil. Large area bands for FFA from the Canola and coffee oils could disrupt transesterification reactions as the presence of high FFA could divert the reaction to undesirable soap formation or require pretreatment with sulfuric acid transesterification reaction before KOH reaction. Feedstocks containing greater than (>2% wt) dry weight FFA are generally not recommended for biodiesel production due to a propensity to form soap and requirement for pretreatment increases production cost astronomically (Rapaka, 2012). In situations where high free fatty acids are inevitable, a two step transesterification process is required which can add to production cost. In such a process, an acid catalzed transesterification of FFA precedes an alkali based catalyzed conversion of triglycerides (Canakci & Van Gerpen, 2001). It can be deduced, that algae biomass present a high quality and relatively low FFA making it an excellent feedstock over waste cooking oils which has high amont of FFA. In the case of coffee oil, oil from new coffee grounds has low to no FFA acid compared to old coffee, hence an improved feedstock for biodiesel.

6.7.3 SONICATION EFFECT ON LIPID EXTRATION

Lipid extraction is maximized when mechanisms to break open algae cell walls known as cell disruption is incorporated into the extraction process. Ultrasonication is one method among grinding, bead vortexing, osmotic shock, waterbath and shakemill which can be utilized to break cellwalls to make fatty acids accessible for extraction. In one study, sonication extracted 31% of lipids where as osmotic shock and grinding with liquid nitrogen increased lipid yield by 2 to 3 folds (Byreddy, Gupta, Barrow, & Puri, 2015). In this study, harvested algae were subjected to varying degrees of ultrasonication before lipid extraction as shown in fig 6.1. No significant differences were observed across the TLC plates from the sonicated algae biomass which could be an indication that *Chlorella sp* do not respond positively to this type of cell wall disruption. A higher magnitude of sonication was further applied in the hope of increasing lipid yield and components bands as shown in fig 6.2. A 480sec, 960sec, and 1440sec with a 0 sec of sonication still produced no significant difference in band appearance except that phospholipid bands appeared more visible this time compared to previous experiments. Reddy et al. (2015), propossed that not all algae responded the same way to cell disruption method and found percentage yield of lipids to be relatively higher with *Chlorella sp* when grinding with liquid nitrogen was used as a cell disruption method. Cell disruption entirely depended on algae species type, cellwall compostion, and age.

6.7.4 CARBOHYDRATE EXTRACTION, QUANTIFICATION AND FERMENTATION

Yeast can ferment sugars from algal biomass into bioethanol which can be a good substititute to corn ethanol. Before this research, the use of sulphuric acid to pretreat algae biomass has been previously documented to be effective and enhance sugar extraction and fermentation (Boonprab, Matsui, & Kataoka, 2018). From the results in fig 6.6, it was

established that the sonicated dry algae of *Chlorella sp* and coffee chaff contained a significant amount of sugars. From the absorbence and standard curve, sonication did not have any significant impact neither did it enhance carbohydrate yield in any way. However, there seem to be significantly higher glucose levels in *Chlorella sp* compared other algae types and coffee chaff making Chlorella sp a good prospect for biomass to energy conversion. An average of 30g/L of total sugars was obtained from *Chlorella sp* compared to only a yield of 10 g/L from Coffee chaff and other consortium of algae.

From fig 6.7a &b, it was evident that increasing the magnitude and duration of the ultrasonication did not enhance sugar concentration in any way and but may have slightly reduced average suger concentration to 15%-25% as compared to 20%-30% in earlier experiments. Future work could investigate other pre-treatment methods and impacts on resource recovery. However, preceeding sugar extraction with lipid extraction boosted sugar extraction by 2 fold which could indicate that the presence of fatty acids could interfer with carbohydate yield. A later experiment in our lab, however, showed the contrary in favor of lipids when glucose extraction preceeded lipid extraction (data not shown). This time, it appeared the pre-treatment with sulphuric acid during the sugar extraction opened up the cell wall contents to make lipids more accessible. Moreover, Miranda et al. (2012) subjected biomass from *Scenedesmus obliquus* to different pretreatment methods such as sonication, bead beating, autoclaving, alkaline and acid hydrolysis and found that pretreatment with acid hydrolysis exerted the most impact on algae cell wall and boosted sugar yield compared to other mechanical techniques. Therefore, depending on the goal of a biofuel project, the order, and the type of processing technique is quiet crucial to maximizing the yield of the biochemical component of the algae desired.

Algae sugars were fermentable by *Kluveromyces* and *Saccaromyces sp* at 85-95% which, therefore, make algae a promising feedstock for bioethanol. Moreover, the the absence of lignin in algae cell walls makes cell wall sugar more accessible to the yeast for fermentation at lesser cost since no rigorous pre-treatment stage is needed (Daroch, Geng, & Wang, 2013). The use of *Saccharomyces sp* in fermentation is very popular amoung biotechnologist, despite the strain's inability to ferment pentoses. However, many experimental outcomes find *S. cerevisiae* to be highly productive compared to other organisms which can convert xylose to ethanol. Although the presence of peptone might provide some vitality to the yeast, the overall impact of the peptone was not that great to warrant the additional economic cost of continuesly boosting fermentation with peptone on a commercial scale.

6.7.5 ALGAE BIOMASS IN METHANE PRODUCTION

From fig 6.9 it is evident that the double (lipid-carbos) extracted algae biomass increased methane production significantly compared to the single (Lipid extracted) and non-extracted algae. Lipid only extraction increased biomethane potential (BMP) only by 1 fold within 5 days run time (RT) compared to double lipid-carbo extraction which increased yield (BMP) by 5 fold within the same duration. The pre-extractions of lipids and sugars from algae was a good pretreatmnent step in enhancing methane production while achieving sustanability goals as lipids can be esterified into biodiesel and carbohydrates can be fermented by yeast to bioethanol. The algae biomass from the extraction can then be co-digested in large scale industrial processing to boost methane production.

6.8 CONCLUSION

Algae oils contain quality triglycerides with minimal FFA content.. The high triglyceride to FFA ratio in microalgae lipids makes it a preffered choice for biodiesel production. While ultrasonication had very little influence on lipids yield, exposing algae biomass to thermochemical processes like acid digestion during sugar extraction and organic solvent based extractions generally weaken cell walls and influenced lipid and sugar yields. Sugar yield from non-lipid extracted algae is about 10g/L of dry biomass, unlike lipid extracted biomass which yielded 15-25 g/L of biomass. It is therefore imperative to preceded sugar extraction with lipid extraction to boost sugar yield. Sugar from the biomass was fermentable by both *K. marxianus* and *S. cerevisiae* which confirms their suitability for bioethanol production.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK.

7.1 CONCLUSIONS

- All the various wastewaters assessed showed high pollutant and nutrient levels which exceed regulatory limits but can serve as suitable growth media for microalgae cultivation. The whey was characterized by low pH and relatevely high P and N where as egg wastewater and ADE contain extremely high ammonia and nitrate.

- Growth studies confirmed the ability of microalgae to utilize nutrient in both food effluents and synthetic nutrients for growth. White light spectrum was found to be the

most effective for microalgae growth compared to blue and red light spectra. *Chlorella sp* and *Botyroccocus sp* exbited high growth rates where as *Scenedesmus sp* exhibited early stress behaviour.

- ADE treatment with microalgae was successful with a 98% NH_3-to- NO_3 conversions expedited by microbiological activity and constant aeration. Nitrate removal was 95-98% for all algae strains except Web3 which only reduced nitrate by 57% given the same treatment duration. Nitrate removal is influenced by factors such as concentrtion gradients among the various pollutants, CO_2 concentrations in system and microalgae physiology. PO_4 reduction ranged from 40%- 75% and affected by factors such as pH fluctuations and stressor hormones. Also, N:P ratios have occassionally influenced P removal. On average, all nutrient reduction targets were achieved in less than 12 days RT.

- Nitrate removal was 90-95% with egg wastewater treatment by most algae strains. PO_4 reduction was highest with Anabaena sp recording an impressive 90% compared to Nostoc sp with only 50% removal. Most nutrients were removed within relatively shorter RT and required occassional top-up to keep the system running. On the whole, nutrient removals in open pond systems were much efficient compared to biorector system due to the former exposed to adequate sunlight and conducive temperatures.

- Tofu, cheese and greek yogurt whey all recorded about 80% nitrate reductions and 70% phosphate reduction. Also, treatment of mixed ADE-Whey effleunts recorded high N removal moderate P reductions.

- Diluting effluents before treatments are highly recommended since highly turbid wastewaters can impede light penetrations and hence reduce treatment efficiencies.

- Post treatment biomass assessment revealed a good triglyceride content and low FFA in algae biomass which is ideal for biodiesel production. Algae sugars were also found to be fermentable by yeast and hence a good feedstock for bioethanol. Also, algae biomass was found to increase methane yield by at least 25% and sequential extraction of resources from biomass directly impacted methane yield.

7.2 RECOMMENDATIONS FOR FUTURE RESEARCH

This study recorded excellent nitogen removal. However, there is more to uncover the mechanism of phosphate removal to streamline algae treatment technologies. Fututre work might focus on studying the dynamics of P removal to overhaul and perfect this technolgy. Also, research efforts should be directed on assessing the effectiveness of monoculture versus polyculture microalgae in waste remediation projects.

Future work can also be focused on investigating other biomass pretreatment techniques as a suitable alternative to ultrasonication to maximize resource yield. Fig 7.1 summerizes the entire waste treatment process and possible products recovered.

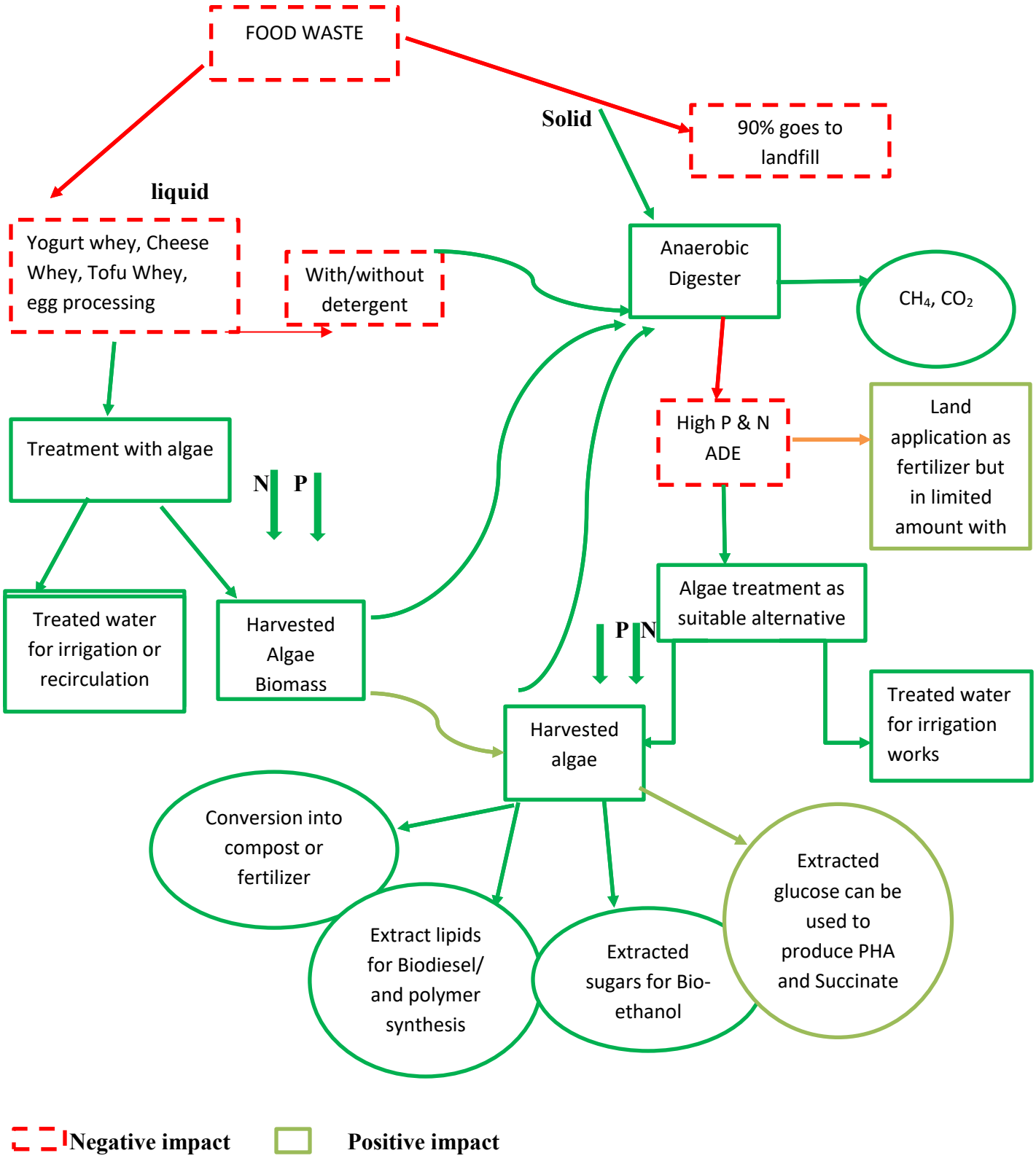


FIGURE 7.1: THE FIGURE ABOVE DEMONSTRATES THE VARIOUS PATHWAYS FROM WASTEWATER REMEMDIATION TO FINAL PRODUCTS REVOVERY.

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