A scalable and sustainable wastewater treatment technology using a methanotroph-microalga co-culture

by

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Abstract

Rapidly growing human population generates large volumes of industrial, municipal, and agricultural wastewater. Traditional wastewater processes face major drawbacks with high energy consumption, inefficient nutrient recovery, excess sludge disposal, and carbon emissions. However, microalgal-based technologies have received more focus in recent years as an alternative wastewater treatment process as they can address significant challenges faced in conventional multistep wastewater treatment process. Specifically, researchers have demonstrated the feasibility of using microalgae as an efficient and cost-effective recycling method for rapid nutrient recovery. Microalgae can also reduce carbon emission through biogas upgrading to biomethane while generating biomass that can be used as a sustainable feedstock for producing valuable products such as biofuels. Despite these advantages, microalgal technology for tertiary wastewater treatment has its own unique challenges. There exists a safety risk when oxygen gas produced during photosynthesis is mixed with methane in biogas. In addition, small-scale water resource recovery facilities (WRRFs) may not utilize the upgraded biogas for combined heat and power (CHP) as several clean up steps render the process uneconomical.

In this research, a highly promising methanotroph-microalga co-culture technology is presented for upgrading biogas while simultaneously bioremediating wastewater effluents using *Methylococcus capsulatus a*nd *Chlorella sorokiniana* as the model co-culture pair. A fast, online experimental-computational protocol was developed for frequent characterization of the co-culture without expensive equipment and time-consuming methods. The developed protocol allowed individual strain biomass estimations as well as O_2 and CO_2 gas consumption and production rates

using yields coefficients. As compared to reducing pollutant levels with freshwater, cultivation of the co-culture on unsterilized municipal anaerobic liquid digestate diluted with secondary clarifier effluent (CLE) has demonstrated higher biomass production and faster recovery of nitrogen and phosphorus where all wastewater samples were acquired from the South Columbus Water Resource Recovery facility. Additionally, the co-culture technology grown on wastewater did not require micronutrient supplementation, as the co-culture biomass productivities between unsterilized AD diluted with CLE and the co-culture grown on defined ammonium mineral salts were not statistically different. Consequently, these results demonstrate the potential for significantly reducing the operating costs of the co-culture technology when the process is scaled up.

Furthermore, studies comparing the sequential *C. sorokiniana* and *M. capsulatus* single cultures to the co-culture has demonstrated that the co-culture has advantages over the single cultures. The metabolic coupling of the co-culture enables a significant increase in biomass production and nutrient recovery as compared to the sequential single cultures. Also, the co-culture can co-utilize both CH_4 and CO_2 in biogas through bioconversion into microbial biomass without external oxygen supply. Thus, process safety is enhanced as the *in situ* produced O_2 is consumed by *M. capsulatus* before it can be mixed with CH_4 in biogas. More importantly, the results suggest the co-culture can play a critical role in reducing air pollution as the co-culture simultaneously and completely captured both CH_4 and CO_2 in the vial experiments. Growth under an analogous amount of biogas substrate demonstrated the co-culture recovered up to 100% of the inorganic nutrient while the sequential single cultures recovered up to 55% and neither the sequential single cultures nor the co-culture was able to recover the organic nutrient fraction in the diluted AD effluent.

Using a bench-scale photobioreactor, the co-culture converted biogas into microbial biomass to achieve steady state co-culture biomass productivity of 0.818 g/L/day. Under continuous growth, steady state was reached due to irradiance and O_2 limitations on the co-culture. Despite these limitations, the co-culture achieved good illuminated areal productivity of 22.8 g/m^2/day under chemostat cultivation and the estimated CH_4 and CO_2 gas consumption rates were 0.634 and 0.658 mmol/g/h, respectively. Ammonia-nitrogen (NH_3-N) and orthophosphate (PO_4^{3-}-P) was continuously recovered during continuous cultivation with the outflow residual NH_3-N and PO_4^{3-}-P reaching as low as 0.038 mg/L and 3.1 mg/L, respectively. Overall, the results indicate that the co-culture platform can be an economical technology for upgrading biogas into microbial biomass and mitigating AD effluent pollution by recovering nutrients from unsterilized wastewater.

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Table of Contents

Abstract ii
Acknowledgments v
Table of Contents vii
List of Tables xi
List of Figures xii
List of Abbreviations xv
Chapter 1 : Introduction 1
1.1 Motivation of this work 1
1.2 Municipal wastewater 3
1.3 Wastewater treatment 3
1.3.1 Primary treatment 3
1.3.2 Secondary treatment 4
1.3.3 Challenges of anaerobic-anoxic-oxic treatment processes 8
1.3.4 Tertiary treatment and advanced processes 10
1.3.5 Disinfection 10
1.4 Anaerobic digestion 11
1.4.1 Anaerobic process for biosolids reduction 11
1.4.2 Biochemical process 11
1.4.3 Biogas production 12
1.4.4 Biogas utilization and challenges 12
1.5 Current treatment of AD-derived biogas and liquid digestate 13
1.6 Microalgae 15
1.6.1 Microalgae as municipal wastewater treatment 17
1.6.2 Microalgal biogas upgrading 18
1.6.3 Microalgal cultivation systems for wastewater treatment 18
1.7 Co-culture biomass as feedstock for valuable products 20
1.7.1 Biofuel potential 20
1.7.2 Single cell protein 24
1.7.3 Biofertilizers 29

1.7.4 Bioplastics 31
Chapter 2 : Co-culture technology for treating wastewater 34
2.1 Brief review on methanotroph-microalgal co-cultures 34
2.2 Proposed co-culture technology 35
2.3 Co-culture strains 37
2.3.1 Chlorella sorokiniana as model photoautotroph 37
2.3.2 Methylococcus capsulatus as model methanotroph 37
2.4 Objectives of this study 38
Chapter 3 : Mathematical Modelling of the co-culture 40
3.1 Materials and methods 40
3.2 Experimental-Computational protocol 41
3.2.1 Experimental-computational equations 44
3.3 Results and discussion 44
3.4 Conclusions 45
Chapter 4 : Co-culture performance on minimally pretreated wastewater 47
4.1 Introduction 47
4.2 Materials and methods 50
4.3 Co-culture experimental design 51
4.4 Analytical Measurements 52
4.5 Data analysis and statistics 53
4.6 Results and discussion 54
4.6.1 Co-culture performance on different AD mixtures 54
4.6.2 Co-culture performance on differently pretreated AD effluent diluted with CLE 60
4.6.3 Co-culture microalgal biomass to biocrude 65
4.7 Conclusions and future perspectives 66
Chapter 5 : Comparisons between sequential single cultures and the co-culture for wastewater treatment 68
5.1 Introduction 68
5.2 Microalgae-based wastewater treatment and biogas upgrading 70
5.3 Microalgae-methanotroph co-culture platform for simultaneous biogas conversion and nutrient recovery 70
5.4 Materials and methods 72
5.4.1 Wastewater collection and growth media 72

5.4.2 Methanotroph and microalga growth conditions 73
5.4.3 Analytical Measurements 73
5.5 Case study rationale 74
5.6 Experimental design 74
5.7 Experimental results and discussion 75
5.7.1 Case study 1: Sequential single cultures and co-culture comparison under nutrient replete but carbon limited condition 75
5.7.2 Case study 2: Single cultures and co-culture comparison under carbon-and nutrient-limited condition 80
5.7.3 Case study 3 and 4: Sequential ingle cultures and co-culture comparison under carbon-replete but nutrient limited condition 88
5.8 Conclusions 96
Chapter 6 : Simultaneous Biogas and Nutrient Recovery by the Co-culture under Chemostat operation 98
6.1 Introduction 98
6.2 Material and methods 100
6.2.1 Preculture and wastewater medium 100
6.2.2 Analytical methods 100
6.2.3 Biomass estimation 101
6.2.4 Considerations for recovery of NH_3-N and PO_4^{3-}-P in the PBR 102
6.2.5 Photobioreactor development and setup 103
6.2.6 Photobioreactor (PBR) considerations: 105
6.2.7 Objectives and experimental procedure 108
6.3 Results and Discussions 111
6.3.1 Co-culture biomass production 111
6.3.2 Biogas conversion into microbial biomass 115
6.3.3 Nutrient recovery in bench-scale chemostat 117
6.4 Conclusions and future perspectives 122
Chapter 7 : Overview of conclusions and Future work 125
7.1 Summary of work and objectives completed 125
7.2 Recommendations for future work and closing statement 126
Appendix A : N:P uptake ratio under batch cultivation in vial experiments 129
Appendix B : Additional plots for Case 3 130

Appendix C : Initial evaluation of co-culture protein and carbohydrate content 132
Appendix D : Individual co-culture species estimation and biomass development in photobioreactor 134
Appendix E : Co-culture nutrient recovery in the PBR 136
References 138

List of Tables

Table 1: Cultivation of different microalgae strains in municipal wastewater.....21

Table 2: Equations used for estimating the biomass concentration, and gas consumption and production rates.....44

Table 3: Overall gas substrate consumption by co-culture on different AD mixtures over 72 hour cultivation period. 58

Table 4: Biomass production comparison between the single cultures and individual strain within the co-culture. 76

Table 5: Biomass concentrations and productivity comparison for single culture and the co-culture. 81

Table 6: Summary of photobioreactor experimental conditions. 111

Table 7: Steady-state chemostat biomass productivity of *C. sorokiniana*, *M. capsulatus* and the overall co-culture. 114

Table 8: Total protein and total carbohydrate content comparison between the single cultures and the co-culture. 133

List of Figures

Figure 1: Nitrification and denitrification by the nitrite pathway where ammonia is only partially oxidized to nitrite. 8

Figure 2: Current process of handling AD produced biogas and digestate from the South Columbus Water Resource Recovery Facility. 14

Figure 3: Recent reports of the protein content of some algae that are of interest (Ritala et al, 2017). 27

Figure 4: Proposed wastewater treatment process using the co-cultlure. 36

Figure 5: Schematic illustration of the experimental-computational protocol. 43

Figure 6: (A) Comparison of the measured co-culture and estimated co-culture biomass, (B) time-course residual gas concentrations in the co-culture, (C) estimation of O_2 consumption and production rates by *M. capsulatus* and *C. sorokiniana* respectively; (D) CO_2 production and consumption rates by *M. capsulatus* and *C. sorokiniana* respectively. 46

Figure 7: Growth profiles of co-culture cultivated on different AD mixtures. (A) Co-culture growth, (B) Individual strain growth of *M. capsulatus* and (C) *C. sorokiniana* in co-culture and (D) co-culture biomass productivity. 55

Figure 8: Time-course profiles of biogas composition in the headspace for co-cultures on different AD mixtures: AD effluent diluted with (A) tap water (AD-TW), (B) secondary clarifier effluent (AD-CLE), and (C) AMS medium (AD-AMS) (D) are plotted. Arrows indicate the points at which each bottle was refed with biogas. 58

Figure 9: Residual inorganic (A) nitrogen, (B) phosphorus and (C) overall nitrogen and phosphorus recovery by the co-cultures on different AD mixtures over the cultivation period. 60

Figure 10: Time-course biomass concentration profiles of (A) co-culture, (B) microalga, and (C) methanotroph within the co-culture and (D) co-culture biomass productivity on AD pretreated by different methods. 62

Figure 11: Residual biogas time-course profiles for co-cultures on (A) ammonium mineral salts (AMS) and AD pretreated by (B) microfiltration and autoclaving (FA), (C) microfiltration (F), and (D) gravitational settling (S). 63

Figure 12: Nutrient profiles (A) NH_3-N and (B) PO43- -P of co-cultures on different AD pretreatments. 64

Figure 13: Potential interactions within microalgae-methanotroph co-culture. 71

Figure 14: Biomass profiles of (A) *C. sorokiniana* and (B) *M. capsulatus* in co-culture compared with the sequential single cultures. 76

Figure 15: Comparison of biomass production between sequential single cultures (*C. sorokiniana* – first column, *M. capsulatus* – second column, total biomass or summation of the first two columns – third column) and the co-culture (last column). 77

Figure 16: Gas phase profiles for (A) co-culture, sequential single culture of (B) *C. sorokiniana* and (C) *M. capsulatus*. 79

Figure 17: Time-course profiles inorganic nitrogen (A) and inorganic phosphorus (B) during the single cultures and the co-culture growth.....80

Figure 18: Biomass growth for *C. sorokiniana* single culture, *M. capsulatus* single and the co-culture. 81

Figure 19: Residual gas composition of (A) co-culture (B) *C. sorokiniana* single culture and (C) *M. capsulatus* single culture. 84

Figure 20: Nutrient profiles for (A) total nitrogen (TN), (B) NH_3-N, (C) total phosphorus (TP), and (D) PO_4^{3-}-P of the single cultures and by the co-culture. 85

Figure 21: Percent nutrient recovery by summation of the single cultures and the co-culture for total nitrogen (TN), ammonia nitrogen (NH_3-N), total phosphorus (TP), and orthophosphate (PO_4^{3-}-P). 86

Figure 22: Correlation of the biomass produced with the recovery of (A) NH_3-N and (B) $PO_4^{3—}$P. 87

Figure 23: Biomass comparison between (A) *C. sorokiniana* single culture and *C. sorokiniana* growth in the co-culture; (B) *M capsulatus* single culture and *M. capsulatus* in the co-culture and (C) the overall co-culture and summation of the single culture biomass. 89

Figure 24: Residual profiles of (A) the co-culture, and the (B) *C. sorokiniana* and (C) M. capsulatus single cultures. 91

Figure 25: NH_3-N (A) and PO_4^{3-}-P (B) recovery comparison among culture of *C. sorokiniana*, *M. capsulatus* and the co-culture. 93

Figure 26: Correlation of the biomass produced with the recovery of (A) NH_3-N and (B) PO_4^{3-}-P under carbon unlimited growth. 94

Figure 27: Case 4: *C. sorokiniana* biomass comparison. 96

Figure 28: Photobioreactor: Modified BioFlo 110 with light panel and flexible heating pad for bench-scale co-culture investigations. 106

Figure 29: Light attenuation inside the PBR caused by self-shading effect of co-culture cells. (A) The light intensity measured at the inner wall of the glass vessel and (B) geometric center of the PBR at different co-culture optical densities. Incidental irradiances tested were 400 and 800 μmol/m^2/s.....108

Figure 30: Dynamics of co-culture growth during batch and chemostat operation of the photobioreactor. 112

Figure 31: Biomass plots of C. *sorokiniana*, M. *capsulatus* and the co-culture. 113

Figure 32: Co-culture illuminated areal productivity of co-culture during chemostat mode.....115

Figure 33: Residual gas profiles of co-culture during chemostat operation.....116

Figure 34: Residual nutrient concentration in photobioreactor. 118

Figure 35: N:P recovered by co-culture during batch and chemostat operation. 120

Figure 36: Dynamics of molar N:P recovery (uptake) ratio of the co-culture during batch and chemostat operation as compared to the molar N:P ratio of the residual nutrients in the photobioreactor. For complete recovery of both N and P in the bioreactor both the N:P recovery ratio of the co-culture and the N:P ratio in the bioreactor must follow the green arrow. 121

Figure 37: Nutrient uptake ratio of co-culture in vial experiments of (A) differently AD mixtures and (B) differently pretreated AD effluent diluted with CLE. 129

Figure 38: Case 3: TN (A) and TP (B) plots for *C. sorokiniana*, *M. capsulatus* and the co-culture. 130

Figure 39: Biomass concentration of *C. sorokiniana* on day 1 when grown under different light intensities. 131

List of Abbreviations

ADP	Adenosine diphosphate
AD	Anaerobic digestion
AMS	Ammonium mineral salts
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCOD	Biochemical oxygen demand
BNR	Biological nutrient removal
BOD	Biological oxygen demand
CHP	Combined heat and power
CLE	Secondary clarifier effluent
COD	Chemical oxygen demand
CWW	Columbus Water Works
DI	Deionized water
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EPA	Environmental Protection Agency
EU	European Union
FAO	Food and Agriculture organization
FOG	Fats, oils and grease
GC	Gas chromatograph
GHG	Greenhouse gases

HRAP	High rate algal pond
HRT	Hydraulic retention time
HSD	Honestly significant difference
HTL	Hydrothermal liquefaction
LED	Light-emitting diode
MGD	Million gallons per day
MMO	Methane monooxygenase
MPBR	Membrane photobioreactor
NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PAO	Phosphorus accumulating organisms
PAR	Photosynthetically active radiation
PBR	Photobioreactor
SCP	Single cell protein
TCD	Thermal conductivity detector
TIC	Total inorganic carbon
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
VFA	Volatile fatty acids
VOC	Volatile organic acids
WRRF	Water resource recovery facility
WWTP	Wastewater treatment plant

Chapter 1: Introduction

1.1 Motivation of this work

To sustain a growing world population with increased demand for food, energy and water, global nitrogen fixation has increased. Unreactive nitrogen (N_2) in the air must be converted to a reactive form (such as ammonia and nitrate) for plants to use. Fertilizer production is one of the main sources for producing reactive nitrogen for increasing food production. Food production relies heavily on nitrogen fixation as bacteria convert dinitrogen in the air to ammonia for plant use. However, excess reactive nitrogen in the environment has resulted in significant air and water pollution, leading to negative consequences such as acid rain, eutrophication, worsening of the greenhouse gas effect and reduction of the protective ozone layer, among others (Driscoll et al., 2003). Release of excessive nitrogen into the aquatic environment also affects human health and the economy by lower water quality through the release of toxins by algal blooms. Thus, to mitigate environmental pollution caused by this disruption to the nitrogen cycle, anthropogenic sources containing high loads of nitrogen must be managed.

Agricultural, domestic, and industrial processes generate large amounts of wastewater which contain high loads of organic content. In order to reduce the organic content, wastewater treatment plants utilize anaerobic digestion which is thought to be the best practice for reducing organic content, pathogens and handling odor. During anaerobic digestion, biogas which contains mainly methane (CH_4) and carbon dioxide (CO_2) is generated. Both CH_4 and CO_2 are greenhouse gases and poor utilization of biogas for power and/or energy results in the release of biogas into the environment, further contributing to global warming. Complex organic material is broken down to volatile fatty acids (VFAs) and organic nitrogen and organic phosphorus is converted to reactive/inorganic forms of nitrogen (ammonia (NH_3), nitrate (NO_3^-) and nitrite (NO_2^-)) and

phosphorus (orthophosphate (PO_4^{3-}-P)). As a result, high concentrations of ammonia and orthophosphate contained in anaerobic digestate effluents which must be recovered before being released into aquatic systems. Biological processes such as aerated lagoons, fixed-bed reactors, activated sludge with anoxic tank and activated sludge with intermittent aeration have been shown to exhibit varying levels of efficiency to recovery the nitrogen and phosphorus. Activated sludge with intermittent aeration is considered more adaptable to variable influent loads. Consequently, it is employed at most full scales processes (Bernet and Beline, 2009). The drawbacks of this conventional process are: 1) the low nitrogen (N) and phosphorus (P) recovery and 2) the large energy utilization due to the aeration requirement. Unrecovered nitrogen and phosphorus from wastewater treatment processes that is released into aquatic systems can result in eutrophication. As a result, there is increased research for new technology capable of utilizing biogas and increasing the N and P recovery, while reducing the energy demand for the process.

Furthermore, scientists are encouraging immense research on technology that increases economic and environmental sustainability. The development of sustainable wastewater technology that is environmentally responsible (i.e. reducing carbon footprint) while balancing the economic and societal impact is crucial. While current water resource recovery facility (WRRF) treat wastewater to meet permissible levels for discharge into water bodies, an imbalance in water and nutrient fluxes can alter ecological systems (Peñuelas et al., 2013). In addition, these facilities also consume considerable amounts of energy which contributes to carbon emissions. Thus, increasing environmental and economic sustainability at WRRFs require alternative technology that reduce carbon emissions, energy requirement and augments current wastewater treatment processes.

1.2 Municipal wastewater

Municipal wastewater originates from different sources such as domestic, industry and commercial establishments, even surface or "run-off" water and contains different chemical, physical, and biological contaminants. These pollutants are quite diverse and requires different processes to effectively reduce pollutants to permissible levels at water resource recovery facilities (WRRFs). Chemical pollutants include heavy metals, lead, mercury, chromium, pharmaceuticals, toxins (pesticides, herbicides and poison) among others. There are different types of wastewater processes for reducing pollutant levels in wastewater and these processes employ various chemical, physical, and biological methods for treating the influent water before discharge into the environment or reuse. While sand, grit, metal particles, paper and plastics are physical pollutants, biological pollutants comprise of different pathogens such as bacteria, viruses, protozoa and parasites especially as raw sewage are treated by municipal wastewater treatment facilities. WRRF waste gas and liquid streams contain essential carbon substrate (CH_4 and CO_2), macronutrient (nitrogen (N), phosphorus (P), potassium (K)) and trace metals that can be the driving factor for sustainable and cost-effective microalgal and methanotrophic cultivation resulting in simultaneous bioremediation and biomass for potential value-added products.

1.3 Wastewater treatment

In general, the treatment processes to handle the complex wastewater matrix are categorized into three stages: 1) primary, 2) secondary, and 3) tertiary treatments.

1.3.1 Primary treatment

Primary treatment mostly involves physical pretreatment of influent wastewater to remove debris and other relatively large objects (wood, cloth, plastics, garbage etc.) through screens with

varying sieve sizes (EPA, 2004). The screens often allow easy removal floating and settleable solid debris. Generally, primary treatment processes remove particles no smaller than 30-80 μm (Sonune and Ghate, 2004). After removal of large physical particles, the wastewater flows to grit chambers where smaller particles like sand and gravel settle at the bottom of the tank. Subsequently, this wastewater is fed to the primary settling basin where the suspended solids settle through gravitational settling to form sludge at the bottom of the basin, leaving dissolved solids in the effluent. At some wastewater treatment facilities, primary treatment can involve the addition of chemical additives that enhance flocculation of smaller media and therefore enhance removal in longitudinal or circular sedimentation tanks. Coagulants (aluminum, lime or iron) are also used as appropriate additives (Väänänen, 2017) (Nieto et al., 2011) which cause pollutants to floc together for removal by physical process. A combination of filtering media/screens and additive pretreatment that enhances sedimentation can also be used. At this point, the wastewater still contains dissolved organics and nutrients in the primary effluent (effluent from primary settling basin) and degradation of these pollutants is achieved by secondary treatment processes.

1.3.2 Secondary treatment

In general, secondary treatment involves biological processes that breakdown organic content and remove harmful pathogens, inorganic chemicals and recover inorganic nutrients from the primary effluent. Usually, this is accomplished through suspended growth system such as biologically activated sludge, oxidation ditches, and sequencing batch reactors. As biological methods are inexpensive and more effective, they are preferred for nitrogen removal and biochemical oxygen demand (BCOD) as compared to physical-chemical methods. In suspended growth process such as activated sludge, bacteria and other microorganisms (archaea, fungi and protist) capable of removing ammonia-nitrogen (NH_3-N) and BCOD are utilized. At this stage, up

to 90% of the biological oxygen demand (BOD) and suspended matter may be removed by providing an aerobic environment to the aerobic bacteria (EPA, 2004). While there are different wastewater processes including conventional activated sludge, anaerobic-anoxic-oxic (A^2O), anaerobic-oxic (A/O), sequencing batch reactor (SBR) which are efficient at BOD removal, these processes can suffer low removal nitrogen and phosphorus removal rates.

1.3.2.1 Anaerobic-anoxic-oxic (A^2O) process in wastewater

This process combines nitrogen, phosphorus and BOD removal with difference oxygenated regions. Each stage in the A2O process removes a certain pollutant and multiple configurations of these three processes have been utilized based on the size of the plant, nature of the wastewater and the obligations to be environmental regulations (Grissop, 2010).

1.3.2.2 Anaerobic process

In the absence of oxygen, anaerobic microorganisms breakdown organic nitrogen and organic phosphorus into inorganic forms (NH_3-N and PO_4^{3-}-P). Heterotrophic bacteria degrade nitrogen-rich organic molecules (e.g. proteins) into amino acids, which are then converted to ammonia, carbon dioxide and water. Wastewater facilities that treat animal waste may have especially higher ammonia concentrations during this process due to the higher protein content. In anaerobic tanks, organic phosphorus is broken down by phosphorus accumulating organisms (PAOs) that uptake volatile fatty acids (VFAs) and convert them to intracellular stored polyhydroxy butyrate (PHB). PAOs then break the energy rich polyphosphate bond to produce the energy required for storing PHB and release orthophosphate into solution.

1.3.2.3 Aerobic process

In this process, the removal of inorganic nitrogen species (NH_3-N, NO_3^--N) and orthophosphate is achieved through dissolved free oxygen present in the aerobic tanks. Removal of the nitrogen in the wastewater influent is often achieved by nitrification processes (Farazaki and Gikas, 2019) while inorganic phosphorus can be removed further by biological means. Nitrification is a two-step process and occurs under the oxidative conditions; thus, aeration is required for maintaining dissolved oxygen. During nitrification, reduced nitrogen (NH_3-N) are sequentially oxidized to nitrate. In the first step of nitrification, autotrophic nitrifying bacteria are responsible for oxidizing ammonia-nitrogen to nitrite. These bacteria are frequently identified as belonging to the *Nitrosomonas* genus. In the second step of nitrification (Equation (1)), nitrite is converted to nitrate by nitrite-oxidizing bacteria (*Nitrobacter* genus) (Farazaki and Gikas, 2019).

Nitrification

$$NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^- \quad (1)$$

$$NH_4^+ + 2O_2 \rightarrow NO_3^- 2H^+ + H_2O \quad (2)$$

Orthophosphate removal occurs in this process as well. Heterotrophic bacteria uptake orthophosphate while metabolizing stored PHB for producing new cells and any excess uptake of orthophosphate is stored as polyphosphates.

1.3.2.4 Anoxic process

Denitrification occurs during the anoxic (lack of free oxygen) process where the nitrate produced during nitrification is reduced to gaseous nitrogen, in the presence of organic carbon.

Denitrifying bacteria breakdown BOD through respiration and utilize the oxygen from nitrate producing gaseous nitrogen through a series of reduction steps shown in equations (3) and (4).

Denitrification

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (3)$$

$$NO_3^- \ 0.1C_{10}H_{19}O_3N \rightarrow 0.5N_2 + CO_2 \ 0.3H_2O + 0.1NH_3 + OH^- \quad (4)$$

In denitrification, conversion of nitrate to nitrite occurs rapidly while nitrite conversion to nitrogen gas is much slower and can be severely affected by temperature (Fan et al., 2015). As seen in the equations above, nitrite is an intermediary nitrogen specie in both nitrification and denitrification. Efforts to increase efficiency and reduce large aeration process have focused on removing nitrogen through the nitrate pathway shown in Figure 1 (Peng and Zhu, 2006). In this pathway, partial nitrification to nitrite and nitrite denitrification is reported to be technically and economically feasible; however, operating conditions (high temperature, high ammonium concentration, low dissolved oxygen concentration (Blackburne et al., 2008), controlling of hydraulic retention times or aeration time (Gao et al., 2009)) have to be manipulated to favor ammonia oxidizing bacteria and inhibit nitrite oxidizing bacteria which can make controlling the process complex (Naseer et al., 2013). After nutrient removal, the effluent in the final process from the anoxic tanks is fed to clarifier tanks which allow settling and sedimentation of particulates and recovery of low residual nutrients. In the clarifier effluent the settled sludge from the A^2O process is further processed in the anaerobic digester.

Nitrification **Denitrification**

$$NH_4^+ \rightarrow NO_2^- \rightarrow \cancel{NO_3^-} \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (5)$$

Figure 1: Nitrification and denitrification by the nitrite pathway where ammonia is only partially oxidized to nitrite.

1.3.3 Challenges of anaerobic-anoxic-oxic treatment processes

While secondary treatment has been employed in nitrogen removal for more than half a century, there still exist challenges of high energy consumption during the process and controlling efficient nitrogen recovery by manipulating recycle streams and addition of exogenous compounds and maintaining operating conditions.

1.3.3.1 Operating conditions

To achieve high efficiencies in the nitrification and denitrification processes operating conditions must be maintained at certain preferred levels. For example, nitrification problems occur if dissolved oxygen is not above 3 mg/L. Low temperature can reduce growth rate of nitrifiers, but high temperatures reduce dissolved oxygen. Insufficient alkalinities can also reduce ammonia removal. pH swings and toxins from the influent wastewater can also have an adverse effect of nitrification.

1.3.3.2 Energy consumption

Nitrification requires an aerobic environment (dissolved oxygen) for oxidation of ammoniac nitrogen to nitrate. Heterotrophic bacteria also use oxygen as a terminal electron acceptor for degradation of organic compounds. Aeration is typically achieved by diffused air aerator as high rates of oxygenation is achieved. However, aeration can account for at least 50%

of the total energy costs of a wastewater plant (Siegrist et al., 2008; Drewnowski et al., 2019). Oxygen has low solubility in water and its solubility in aerobic tanks is affected by various properties of the mixed liquor (water containing activated bacteria, BOD and pollutants in aerobic tanks). Consequently, long aeration times might be required to maintain minimum levels of dissolved oxygen.

1.3.3.3 Low C:N ratio

Anaerobic digestate effluent is recycled back to the headworks of the plant. The AD effluent contains high concentrations of ammonia and orthophosphate which will be recovered during secondary treatment. This can often upset the A^2O process and cause phosphorus accumulation over time. Wastewater treatment plants also face challenges of low C/N ratio which results in inefficient nutrient recovery due to inadequate BOD (Peng et al., 2006). To address this problem, aerobic effluent is recycled with the anoxic tank to meet the BOD requirement. As a result, controlling the operation can become complex. Besides recycling the aerobic effluent, exogenous carbon sources (methanol, etc.) can be introduced into the effluent to enable complete denitrification. Consequently, operational cost is increased. To improve biological nitrogen removal through denitrification, recent studies have focused on different approaches such as partial nitrification of ammonia to nitrite (Peng and Zhu, 2006) and step feed operation (Hu et al., 2014). Peng et al suggest that two main advantages are lower oxygen consumption, higher denitrification rates and reduced CO_2 emissions but full scale application is limited (STOWA, 1995; Peng and Zhu, 2006). Furthermore, nitrification and denitrification tanks occur in series, requiring sufficient residence time for ammonia oxidation and prevention of dissolved oxygen transfer to anoxic tanks. Thus, operation of A^2O systems can be complex and some facilities cycle aerators on and off in order to achieve the desired oxygenation level of the mixed liquor.

1.3.4 Tertiary treatment and advanced processes

Tertiary wastewater treatment aims to remove specific wastewater pollutants which remain after secondary treatment (Abdel-Raouf et al., 2012). Tertiary treatment is accomplished through either chemical or biological methods. In chemical treatment, undesired compounds and heavy metals are removed through precipitation but some chemicals remain unaffected. Another disadvantage of this process is the costs associated with the chemical additives and disposal of the chemical sludge (Samer, 2015). Advanced treatment processes are more complex than chemical tertiary treatment; however, advanced treatment also serves the similar purpose of further removal of nitrogen, phosphorus, metals or fine particulates. The advanced processes are varied in the underlying principles for pollutant removal and they include chemical precipitation, ozonation, reverse osmosis or carbon adsorption.

1.3.5 Disinfection

Disinfection is usually the last step in wastewater treatment. The process is designed to inactivate pathogenic microorganisms such as bacteria, viruses or cysts especially as wastewater is discharged into waterbodies that can be used for human activity. Three methods used are chlorination, ozonation and ultraviolet radiation. However, chlorination is the most commonly used method as it is known to be effective in destroying various types of harmful bacteria, viruses and protozoa.

1.4 Anaerobic digestion

1.4.1 Anaerobic process for biosolids reduction

The sludge collected from primary treatment is pumped to anaerobic digesters (AD) for degradation of the organic nitrogen and phosphorus. During anaerobic digestion, acetic acid, volatile fatty acids (VFAs) and biogas are the main products. Organic nitrogen and organic phosphorus are converted mainly to inorganic nitrogen as ammonia (NH_3) and inorganic phosphorus as orthophosphate (PO_4^{3-}) while very low concentrations of nitrate and nitrite may be present. Consequently, high concentrations of NH_3 and PO_4^{3-} are generated in AD processes due to the high organic content. Nitrogen and phosphorus levels found in anaerobic digester effluents at municipal wastewater plants can vary between 100 – 3500 mg/L of N and 18 – 400 mg/L P (Cai et al., 2013). Subsequently, the AD effluent is sent to gravity belt or centrifuges to separate the liquid and solid fractions. At some facilities the liquid fraction of the digestate is recycled back to the headworks of the plant while others utilize the liquid fraction for plant fertilizer. By recycling the liquid fraction to the headworks of the plant, the ammonia and phosphorus generated during the anaerobic process is removed during aerobic and anoxic processes.

1.4.2 Biochemical process

Anaerobic digestion refers to a series of complex process that degrade organic compounds in the absence of oxygen. It is considered the best practice for handing organic material and reducing odor and pathogens. Generally, there are four stages of this process, namely: hydrolysis, acidogenesis, acetogenesis/dehydrogenation and methanation and each step is carried out by different microorganisms. During hydrolysis various microorganisms convert polymeric compounds to monomers (long chain fatty acids, amino acids, sugars) and monomeric molecules can be further degraded to volatile fatty acids (acetate, butyrate, etc.), hydrogen and carbon

dioxide. Acetogenic bacteria produce acetic acid from volatile fatty acids and methanogens are responsible for the production of methane from acetate or hydrogen and carbon dioxide.

1.4.3 Biogas production

Biogas is a renewable and valuable energy resource as a byproduct of anaerobic digestion. Typically, biogas contains 50-70% methane and 30-50% carbon dioxide and the ratio of these two gases in biogas depends on the substrate, operating conditions and pH among other factors. At WRRFs, the anaerobic digester substrate usually comprises of the sludge from the primary settling basin as well as waste activated sludge from the secondary clarifier tanks. However, co-digestion of organic wastes with sludge is applied as a strategy for increasing the digester gas production and yield (Shen et al., 2015). Fats, oil and grease (FOG) is a lipid-rich organic waste that that is currently utilized in numerous WRRFs across the United States. As a result, biogas production at WRRFs will benefit from improved efficiency and conversion of waste to energy.

1.4.4 Biogas utilization and challenges

When captured and utilized efficiently, biogas can yield significant energy and economic value to WRRFs. Methane and carbon dioxide can be valorized through bioconversion to value-added products. Additionally, methane can be recovered for power generation, heat and vehicle fuel. Combined heat and power (CHP) technology can reduce the energy consumption of WRRFs, reduce their carbon footprint by decreased fossil fuel consumption and mitigate methane emissions. In addition, the potential reduction of fossil fuel use for energy generation will low the carbon footprint wastewater treatment facilities. Combustion of biogas for heating releases CO_2 in the atmosphere, further contributing to GHG emissions.

Technical and economic barriers have limited the utilization of biogas for power generation. One such barrier is the expensive equipment and costly processes that are necessary for removing contaminants such as carbon dioxide (CO_2), hydrogen sulfide (H_2S) and siloxanes. These impurities must be cleaned up to meet biogas quality requirements for use with CHP technology. As a result, only 10% of plants utilize biogas for heating or electricity generation(Shen et al., 2015). Furthermore, it was suggested for economic feasibility with CHP technology the wastewater influent should be 5 million gallons per day (MGD). To address some of these challenges, both methanotroph and microalgal biotechnology has emerged as promising approaches for recovering the carbon and energy through microbial biogas upgrading. Methanotroph and microalgal biotechnology has proven to be scalable and provides this advantage over CHP technologies.

1.5 Current treatment of AD-derived biogas and liquid digestate

Raw municipal wastewater was acquired through the collaboration with the South Columbus Water Resource Recovery Facility (SC WRRF) in Columbus, GA. Figure 2 below illustrates how the biogas and digestate produced from the AD are handled. Briefly, SC WRRF operates one thermophilic and two mesophilic digesters. The biogas produced in these digesters must be cleaned by passage through the gas treatment pad to remove hydrogen sulfide (H_2S), siloxanes and any other impurities before being used for combined heat and power (CHP). While SC WRRF utilizes this technology, the gas treatment pad is costly to operate in small wastewater treatment facilities; as a result biogas is mainly underutilized and used for heating at small facilities (Shen et al., 2015). The liquid digestate is sent back to the head of the plant for nutrient recovery by secondary treatment. At some wastewater facilities, the biosolids and/or liquid digestate can

also be used for liquid fertilizer. However, excessive application can lead to surface runoff of pollutants; further contributing to environmental pollution.

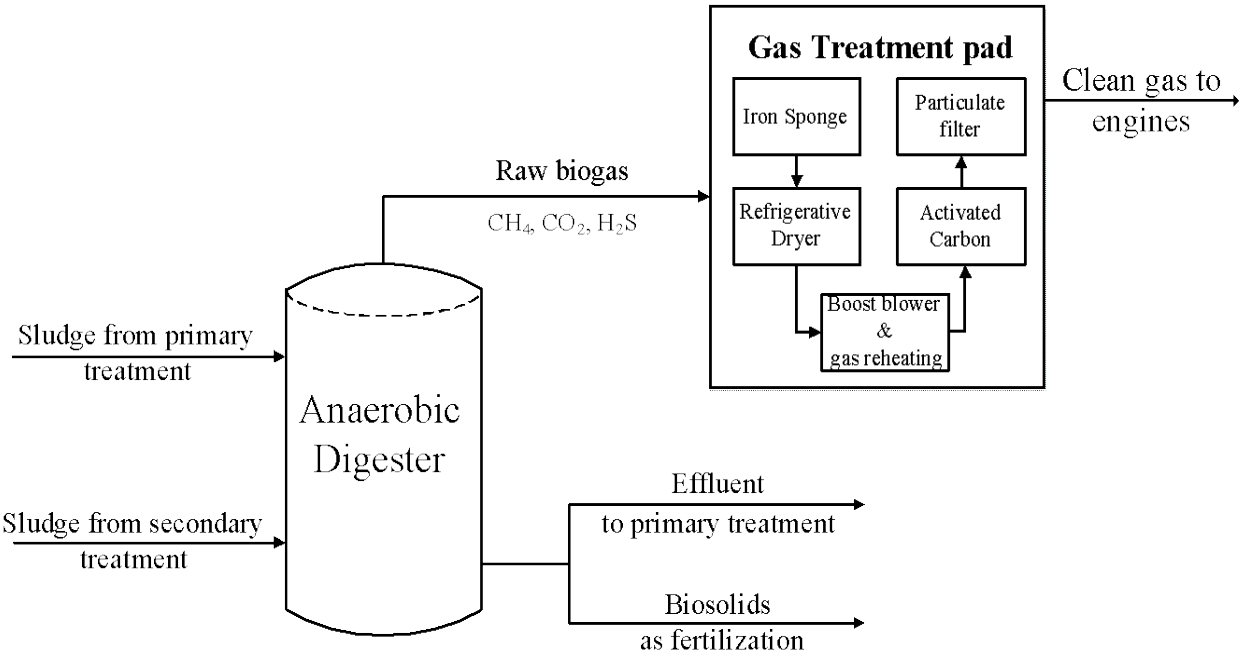


Figure 2: Current process of handling AD produced biogas and digestate from the South Columbus Water Resource Recovery Facility.

1.6 Microalgae

Microalgae are photosynthetic prokaryotic or eukaryotic microorganisms below 100 – 200 μm, mostly unicellular and are classified based on their pigment composition. They can grow rapidly and live in harsh conditions due to their unicellular or multicellular shape. As a result, the large diversity of microalgal species have enable wide research in their use for wastewater applications (Li et al., 2019).

Carbon metabolism: Autotrophic

Oxygenic photosynthesis falls under the autotrophic metabolism and is key survival mode of all photoautotrophs, including microalgae. During photosynthesis, light energy is captured and converted into various organic compounds for growth, storage and different metabolic processes. Specifically, photosynthesis involves conversion of carbon dioxide and water into carbohydrates and water through redox reactions that are driven by energy harnessed from light (Masokidek et al., 2013). The process is divided into two stages: light-dependent and light independent reactions. In the light dependent reactions, the light energy is captured by photosynthetic pigments (carotenoids, chlorophyll a and b, phycocyanin, phycoerythrin (MacIntyre et al., 2002)) that comes from the photosynthetically active radiation (PAR).. In general, the PAR region falls between 400 -700 nm which are the wavelengths of light utilizable for photosynthesis. In the thylakoid membranes of microalgae, light energy is trapped by light-harvesting pigment-proteins called photosystems I and photosystems II. Two electrons are extracted from water and transported through the electron chain through a series of carriers in order to produce the biochemical reductant nicotinamide adenine dinucleotide phosphate ($NADPH_2$) and energy molecule adenosine triphosphate (ATP) evolving oxygen in the process. Protons are also transferred from the stroma to the lumen of the cell to create a pH gradient which drives ATP synthesis. In the dark reaction,

the $NADPH_2$ and ATP produced in the light reactions will be used for fix carbon fixation into the biomass. The conversion of CO_2 into carbohydrates (sugars) occurs by the so-called Calvin-Benson cycle (Benson and Calvin, 1947; Calvin, 1956).

Nitrogen metabolism

Nitrogen (N) is an essential and major component of microalgal cells apart from carbon, hydrogen and oxygen. Elemental nitrogen can contribute up to 10% of the dry mass. Nitrogen is incorporated into various amino acids that are then used for producing proteins. Microalgae can uptake different reactive forms of nitrogen (NH_3-N, NO_3^--N, NO_2^-) for assimilation into various biochemical molecules, amino acids and nucleotides that are then used to produce proteins and nucleic acids (DNA and RNA), respectively. Ammonia is the preferred form of nitrogen because a redox reaction is not involved, and it can be directly assimilated into their nitrogen metabolism to produce glutamine, glutamate and aspartate. Ammonia assimilation is directly coupled to CO_2 fixation because the carbon skeletons formed during photosynthesis are used for amino acid synthesis (Flynn, 1991). When ammonia is not present, microalgae can reduce nitrate to nitrite and subsequently ammonia before assimilation as described above. However, reduction from nitrate to ammonia (NO3- → NO2- → NH3) requires energy as two redox reactions are must occur. While some microalgae are capable of assimilating specific molecules containing organic nitrogen it has been reported that organic nitrogen uptake is quite small (Pehlivanoglu and Sedlak, 2004; Liu et al., 2012).

Phosphorus metabolism

Phosphorus is also an essential macronutrient for microalgae cells as it is a component of nucleic acids, phospholipids, proteins, sugar phosphates and energy production (ATP).

Furthermore, microalgae can also store phosphorous as organic polyphosphate (Poly-P) compounds (Solovchenko et al., 2019) especially under replete conditions (>5 mg/L) (Powell et al., 2009). Poly-Ps are linear, unbranched internal storage polymers of three to several hundreds of orthophosphate residues linked by phosphoanhydride bonds (Harold, 1966). Orthophosphate is readily taken up by microalgae and as in the case organic nitrogen, uptake of organic phosphorus is negligible.

Minerals: Trace metals or micronutrients

Trace metals such as cobalt and zinc are essential at low/trace concentrations and other metals such as iron, magnesium and copper are required in small amounts. Trace metals and micronutrients are required for different metal complexes in microalgal biomolecules. For example, magnesium occupies the central position in the chlorophyll pigment which is affects the photosynthetic activity of the cells(Ben Amor-Ben Ayed et al., 2016). Additionally, copper serves as a cofactor in enzymes that regulate photosynthetic redox reactions (Gonzalezdavila, 1995).

1.6.1 Microalgae as municipal wastewater treatment

Regulatory obligations require that wastewater pollutants are reduced to safe limits before use or discharge into freshwater or land. Traditional wastewater treatment is not effective, as a result, advanced chemical treatment processes are being implemented to meet stricter discharge regulations. One challenge to the wide implementation of chemical processes as tertiary treatment is the high implementation cost and secondary pollution contribution. In contrast, biological methods are potentially more cost effective for bioremediation with reduced waste and the biomass produced can be valorized for value-added products (Osundeko et al., 2019). For several decades, microalgae-based wastewater technology has been investigated as a promising platform for

bioremediation of wastewater effluent streams (Oswald et al., 1957; McGriff and McKinney, 1972; Gupta et al., 2017) as these streams contain the required nitrogen, phosphorus and minerals required for microalga proteins, nucleic acids and other storage compounds. Thus, wastewater streams can serve as an economical and sustainable growth medium for culturing microalgae. Due to this reason, microalgal-based technology continues to maintain research interest as a promising tertiary treatment method from reducing the high N and P concentrations in wastewater streams such AD effluent and studies are being conducted with pilot studies to further prove feasibility of this approach.

1.6.2 Microalgal biogas upgrading

In addition to prohibitive economic gain from using different physical and chemical methods to remove CO_2 from biogas, other disadvantages include the requirements for large amounts of energy and the generation of wastes (Ryckebosch et al., 2011). Oxygenic photosynthesis has long been known as nature's way of capturing light energy for CO_2 fixation. Microalgae are fast growing microorganisms with higher carbon fixation through photosynthesis and studies have demonstrated microalgae as a promising technology for biogas upgrading. Microalgal based technology is seen to be more favorable as expensive equipment is not required and its cultivation can occur under ambient conditions. However, there exists the process safety risk of mixing methane with photosynthetically produced oxygen which can create potential explosive conditions. (Bahr et al., 2014; Rasouli et al., 2018).

1.6.3 Microalgal cultivation systems for wastewater treatment

Technologies for microalgal production at pilot-scale or large scale operation can be classified into three groups: Traditional open systems, enclosed photobioreactors (PBRs) and new

designed multi-technology hybrid systems (Li et al., 2019). One of the most commercialized industrial culture systems is the high algal rate pond (HRAP) which is an enhanced type of raceway pond that maximizes the algal biomass concentration to increase wastewater treatment efficiency (Young et al., 2017). HRAP are shallow ponds (0.2-0.4 m deep) that allow light penetration and paddle wheel circulation for mixing. CO_2 is requirement is provided by surface air or submerged aerators for CO_2 addition. However, HRAP system have a high rate loss of CO_2, relatively low biomass productivity and high cost for harvesting which limits wide-spread application of this technology. Closed PBRs are tubular or flat plate photobioreactors arranged into different configurations as vertical, horizontal, helical or inclined. Mechanical pumps are used for circulating CO_2 and suspended biomass. Lower volume /surface ratios in PBRs enable higher biomass concentrations than HRAP but PBRs are still limited by energy consumption and biomass productivity, harvesting costs (Li et al., 2019) and the self-shading effect. Membrane PBRs (MPBRs) is a considered a hybrid cultivation system which enable higher biomass concentration with higher nutrients removal rates and a smaller footprint. The operational costs were calculated to be 0.113 US$/m^3 in comparison with 0.65-0.96 $/m^3 in conventional PBRs (Sheng et al., 2017). Unfortunately, membrane fouling contributes to more than 50% of the costs (Li et al., 2019). Finally, microalgae biofilm hybrid systems are gaining research interest as a more efficient algal cultivation system that could enhance biomass productivity (higher number of cells per unit volume) and harvesting procedures. Microalgal biofilms can be constantly submerged, intermittently submerged or perfused systems. There is also a reduction in the growth medium when compared to similar biomass amounts in open systems. Consequently, this will lead to potential savings due to a reduction in harvesting and dewatering cost (Berner et al., 2015). Carbon substrate can be acquired from both the liquid and headspace in the different biofilm reactor;

thereby, decreasing mass transfer limitations and manipulation of hydraulic retention times for CO_2 fixation as in fully suspended algal systems.

1.7 Co-culture biomass as feedstock for valuable products

Microalgae and methanotrophs have immense potential as renewable feedstocks as the biomass can be used for producing a variety of products. Under different operating conditions, both microalgae and methanotrophs can accumulate various energy compounds (carbohydrates, proteins and lipids) to use as feed, other products and especially for energy products as biofuels. Moreover, cultivation on waste streams such as wastewater is a major cost reduction step towards a more economically feasible co-culture cultivation.

1.7.1 Biofuel potential

The limited supply of fossil fuel as well as its negative effects on the environment (contribution to global warming) has led to the reduction of fossil fuels for energy. Consequently, balancing the increasing global energy demand while decreasing use of traditional fossil fuel has prompted continued research into alternative fuels from sustainable feedstock. Microalgae has attracted significant interest to the scientific community as a renewable resource of energetic compounds (carbohydrates, lipids) which can be converted to biofuels(Lakatos et al., 2019). Biofuels consist of biodiesel, bio-oil or bioethanol. First generation biofuels are acquired from crops. Three major challenges of first generation biofuels are: 1) reduced human food production and increased use of arable land and environmental damage(Lakatos et al., 2019).

Table 1: Cultivation of different microalgae strains in municipal wastewater.

Wastewater type (Cultivation mode)	**Species**	**Initial TN concentration (mg/L)**	**Initial TP concentration (mg/L)**	**Biomass productivity (mg/L/day)**	**TN removal (%) TP removal (%)**	**References**
Centrate (Batch)	*Chlamydomonas reinhardtii (microalga)*	128.6	120.6	2000	43.4 14.4	(Kong et al., 2009)
Filtered secondary effluent (Batch)	*Chlorella sp. (microalga)*	18.9	1.7	74	92 86	(Cho et al., 2011)
Secondary treated (batch)	*Scenedesmus obliquus (microalga)*	27.4	11.8	26	94 98	(Martínez et al., 2000)
Centrate (batch)	*Chlorella sorokiniana (microalga) + Methylococcus capsulatus (methanotroph)*	140	25	720	80 99	This study
Centrate (batch)	*Chlorella sorokiniana (microalga) + Methylococcus capsulatus (methanotroph)*	120 (NH_3-N)	18 (PO_4^{3-}-P)	700	100 100	This study

Second generation biofuels utilized non-crop feedstocks such as agricultural and forest residue, grass and waste oil; however, its production has is not profitable as it requires expensive technology. Third generation biofuels are promising in overcoming the challenges posed by 1st and 2nd generation biofuels(Behera et al., 2014; Chew et al., 2017). Advantages of third generation biofuels include:

1) Rapid growth rate of microalgae as compared to plants as feedstock
2) Cultivation of microalgae on various waste nutrient sources promotes bioremediation through efficient removal of mainly inorganic N and P.
3) Higher photosynthetic efficiencies (4-5%) can be reached as compared to plant (1-2%)(Lakatos et al., 2019)

1.7.1.1 Bioethanol

Microalgae contains carbohydrates that serve as the feedstock for fermentative bioethanol production. Carbohydrates are present in microalgae as either stored (glycogen, starch) or as a structural component of the cell wall (cellulose, sulphated polysaccharides). Different physical (bead milling, freeing, agitation, high pressure homogenization), chemical (acidic or alkaline hydrolysis), and enzymatic pretreatments are required to release the carbohydrate content. It is important to note that the lack of lignin in microalgae requires less harsh pretreatment as compared to 1st generation bioethanol. Then microalgal biomass is hydrolyzed to convert polymeric carbohydrate to glucose (most abundant monomeric sugar) for subsequent fermentation by yeast or bacteria. Low carbohydrate content in microalgae will not favor economic production of bioethanol; thus, there are increasing efforts to enhance and optimize the carbohydrate content of microalgal cells through manipulation of the culture conditions (irradiance, temperature, carbon dioxide supply, pH and nutrients)(Khan et al., 2018; Lakatos et al., 2019; Qu et al., 2019). Some

of the challenges of third generation bioethanol are dewatering algae culture, pretreating biomass for releasing carbohydrates, and optimizing the fermentation process.

1.7.1.2 Bio-oil

Microalgal biomass have gained ground as feedstock for high-value biofuels due to their energy-rich biomolecules (carbohydrate, protein and lipids). However, high nutrient cost coupled with the use of limited freshwater sources pose significant challenges for commercial application. The AD effluent and biogas produced at WRRFs is a convenient and sustainable source of essential nutrients (nitrogen, phosphorus and trace metals) and carbon substrate (CO_2) required for high growth rates and biomass production. The significant advantages offered through this approach are 1) reduction in environmental pollution by capturing the nutrients and 2) low cost biomass production which can be used for production bioenergy such as biofuels. However, the energy and cost-intensive downstream processing of microalgal biomass for biodiesel production is a major drawback for the economic feasibility of converting microalgae to biofuels. Producing biodiesel from lipids by the conventional transesterification process is energy intensive as the biomass has to be dried and the residual biomass containing proteins and carbohydrates are not utilized(Goswami et al., 2019). The traditional transesterification process also utilizes hazardous organic solvents(Cao et al., 2013) which can increase operating costs. Furthermore, acquiring high lipid content of microalgae is usually stimulated to nutrient depletion which in turn would affect microalgal growth rates and biomass productivity. In order to address these shortcomings, researchers are focusing on converting microalgal biomass to biofuels through hydrothermal liquefaction (HTL). HTL greatly reduces the energy input as wet biomass can be directly converted to biocrude and all components of the biomass can be converted to biocrude. HTL is more ecofriendly as it does not involve harmful solvents for oil extraction. Thus, integration of the

methanotroph-microalga co-culture for bioconversion of wastewater treatment to biocrude can yield significant economic and environmental benefits while addressing the shortfalls of the microbial biomass to biodiesel process.

1.7.2 Single cell protein

Global consumption demands of animal-derived protein is predicted to require 1,250 million tons of meat and dairy to be produced annually by 2050(Ritala et al., 2017). However, increasing meat production faces the major limitation of low feed conversion ratio by beef, pork and poultry(Ritala et al., 2017; Jones et al., 2020). Additionally, meeting increased protein demands from plant-based sources such as bean will be limited by the arable land and water requirements. Seafood, wild-catch and aquaculture is reported as the largest animal protein industry in the world and over the past two decades the largest increase in animal protein sectors results from aquaculture. By lowering the feed production cost, aquaculture has the potential to become more a sustainable animal protein industry towards meeting global protein demands.

Single cell protein (SCP) is protein produced by microbial cells and has been investigated for decades for enhancing protein content in animal feed. especially when waste side streams of carbon and nutrients are valorized, and arable land is not required (A.T. Nasseri et al., 2011). By weight percent, the protein in meat, milk and soybean are about 45%, 25% and 35%, respectively (Ritala et al., 2017). Various microorganisms identified as suitable for SCP are algae (cyanobacteria and microalgae), bacteria (methanotrophs) and yeast (Candid, Saccharomyces, etc.) amongst others. Vitamin, amino acid, fat and high protein content(A.T. Nasseri et al., 2011; Ritala et al., 2017) of these microorganisms make them attractive at animal feed supplement. Advantages of SCP processes over conventional plant and animal sources of protein include higher efficiency in substrate conversion and high productivity derived from fast growth rate of

microorganisms. In addition, these microorganisms afford the ability to utilize carbon from waste feedstocks originating from agricultural, municipal and industrial sources while recovering harmful pollutants. For example, agricultural and municipal waste streams have been increasingly investigated as a cheap and economical medium for methanotrophic bacteria, yeast and microalgae cultivation with intended use as single cell protein(Ritala et al., 2017). This approach is intended to produce SCP more economically while utilizing the microorganism for bioremediation. The gaseous carbon (CO_2, CH_4) from waste gas streams of anaerobic digesters serve as substrate for microalgae and methanotrophic bacteria eliminating the threat of release of GHGs. Organic compounds in wastewater has also demonstrated the potential for promoting microalgal growth. Furthermore, microalgae and methanotrophic bacteria are capable of reducing pollutants by assimilating nitrogen, phosphorus and COD from wastewater effluents(Putri et al., 2018; Rasouli et al., 2018). Conversely, the SCP process has a principal disadvantage of high nucleic acid content which can cause health disorders(A.T. Nasseri et al., 2011; Ritala et al., 2017) such as kidney and bladder stones in long-lived animals. Any use in short-lived animals requires further processing(Strong et al., 2015). Secondly, animal feed supplemented with SCP originating from waste feedstock substrates may introduce toxic and carcinogenic compounds into animal feed. Extensive testing of SCP products is performed before being marketed for animal feed(Strong et al., 2015; Ritala et al., 2017). Microalgae can survive in high concentrations of heavy metals whereas excessive concentrations of heavy metals can be detrimental to animals. Another challenge is the technical and economic cost of harvesting microorganisms cultivated for SCP. Lastly, wide-spread commercial application of SCP from wastewater treatment will heavily rely on addressing production cost by reducing downstream product modification and separation processes.

1.7.2.1 Algae as SCP

Microalgal biomass can also contain relatively high protein content in addition to a number of essential micronutrients (i.e. omega-3 fatty acids, beta-carotene, beta-glucan and vitamins A, B, C and E and pigments) that can supplement nutritional value (Jones et al., 2020). Provision of protein through microbial biomass should preferably contain at least 30% protein content. Figure 3 reports the protein content of various algae where the species-dependent protein content is between 30-75%. *Li et al.*(Li et al., 2019) has recently compiled a more comprehensive list of microalgae species commonly used in wastewater treatment. Although, the species selected by Li et al. was based on nutrient recovery, the reported the protein composition of the different species ranging between 60-71% by weight.

While the protein content of microalgae is attractive for use as SCP, production costs would have to be decreased. Feedstock has a major contribution to production cost, hence, to improve the economics of SCP production for animal feed, researchers have turn to utilization of waste streams. Presumably, use of waste streams from sustainable processes like anaerobic digestion support a more circular economy while lowering production costs. Conversely, economic factors (energy, feedstock pretreatment/processing cost). Most definitely, regulatory challenges will present significant barrier for SCP obtained from wastewater to be incorporated into animal feed. The effect of wastewater toxins on animal health as well as the potential bioaccumulation of heavy metals are major concerns. For example, Quorn^{TM} which is a SCP produced from fungus involved 16 years of initial safety testing with more years of testing for use outside the UK (Ritala et al., 2017). Furthermore, public perception of acquiring SCP from wastewater may be difficult to overcome.

Organism	Protein content (%)	References
Aphanizomenon flos-aquae	60–75	https://bluegreenfoods.com/nutritional-analysis/ http://klamathvalley.com/aphanizomenon-flos-aquae/
Aphanothece microscopica	42	Zepka et al., 2010
Arthrospira maxima (*Spirulina maxima*)	60–71	De Oliveira et al., 1999
Arthospira platensis (Spirulina platensis)	46–63	Rafiqul et al., 2005
Chlorella pyrenoidosa	45	Waghmare et al., 2016
Chlorella sorokiana	46–65	Safafar et al., 2016
Chlorella spp.	62–68	Liu et al., 2013
Chlorella vulgaris	42–55	Li et al., 2013; Safafar et al., 2016
Euglena gracilis	50–70	Rodríguez-Zavala et al., 2010
Scenesdesmus obliquus	30–50	Duong et al., 2015; Apandi et al., 2017

Figure 3: Recent reports of the protein content of some algae that are of interest (Ritala et al, 2017).

Heavy metals in algal-based SCP

One regulatory issue is the safe consumption of SCP produced from waste streams. Wastewater streams can contain varying concentrations of heavy metals such as zinc, copper, nickel, cadmium, mercury, iron, aluminum and lead among others(Al-Rub et al., 2004; Aksu and Donmez, 2006; Kurniawan et al., 2006). While some of these metals (zinc and copper) are required by microalgae for biomolecular processes in trace concentrations, biosorption of other toxic metals can result in serious health issues (e.g. accumulative poisoning, cancer and brain damage). Due to the ability of microalgae in bioremediation to uptake and accumulation of toxic heavy metals, utilization of microalgae for SCP has been a major concern. Regulation have been imposed for safe limits of heavy metal concentrations in animal feed. For example, European Union (EU) permitted limits for arsenic, cadmium and lead in aquafeed are <10, <1 and <5 ppm, respectively

(Elliott et al., 2017). Animal feed supplemented with sewage solids (as feed) which contain high concentrations of heavy metals resulted in poor meat quality(Ray et al., 1982). Ray et al.(Ray et al., 1982) studied the quality of meat from cattle fed dried excess. Smaller growth rates in cattle compared to control levels were observed as well as elevated levels of iron and lead in the cattle livers and kidney. In aquaculture, reduction of the developmental growth, increase of developmental anomalies and reduction of fish survival are some of the toxic effects caused by heavy metals.

In addition, the digestibility of algal cell used for SCP have also been a concern due to the cell was rigidity (Tibbetts et al., 2017). Cell-rupture methods such as enzymatic (cellulase), chemical, (acids) or physical and mechanical methods can be applied for releasing energy-yielding compounds (proteins, carbohydrates, lipids). *Tibbetts et al.* (Tibbetts et al., 2017) in their recent study used *Chlorella vulgaris* meal as feed for salmon. The cell-disrupted and whole-cell *C. vulgaris* meal demonstrated the similar protein content but had improved digestibility of cellular compounds in the cell-disrupted meal. Additional downstream processing of microalgal SCP will be a cost factor for consideration in massive industrial scale up of SCP processes.

1.7.2.2 Methanotrophs as SCP

Methanotrophic bacteria is considered a promising source for enhancing the protein content of animal feed. Compared to traditional sources of animal protein (i.e. fishmeal), methanotrophic bacteria is expected to have comparable amino acid content to the Food and Agriculture organization (FAO) recommendations(Erdman et al., 1977; Ritala et al., 2017). Besides, protein content, additional nutritional biomolecules such as lipids and vitamins from the B group can be provided by methanotrophic bacteria (Ritala et al., 2017). Additional considerations for methanotrophic SCP include the digestibility and animal health and performance as is seen with

the commercially used methanotroph *Methylococcus capsulatus* (Strong et al., 2015). Some of the animals for which methanotrophic bacteria has been used as a protein source include salmon, pigs, broiler chicken, Atlantic salmon and trout (Aas et al., 2006; Overland et al., 2010; Kuzniar et al., 2019). Furthermore, methanotrophic bacteria are biocatalysts that utilize methane as their sole carbon source and directly convert methane into cellular compounds. Anthropogenic methane is generated by livestock farming, biomass burning, wastewater treatment and landfilling. Due to the sustainable production of methane as a waste gas at wastewater treatment plants, there is continued interest as this waste gas would provide a cheap carbon substrate for producing methanotrophic SCP. Thus coupling waste methane gas streams with methanotrophic SCP production at even small scale (VTT Technical Research Centre, 2016) is being investigated as there is little or reduce cost for methane as gas substrate obtained from waste gas streams.

1.7.3 Biofertilizers

Microalgae and cyanobacteria have gained interest for use as biofertilizers since research in the last few decades have indicated that these microbes are of significant agricultural importance. Of the various type of biofertilizers, algal-derived biofertilizers have demonstrated considerable benefits such as contributing to the improvements in crop yields, plant growth and soil quality as a result of the stimulation of soil microbial interactions (Renuka et al., 2018). These interactions aid in plant growth by improving soil nitrogen, secretion of essential metabolites and organic carbon, mineralization, release of macro and micro-nutrients and production of growth hormones (Gupta and Lata, 1964; Lu and Xu, 2015; Yilmaz and Sönmez, 2017). Nitrogen-fixing cyanobacteria has shown to enhance the N availability in the soil especially as they do not compete with plants for their N demand. Swarnalakshmi et al. has reported a reduction in chemical fertilizer use when algal cells were inoculated in soil of wheat crop (Swarnalakshmi et al., 2013).

Excessive nitrogen in soil creates an environmental concern of leaching; however, as compared to chemical fertilizer, excessive N is biologically fixed to the soil in complex chemical forms. Consequently, leaching through biofertilizers is thought to be low as leaching is only increased after release of inorganic forms of N (Renuka et al., 2018).

Another contribution of microalgae- and cyanobacterial-based biofertilization is mineralization and solubilization of macro- and micro-nutrients that can improve plant growth (Coppens et al., 2016). Microalgae can also secrete siderophores which are organic compounds that help chelate iron or copper such that they are made available to plants and other microbes. Furthermore, there are reports of increased micronutrients (Fe, Mn, Cu, and Zn) in plants when a consortia including microalgae, bacteria and cyanobacteria was used (Manjunath et al., 2016; Renuka et al., 2017).

Microalgae also has the potential to secrete phytohormones (growth hormones) which can play critical roles in the development of plants. Various hormones such as cytokinin and auxin are reportedly produced intracellularly in green microalgae and some strains can also excrete hormones in the cell broth (Mazur et al., 2001; Manjunath et al., 2016; Romanenko et al., 2016). Besides microalgae, methanotrophs may also play a role in plant growth by shaping bacterial communities in paddy rice root (Minamisawa et al., 2016). Therefore, utilization of microalgae and methanotrophs as biofertilizer can be a good agronomic practice for stimulating plant growth and crop yield.

Cultivation of microalgal and methanotrophic bacteria for biofertilizer require large amounts of nutrients. As growth media can be a large fraction of production costs, utilization of wastewater as low-cost medium is critical for improving economic viability. As discussed in earlier sections, municipal wastewater effluents can contain macronutrients (N, P, K) as well as micronutrients (Fe,

Cu, Mn, Zn) required for good growth of algae and methanotrophic bacteria. By this approach, both wastewater treatment and biomass production can be achieved. However, use of wastewater as cultivation media pose potential challenges. Municipal wastewater contains heavy metals that can accumulate in microalgae and hinder its use as fertilizer. Besides, biomass production on unsterilized wastewater effluents is the most economically viable method, but there is the risk of transferring viruses and other potentially harmful bacteria to crops and plants. The use of algal and methanotrophic fertilizer cultivated on wastewater for crop and vegetable fertilizer will depend on the source of the wastewater and the quality of biomass produced. Thus, growth of the co-culture on appropriate wastewater effluents has the potential to be used as an environmentally friendly biofertilizer when the biomass produced meets the biomass feedstock quality requirements. Lastly, co-culture biomass as biofertilizer can also reduce greenhouse gas emissions through methanotrophic CH_4 oxidation and microalgal CO_2 biofixation. Also, the production of chemical fertilizer is an energy intensive process that result in greenhouse gas emissions. Thus, increasing biofertilizer use will also reduce greenhouse gas emissions from chemical-based fertilizers.

1.7.4 Bioplastics

The world-wide demand for petroleum-based plastics are increasing as a variety of consumer products utilize these plastics due to their strength, low weight and resistance to degradation by water, light and chemicals (Zeller et al., 2013). While these properties make plastics attractive and suitable for use in numerous applications and products, petroleum-based plastics have raised both economic and environmental concerns. Polystyrene is a widely used plastic, but its production is an energy costly process and the use of crude oil as a conventional plastic feedstock consumes an already diminishing resource. Further, the resistance to degradation by petroleum-based plastics

increase the amount of solid waste that has to be managed by landfills and waste generated by resin production can cause air and water pollution (Zeller et al., 2013).

To meet increasing plastic consumption demands in a sustainable manner, bioplastics from natural feedstocks offer an alternative to conventional plastics. Starch and cellulose derived from corn, wheat, oil seeds have been used as a feedstock for bioplastics (Piemonte, 2011). Also, proteins in crops (e.g. soybean and sunflower) also serves as a base material for bioplastics (Zhang et al., 2003). A major limitation of crop-derived bioplastics is the competition of these crops for food and feed. Furthermore, these crops require time to grow, arable land, water and fertilizer to produce sufficient amounts the crop biomass necessary for offsetting petroleum-based plastic feedstocks.

In recent years, microalgae have presented as an attractive, alternative for bioplastics feedstock. Microalgae biomass can reach relatively high fractions of protein (30-70%) that render them suitable for use in bioplastics. Some of the advantages of microalgal feedstock for bioplastics include rapid microalgal growth in comparison to terrestrial crops, elimination competition for food, feed and arable land. Moreover, microalgae can be sustainably cultivated on waste streams where AD liquid digestate serves as a low-cost and economical nutrient (N & P) source and CO_2 in biogas is a gaseous substrate for microalgal growth which renders a more economical process but also remediating water for reuse and reducing greenhouse gas emissions. Recent studies suggest that microalgal biomass with the proper protein content can be used in bioplastics. In addition to microalgae, methanotroph biomass also contains high fractions of protein that make them proper for use in bioplastics.

Consequently, once the methanotroph-microalga co-culture contains a proper fraction of protein that yields the desired bioplastic properties, the co-culture technology grown on wastewater effluents can potentially be a suitable biomass feedstock for bioplastics.

Chapter 2: Co-culture technology for treating wastewater

2.1 Brief review on methanotroph-microalgal co-cultures

Recent studies have demonstrated that methane reduction in wetlands have been due to the coupling of aerobic methanotrophs with *Sphagnum* mosses (Kip et al., 2010). Methane oxidation in peat bogs were driven by the *in situ* oxygen produced by the mosses. CO_2 produced by methanotrophs were then incorporated into the mosses creating this carbon recycling nature. In a laboratory study, Van der ha *et al*. (van der Ha et al., 2012) showed that this principle of carbon recycling coupled to *in situ* oxygenic photosynthesis can be used a biogas sink without external oxygen while transforming the biogas to bioproducts. In this study, a two-stage system was first used where the microalga (*Scenedesmus sp.*) was grown on biogas creating a mixture of methane and oxygen which was then utilized the methanotroph (*Methylocystis parvus*) in the second stage. The microbial biomass produced in each stage could be used for different specialty products. Van der ha et al. also performed a one stage culture with both strains to demonstrate external oxygen was not required due to the *in situ* oxygen production. Hill et al. (Hill et al., 2017) extended the application of the co-culture for biogas conversion on different gas mixtures and showed that the methanotroph-microalga pair in their study grew best on a high methane composition mixture (97% CH_4 , 3% CO_2) but also demonstrated good growth on raw biogas (58% CH_4, 42% CO_2, 0% O_2 and 0.3% H_2S). One recent study also investigated the co-culture for use in treating industrial wastewater from a potato processing plant with high starch content but very low concentrations of total nitrogen (19 mg-N/L ammonium) and total phosphorus (14 mg-P/L). Using the co-culture, they were able to achieve high COD removal (91%) but nutrient removal were only 67% and 43% for TN and TP, respectively.

2.2 Proposed co-culture technology

As discussed extensively above, conventional wastewater treatment technologies have major limitation which cause inefficient and uneconomical operation of wastewater facilities. Microalgal-based wastewater treatments have been researched for many decades as tertiary wastewater with conversion of its biomass to value-added products. However, in wastewater treatment facilities biogas upgrading to biomethane is costly and poses a safety risk when photosynthetic oxygen is mixed with methane. Research studies using methanotroph for treating wastewater through methane mitigation and nutrient recovery are limited even though methanotrophs have been found in different wastewater locations (Ho et al., 2013; Siniscalchi et al., 2015). This limited use of methanotrophs may also be due to the inability to safely scale up the process with methane and oxygen mixtures. For this reason, the methanotroph-microalga co-culture provides a critical advantage. The *in-situ* exchange of oxygen between the methanotroph and microalga will increase process safety as the photosynthetic oxygen produced by the microalga is rapidly assimilated by the methanotroph. In addition, the added benefit of capturing the energy and carbon in biogas and converting it to microbial biomass as feedstock for subsequent biofuel product can convert wastewater processes into an "energy-positive" system.

Thus, the co-culture platform is investigated for AD digestate treatment and biogas upgrading at wastewater treatment facilities. Figure 4 shows the proposed process for integrating the co-culture platform into wastewater treatment plant. The costly biogas treatment pad will be replaced by co-culture for biogas for conversion into microbial biomass. In our preliminary test, both methanotroph and microalgae exhibit tolerance to hydrogen sulfide (H_2S) impurity and multiple studies have shown that microalgal-bacterial technology can removed 100% H_2S (up to 5000 ppm) from the system. The co-culture is capable of co-utilization of both CH_4 and CO_2 without external oxygen supply under ambient pressure and temperature which would reduce

operating cost. To support the co-culture growth, the AD digestate which is rich in macro- and micro-nutrient is attractive as a cost-effective medium for the co-culture biomass production as a feedstock for biofuels. Using the co-culture for bioremediation of AD gas and liquid streams at wastewater treatment plants would significantly reduce emission of two potent greenhouse gases (CH_4 and CO_2) and decreasing the nutrient load at wastewater treatment plants; thereby, providing beneficial environmental, economic and societal value.

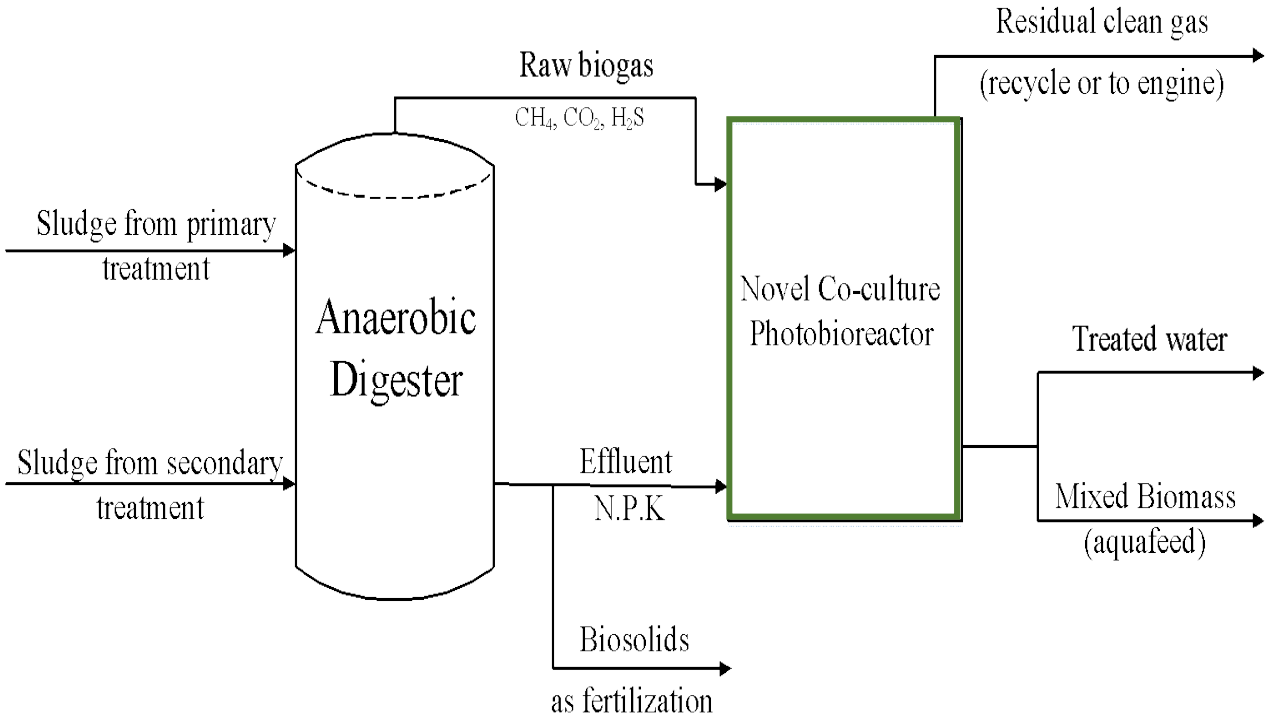


Figure 4: Proposed wastewater treatment process using the co-cultlure.

2.3 Co-culture strains

2.3.1 Chlorella sorokiniana as model photoautotroph

C. sorokiniana is a widely studied chlorella strain for mass cultivation and has been identified as an ideal candidate for biomass production with potential application in bioenergy (Li et al., 2014). Furthermore, there is extensive study on *C. sorokiniana* for bioremediation (Shriwastav et al., 2014; Higgins et al., 2018; Wang et al., 2018; Kimura et al., 2019; Wang et al., 2019; Liu et al., 2020), different trophic modes (Kim et al., 2013; Li et al., 2014) and biomass composition (Takeda, 1988; Banskota et al., 2013). *C. sorokiniana* is also grows rapidly and can tolerate extreme winter (Cuaresma Franco et al., 2012) and summer conditions (de-Bashan et al., 2008). For example, *C. sorokiniana* UTEX 130 was found to grow faster under heterotrophic conditions as compared to autotrophic conditions among 30 chlorella strains (Rosenberg et al., 2014). In addition, after screening of four different microalgae strains for treatment of sewage wastewater, *C. sorokiniana* demonstrated more tolerance to the high total nitrogen concentrations (1.21 g/L) (Vasconcelos Fernandes et al., 2015).

2.3.2 Methylococcus capsulatus as model methanotroph

M. capsulatus was isolated by Foster and Davis in the 1966 (Foster and Davis, 1966) and has emerged to be one of the most studied methanotrophic strains. This strain falls within a new group in type I methanotrophs, called type X. Like type I methanotrophs, *Methylococcus capsulatus* utilizes the RUMP pathway; however, type X was distinguished from type I methanotrophs because 1) they possess enzymes of the serine pathway, 2)they grew at higher temperatures than type I and II and 3) they contain DNA with higher moles percent of guanine and cytosine as compared to type I and type II methanotrophs (Hanson and Hanson, 1996). Extensive research has also been performed on *M. capsulatus* to understand nitrogen assimilation (Murrell and Dalton,

1983; Carlsen et al., 1991; Zhivotchenko et al., 1995), methane monooxygenase (MMO) (Nguyen et al., 1994; Nguyen et al., 1998; Basu et al., 2003; Yu et al., 2003), carbon utilization (Patel and Hoare, 1971; Eccleston and Kelly, 1972), and bioremediation (Rasouli et al., 2018) among other physiological studies. There also exist genetic tools (Williams and Bainbridge, 1971) for manipulation of the strain. Recently, the complete genome of *Methylococcus* has been sequenced (Ward et al., 2004) and genome-scale models have been published (Lieven et al., 2018b; a; Gupta et al., 2019) which allows simulation of metabolic behavior under different conditions. Additionally, *M. capsulatus* is one of the few strains being used commercially (Bothe et al., 2002; VTT Technical Research Centre, 2016). Since *M. capsulatus* is extensively studied along with the available tools and commercial application, this strain is attractive for further application on wastewater effluents.

2.4 Objectives of this study

The aim of this dissertation is to establish a new biological platform for upgrading biogas and treating the liquid digestate from AD systems. Experiments were performed in vials and a bench-scale chemostat to:

1) Develop a protocol that can be used for characterizing the co-culture where characterization refers to estimation of the individual biomass concentrations and the O_2 and CO_2 production and consumption rates.
2) Demonstrate the potential feasibility of the platform by cultivating the co-culture completely on unsterilized wastewater effluents without using freshwater for dilution and energy-intensive pretreatments (sterilization) methods.

3) Evaluate the advantages of using the co-culture technology for biogas and nutrient recovery as compared to the current technology (sequential microalgal and methanotroph single cultures)

4) Demonstrate co-utilization both CH_4 and CO_2 in biogas without external oxygen supply as well as the enhanced process safety of using the co-culture due *in situ* O_2 production and consumption.

5) Evaluate the performance of the co-culture simultaneous biogas and nutrient recovery at steady state in a bench-scale photobioreactor.

Chapter 3: Mathematical Modelling of the co-culture

Co-cultivation of methanotrophs and microalgae are a promising platform with the ability to co-utilize both CH_4 and CO_2 from gas feedstocks such as natural gas and biogas (van der Ha et al., 2012; Hill et al., 2017; Rasouli et al., 2018). The microbial biomass generated has intended application for single cell protein (Rasouli et al., 2018), biocrudes (Chen et al., 2014) and other products. Despite these potential applications, widespread use of the platform will require improving the performance of the co-culture. To do so, further knowledge of the individual strain performance and interactions in the co-culture is required. However, the current methods for estimating the individual strain in mixed cultures can be time-consuming, expense and are not suitable for frequent characterization of mixed cultures. In this chapter, a protocol to quantitatively characterize the methanotroph-microalga co-culture was developed. The protocol addresses the challenges of production and consumption rates of gas species shared by both microorganisms as well as providing estimates of the individual strain concentrations in the co-culture.

3.1 Materials and methods

Biomass was measured via a UV/Vis spectrophotometer (Beckman Coulter DU® 730) after proper dilution with deionized water to obtain the optical density (OD) at 750 nm. The biomass concentration was then calculated through an OD_{750} to biomass concentration calibration curve for each strain in the co-culture. The calibration curve was obtained by cultivating *C. sorokiniana* and *M. capsulatus* and harvesting the biomass in mid exponential phase. After washing three times in deionized water to remove any residual nutrients, the cells were serially diluted, aspirated unto pre-weighed aluminum dishes and then placed in a convection oven overnight at 90 ℃. The dishes with cells were weighed once daily until the mass was constant.

2.5 mL gas samples are withdrawn with a gas-tight syringe and measured using GC (Agilent 7890B customized with FID, TCD, Unibeads IS 60/80 mesh and MolSieve 5A 60/80 SST columns). Gas calibrations were performed by injecting gas mixtures of CH_4, CO_2 and O_2 of known compositions and calibration curves generated through regression analysis in Microsoft Excel. Samples were first centrifuged to remove cell biomass. The supernatant is then filtered through a 0.2 μm filter. The total inorganic carbon (TIC) of the liquid phase is measured via a Shimadzu TOC-VCSN analyzer.

To assess the protocol, a co-culture experiment was performed to measure the biomass produced as well as the changes in biogas concentration. Then using the protocol, the biomass generated, and the gas consumption and production rates was calculated and compared with the measured biomass and CH_4 and CO_2 headspace concentrations.

3.2 Experimental-Computational protocol

The protocol hinges on direct measurements of the different substrate and product concentrations in the gas phase and the liquid phase as well as various yield coefficients for estimating the biomass concentration, O_2 production and consumption rate and CO_2 production and consumption rate. The yield coefficients are either measured through in-house experiments or acquired from literature, but all the yield parameters are kept constant throughout the protocol under the experimental conditions. The equations used for the protocol are detailed in section 3.2.1 and each of the steps in equations (6) – equation (12) are performed between each sampling period. Biogas (CH_4, CO_2) fed to the vial as well as any produced O_2 that is released to the headspace can be directly measured while only dissolved CO_2 measured in the liquid broth. Due to the low solubility of methane, dissolved CH_4 is considered negligible in this protocol. It is important to note that within the co-culture, the methanotroph is the only strain capable of CH_4 oxidation. Thus,

CH_4 consumption can be directly associated with assimilation by the methanotroph strain. Unlike CH_4, both CO_2 and O_2 are produced and consumed. Due to the metabolic coupling of the co-culture, the CO_2 and O_2 production and consumption rates cannot be measured directly but can be tracked through mass balances and stoichiometric growth yields.

Photosynthetically produced O_2 is utilized by the methanotroph to oxidize CH_4 producing methanotrophic biomass and carbon dioxide. Using yield coefficients (shown in red below), the O_2 consumption rate, methanotroph biomass generated and CO_2 produced can be calculated. To properly account for the carbon dioxide mass balance, CO_2 initially dissolved the liquid, CO_2 produced by the methanotroph and CO_2 in the headspace can be assimilated by the microalga through photosynthesis producing O_2, and microalgal biomass. The produced oxygen will be utilized by the methanotroph; however, any excess oxygen that is not consumed by the methanotroph will be measured in the headspace as O_2 is also sparingly soluble. As only the net oxygen production can be measured, and light respiration is sufficiently low during photosynthesis, O_2 consumption by respiration is not considered (Kliphuis et al., 2011). The protocol used two methods to calculate the microalgal biomass generated: 1) CO_2 fixation to produce microalgal biomass; 2) O_2 produced as a result of biomass production. To achieve a well-defined set of equation, the microalgal biomass is calculated as the average of both routes. By doing so, there will be a unique set of equations with 7 unknowns and 7 equations and zero degrees of freedom. To calculate the increase in OD_{750} throughout the growth period, the calculated biomass change between two sampling periods is converted to OD_{750} by using biomass concentration (g/L) to OD_{750} calibration curves specific for each strain. Then, starting with the initial OD_{750} of the co-culture at inoculation, the change in OD_{750} during each period can be cumulatively summed to calculate the OD_{750} at each subsequent sampling point.

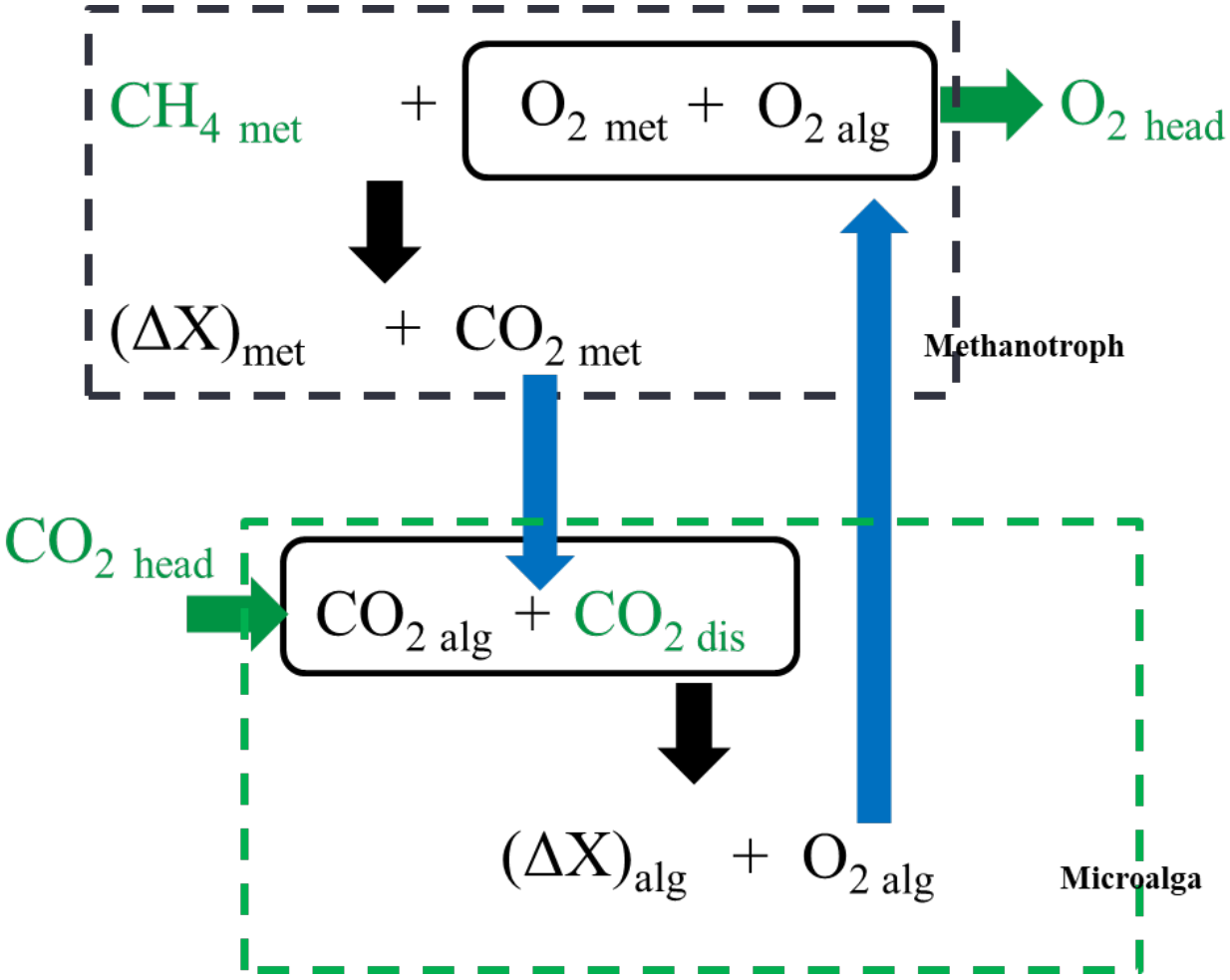


Figure 5: Schematic illustration of the experimental-computational protocol.

3.2.1 Experimental-computational equations

Table 2: Equations used for estimating the biomass concentration, and gas consumption and production rates.

$$(\Delta CH_4)_{met} = (\Delta \boldsymbol{CH_4})_{\boldsymbol{head}} \quad (6)$$

$$(\Delta O_2)_{met} = (\Delta CH_4)_{met} \cdot \left(Y_{\frac{O_2}{CH_4}}\right)_{met} \quad (7)$$

$$(\Delta CO_2)_{met} = -(\Delta CH_4)_{met} \cdot \left(Y_{\frac{CO_2}{CH_4}}\right)_{met} \quad (8)$$

$$(\Delta X)_{met} = (\Delta CH_4)_{met} \cdot \left(Y_{\frac{X}{CH_4}}\right)_{met} \quad (9)$$

$$(\Delta CO_2)_{head} = (\Delta CO_2)_{met} + (\Delta CO_2)_{alg} + (\Delta CO_2)_{dis} \quad (10)$$

$$(\Delta O_2)_{head} = (\Delta O_2)_{met} + (\Delta O_2)_{alg} \quad (11)$$

$$(\Delta X)_{alg} = \frac{1}{2}\left[(\Delta CO_2)_{alg} \cdot \left(Y_{\frac{X}{CO_2}}\right)_{alg} + (\Delta O_2)_{alg} \cdot \left(Y_{\frac{X}{O_2}}\right)_{alg}\right] \quad (12)$$

Measured **Estimated** **Calculated**

Notations:

met: methanotroph; alg: microalga, head: headspace and dis: dissolved

3.3 Results and discussion

To evaluate the accuracy of the experimental-computational protocol, the measured co-culture biomass produced was compared with co-culture biomass estimated by the protocol and

was plotted in Figure 6A. As shown in Figure 6A there is good agreement between the measure co-culture OD_{750} showed good agreement throughout the co-culture cultivation period. It should be noted that other experiments performed in our lab compared protocol to cell counting by flow cytometry (data not shown) and these results also demonstrate that the experimental-computational protocol can provide good accuracy for estimating the biomass concentrations.

Due to the good biomass estimation by the protocol, the next objective was to determine whether the protocol can provide good estimation of O_2 and CO_2 production and consumption rates. The biogas concentration profile throughout the growth of the co-culture is plotted in Figure 6B. The arrows in Figure 6B indicate the times which the bottles were gas feeding and declines in gas measurements indicate consumption of CH_4 by methanotroph and CO_2 dissolution then consumption by the microalga. The gas measurements indicate that there was no residual oxygen as the co-culture grew over 66 hours. In Figure 6C is plotted *M. capsulatus* O_2 consumption rate, the *C. sorokiniana* O_2 production rate and the overall change in O_2. The estimated overall change in oxygen is approximately zero, indicating all the oxygen produced by microalgal photosynthesis was consumed by the methanotroph partner for CH_4 oxidation. This agrees with the gas phase measurements as there was no residual O_2 (Figure 6C). In Figure 6D is plotted CO_2 production by *M. capsulatus*, CO_2 consumption by *C. sorokiniana* and the overall change in CO_2. The change in CO_2 indicated an overall consumption of CO_2 from the gas and liquid phase which is consistent with the decrease in CO_2 concentration between gas feeding periods (Figure 6D).

3.4 Conclusions

An experimental-computational protocol for studying the dynamic growth and interactions of the co-culture was presented in this chapter. Using mass balances and yield coefficients, the co-culture biomass concentration can be accurately estimated over the cultivation period. Although

O_2 and CO_2 are produced and consumed by both strains in the co-culture, the protocol can provide good estimation of the individual species gas production and consumption rates through the yield coefficients.

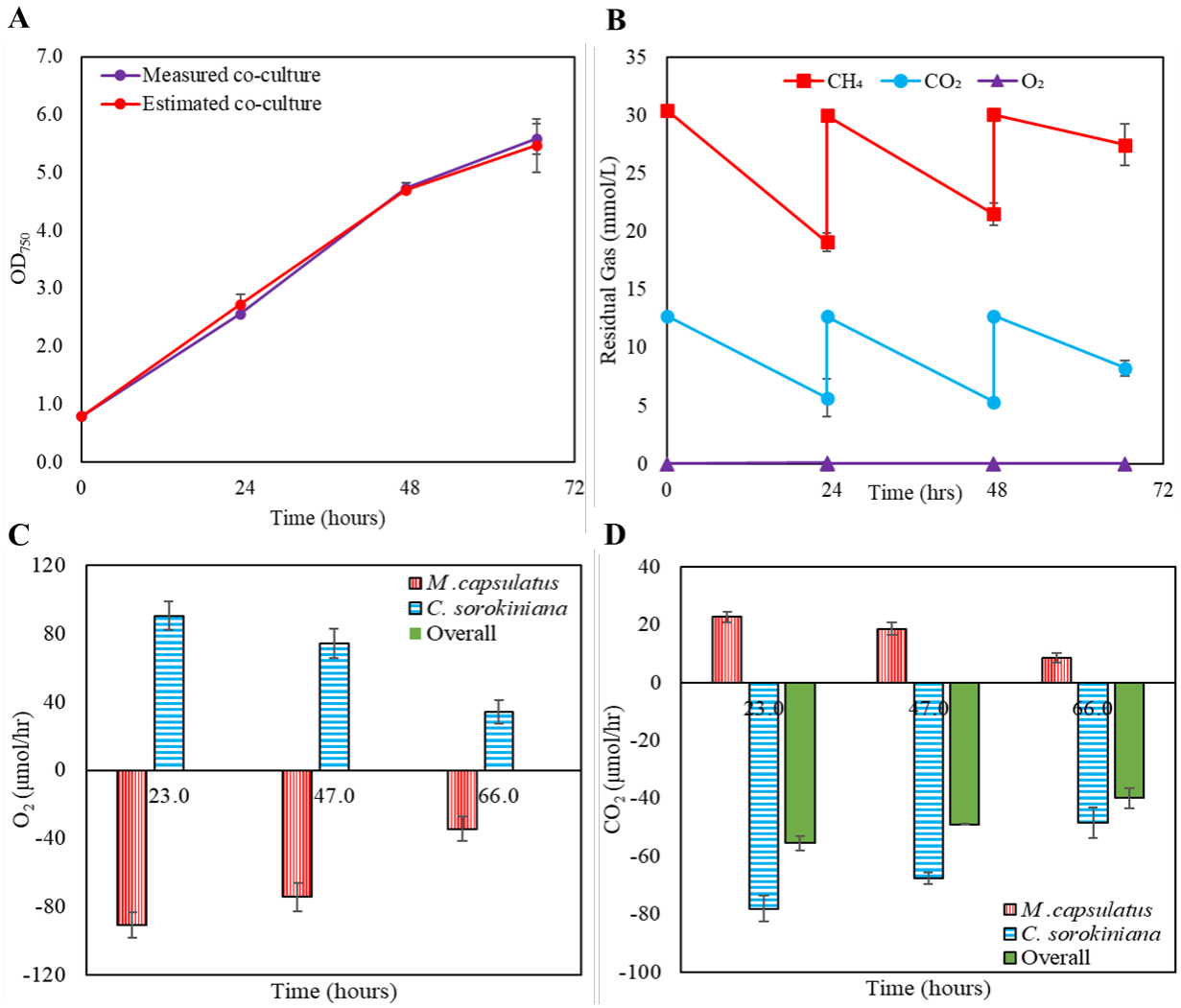


Figure 6: (A) Comparison of the measured co-culture and estimated co-culture biomass, (B) time-course residual gas concentrations in the co-culture, (C) estimation of O_2 consumption and production rates by *M. capsulatus* and *C. sorokiniana* respectively; (D) CO_2 production and consumption rates by *M. capsulatus* and *C. sorokiniana* respectively.

Chapter 4: Co-culture performance on minimally pretreated wastewater

4.1 Introduction

Large volumes of municipal, industrial and agricultural wastewater are generated due to increased water demand by a rapidly growing human population. Wastewater contains nutrients (N and P) that can have negative consequences on the environment, public health and local economy if excessive nutrients are released into waterbodies. One well established environmental consequence is eutrophication, which is characterized by excessive algal blooms and hypoxic water that degrade water quality and aquatic ecosystems when excessive N & P are present in aquatic bodies of water. Algal blooms also create serious health concerns due to the contamination of waste bodies by algal toxins. Therefore, effective and economical treatment technologies for treating wastewater has become a major concern and is an urgent need for reducing the impact of nutrients in wastewater.

At wastewater recovery facilities, anaerobic digestion is a mature technology that is commonly applied for treating various waste streams from municipal, industrial and agricultural industries. It has been shown that AD is advantageous in handling organic waste and mitigating pathogens and odors. After AD treatment, biogas (50-70% CH_4, 30-50% CO_2) is produced with trace amounts of impurities such as H_2S and siloxanes. Due to these impurities, biogas clean-up process is expensive, thus, limiting wide usage of biogas. In addition, the AD liquid effluent contains high concentrations of inorganic nitrogen and phosphorus which must be removed before discharge into bodies of water. Usually, the AD effluent is recycled to the headworks of the plant for nutrient recovery by biological nutrient removal (BNR) processes which employ conventional A^2O (anaerobic-aerobic-anoxic) tanks. Nitrification and denitrification processes remove nitrogen in the aerobic-anoxic tanks while phosphorus is removed by anaerobic-aerobic processes.

However, denitrification process is often limited due to competition for chemical oxygen demand (COD) with the phosphorus removal process. Further, when high concentrations of inorganic N and P contained in the AD effluent are sent to the nitrification and denitrification process, the C:N ratio is lowered below an optimal ratio of 8, causing disturbances to the nitrification-denitrification processes. As a result, the traditional BNR facilities are not always efficient in recovering nitrogen and phosphorus. In addition, operation of the aeration tanks is reported to account for at least 50% of the total energy costs at a WRRF (Siegrist et al., 2008; Drewnowski et al., 2019).

To combat these challenges, the methanotroph-microalgal wastewater treatment technology is proposed. Microalgal-based wastewater treatment has shown promise as tertiary wastewater treatment due to the more economical recovery of nitrogen, phosphorus, and trace metals and higher efficiency (Hoffmann, 1998; Christenson and Sims, 2011) as compared to conventional wastewater treatment (BNR processes) and chemical methods which have high implementation costs and their waste may contribute to secondary pollution (Osundeko et al., 2019). Thus, the methanotroph-microalga co-culture technology can be used to convert biogas into microbial biomass while simultaneous recovering the nutrients in the AD effluent. Further, due to the individual co-culture strain capability for storing energy compounds, microalgal biomass can be used as a source of carbohydrates for bioethanol production in biorefineries, lipids for biodiesel or, proteins and other nutritional compounds as single cell protein.

While the AD effluent is attractive as an alternative low-cost medium, the high loads of ammonia, organic compounds and high turbidity of AD effluent have been reported to significantly inhibit both microalgae and methanotroph growth and cause low nutrient recovery rates (Crofts, 1966; Azov and Goldman, 1982; Carlsen et al., 1991; King and Schnell, 1994; Dunfield and Knowles, 1995; Wieczorek et al., 2011). As a result, most microalgae applications require large

dilutions of AD with freshwater to reduce the nutrient and pollutant concentrations to tolerable levels (Wang et al., 2018). The use of freshwater as a diluent (Ding et al., 2015) consumes an already clean water sources and freshwater is limited in many regions. Besides utilization of freshwater as a diluent, some applications utilize energy-intensive and costly sterilization methods such as filtration, centrifugation and autoclaving (Ding et al., 2015) to alleviate pollutant stress on the cells and achieve satisfactory biomass growth when treating wastewater. For instance, Marjakangas et al. observed a 15% increase in final biomass when *C. vulgaris* was cultivated on autoclaved piggery wastewater as compared to non-sterilized piggery wastewater. However, large-scale filtration can increase pumping costs and the complex matrix (i.e. organic molecules and sediment) of the wastewater will cause membrane fouling and autoclaving wastewater at high temperature and pressure is not economically feasible due to the energy costs. Thus, practical application of microbial platforms for wastewater treatment investigated on wastewater effluents (i.e. AD effluent diluted with secondary clarifier effluent) to reduce freshwater use and require minimal pretreatment (e.g. gravitational settling) to significantly reducing operating costs.

In this study, a novel biological platform was proposed for wastewater treatment using *Chlorella sorokiniana* and *Methylococcus capsulatus* as the model co-culture pair. The commercial feasibility of the co-culture on raw wastewater is demonstrated by robust growth on different wastewater mixtures and on minimally pretreated AD effluent which could significantly reduce the costs associated with wastewater treatment while the co-culture biomass can potentially serve as feedstock for value-added products.

4.2 Materials and methods

Wastewater collection and pretreatment

Municipal wastewater was collected from South Columbus Water Resources Facility in Columbus, GA. This facility treats an average of 45 million gallons of wastewater that comes from homes, businesses and industries. Anaerobic digestate samples were collected in clean plastic containers from the mesophilic digester #2 through sampling ports. Secondary clarifier effluent (CLE) was also collected from the top of clarifier #2 (water before discharge into water bodies). These wastewater samples were then stored on ice for transportation to the lab where samples were frozen at -20℃.

Before each experiment, wastewater samples were thawed, and three different pretreatment methods were tested in this work – settled (S), filtered (F) and autoclaved (A). For settled samples, the thawed wastewater sample was set aside in refrigerator for 24 hours to allow the solid fraction to settle down, and the top liquid phase was decanted for experiments; for filtered samples, the settled wastewater sample was filtered through a 0.2 μm filter (nylon, VWR) to remove most bacteria and small floating particles; for autoclaved samples, the filtered wastewater sample was further autoclaved to completely remove any bacteria contained in the digestate.

Precultures of the methanotroph and microalga

Cultures of *M. capsulatus* and *C. sorokiniana* were grown in 250 mL serum bottles sealed with a septum and aluminum cap. Pre-cultures of both strains were maintained on sterile-filtered and autoclaved anaerobic digestate diluted with the secondary clarifier effluent to ensure sterile monocultures. For methanotrophic growth, methane was supplied to a final concentration of 70% (v/v) CH_4 and 30% (v/v) O_2 at 200 rpm and 37℃. *C. sorokiniana* was also grown on the same

wastewater media, and CO_2 was supplied to a final concentration of 30% (v/v) CO_2 and 70% (v/v) N_2 at 200 rpm, 37℃, and under continuous illumination at 200 μmol m^{-2} s^{-1}.

4.3 Co-culture experimental design

4.3.1.1 Co-culture growth on different AD mixtures

The AD effluent contains high concentrations of NH_3-N and other potential inhibitors that can reduce co-culture growth. To determine a suitable alternative for using freshwater for diluting the AD effluent, three different diluents were investigated to determine their effect on the co-culture growth. The three diluents utilized were: (1) tap/portable water (TW) and (2) secondary clarifier effluent (CLE) and (3) a modified ammonium mineral salts (AMS) which is the standard AMS medium (Whittenbury et al., 1970) without NH_3-N and PO_4^{3-}-P. The AD mixtures were denoted as AD-TW, AD-CLE and AD-AMS were prepared by diluting the AD ~6 times to a final NH_3-N concentration of 120 mg/L NH_3-N. Gravitational settled AD was used and none of the diluents (TW, CLE, AMS) were sterilized.

Co-cultures were grown in 250 mL serum bottles sealed with a septum and aluminum cap with the different diluted AD effluent mixtures as culture media. Cell were inoculated in a 3:1 ratio based on the optical density at 750 nm. The initial microalga OD was 0.6 and the methanotroph was 0.2. Synthetic biogas of composition 70% CH_4 and 30% CO_2 was sparged through the medium for 10 minutes. Bottles were placed on a rotary shaker at 200 rpm, 37℃, and under continuous illumination at 200 μmol m^{-2} s^{-1}. After inoculation, both liquid and gas samples were taken once per day to measure total OD, gas composition, and individual biomass composition.

4.3.1.2 Co-culture growth on differently pretreated AD effluent diluted by CLE

Co-culture experiments were performed on AD effluent pretreated according to the three methods in "Wastewater pretreatment and collection" to examine the effects of pretreatment on co-culture growth. AD effluent was diluted with CLE pretreated according the three methods described above and were diluted to a final NH_3-N concentration of 120 mg/L. Cells were inoculated in a 3:1 ratio based on the optical density at 750 nm. The initial microalga OD_{750} was 0.6 and the methanotroph was 0.2. Synthetic biogas of composition 70% CH_4 and 30% CO_2 was sparged through the medium for 10 minutes. Bottles were placed on a rotary shaker at 200 rpm, 37°C, and under continuous illumination at 200 μmol m^{-2} s^{-1}.

4.4 Analytical Measurements

Biomass measurement and determination of growth parameters

Biomass was measured via a UV/Vis spectrophotometer (Beckman Coulter DU® 730) after proper dilution with deionized water to obtain the optical density (OD) at 750 nm. The biomass concentration was then calculated through an OD_{750} to biomass concentration calibration curve for each strain in the co-culture. Biomass yield (ΔX) and biomass productivity (*Pr*) were calculated as indicated in equations below

$$\Delta X = X_f - X_i \tag{13}$$

where X_f and X_i are the final and initial biomass amount

$$\mathrm{Pr} = \frac{\Delta C}{\Delta t} = \frac{C_f - C_i}{t_f - t_i} \tag{14}$$

where C_f and C_i are the initial and final biomass concentrations and t_f and t_i represent the initial and final time, respectively.

Gas and inorganic carbon measurements

2.5 mL gas samples are withdrawn with a gas-tight syringe and measured using GC (Agilent 7890B customized with FID, TCD, Unibeads IS 60/80 mesh and MolSieve 5A 60/80 SST columns). Gas calibrations were performed by injecting gas mixtures of CH_4, CO_2 and O_2 of known compositions and calibration curves generated through regression analysis in Microsoft Excel. Samples were first centrifuged to remove cell biomass. The supernatant is then filtered through a 0.2 μm filter. The total inorganic carbon (TIC) of the liquid phase is measured via a Shimadzu TOC-VCSN analyzer.

Nutrient analyses

Total nitrogen, ammonia-nitrogen, total phosphorus and orthophosphate were all measured using Hach kits. Samples were centrifuged at 12,000 rpm for 4 mins then filtered (0.2 μm) and frozen for analysis within a week. Analysis of the freezing protocol shows percent error of 2-4% 14 days after freezing, indicating that only small amounts of ammonia may have been volatized. Percent nutrient recovery (R) was calculated by the following equation:

$$R = \frac{R_0 - R_i}{R_0} * 100\% \quad (15)$$

where R_0 and R_i are the initial and final nutrient concentrations of NH_3-N, PO_4^{3-}P, TN and TP

4.5 Data analysis and statistics

All experimental conditions were performed in duplicate. Analysis and standard deviation calculations were performed in Microsoft Excel. One-way ANOVA and Tukey HSD test were carried out in R using the 'multcomp' and 'agricolae' packages at a significance level of $\alpha = 0.05$.

4.6 Results and discussion

4.6.1 Co-culture performance on different AD mixtures

For practical application of the co-culture technology, the performance of the co-culture on AD effluent must be examined. Undiluted AD effluent containing high concentrations of toxins are reported to significantly reduce cell growth (Wen et al., 2017) and the presence of antibiotics may further inhibit growth of the methanotroph. For this reason, previous studies employed large dilutions (10-20 times) with freshwater to reduce the inhibitory effects on microalgal growth (Xia and Murphy, 2016; Wang et al., 2018) and enable sufficient nutrient removal rates (Wen et al., 2017) but freshwater is not available in many regions. Instead, the use of secondary clarifier effluent as a diluent is more sustainable and more attractive for industrial application. Thus, the co-culture growth performance on AD effluent diluted with CLE was compared to AD effluent diluted with tap water (TP) and AMS medium.

The co-culture performance was evaluated by the biomass production, biogas utilization and the nutrient recovery. The biomass profiles over the 72-hour cultivation period are plotted in Figure 7. The biomass concentration of the co-culture as well as the concentration of each individual strain of *M. capsulatus* and *C. sorokiniana* were calculated using the experimental-computational protocol in Chapter 3. The maximum co-culture biomass concentration of 3.51 g/L and productivity of 1.04 g/L/day obtained on AD-CLE was higher than those obtained on AD-P (2.93 g/L; 0.85 g/L/day) and AD-AMS (3.06 g/L; 0.89 g/L/day) (Figure 7). These results clearly demonstrate CLE is a better diluent as compared to the costly alternatives (TW, AMS) and suggests the minerals and native microorganisms present in the AD and CLE mixture are beneficial to the co-culture and enabled stable and robust growth of both strains in the co-culture which is in agreement with previous studies (Tandon and Jin, 2017; Toyama et al., 2018; Lee et al., 2019; Qu

et al., 2019).Various bacteria exist in wastewater which have been shown to work symbiotically with microalgal species (Chen et al., 2019).

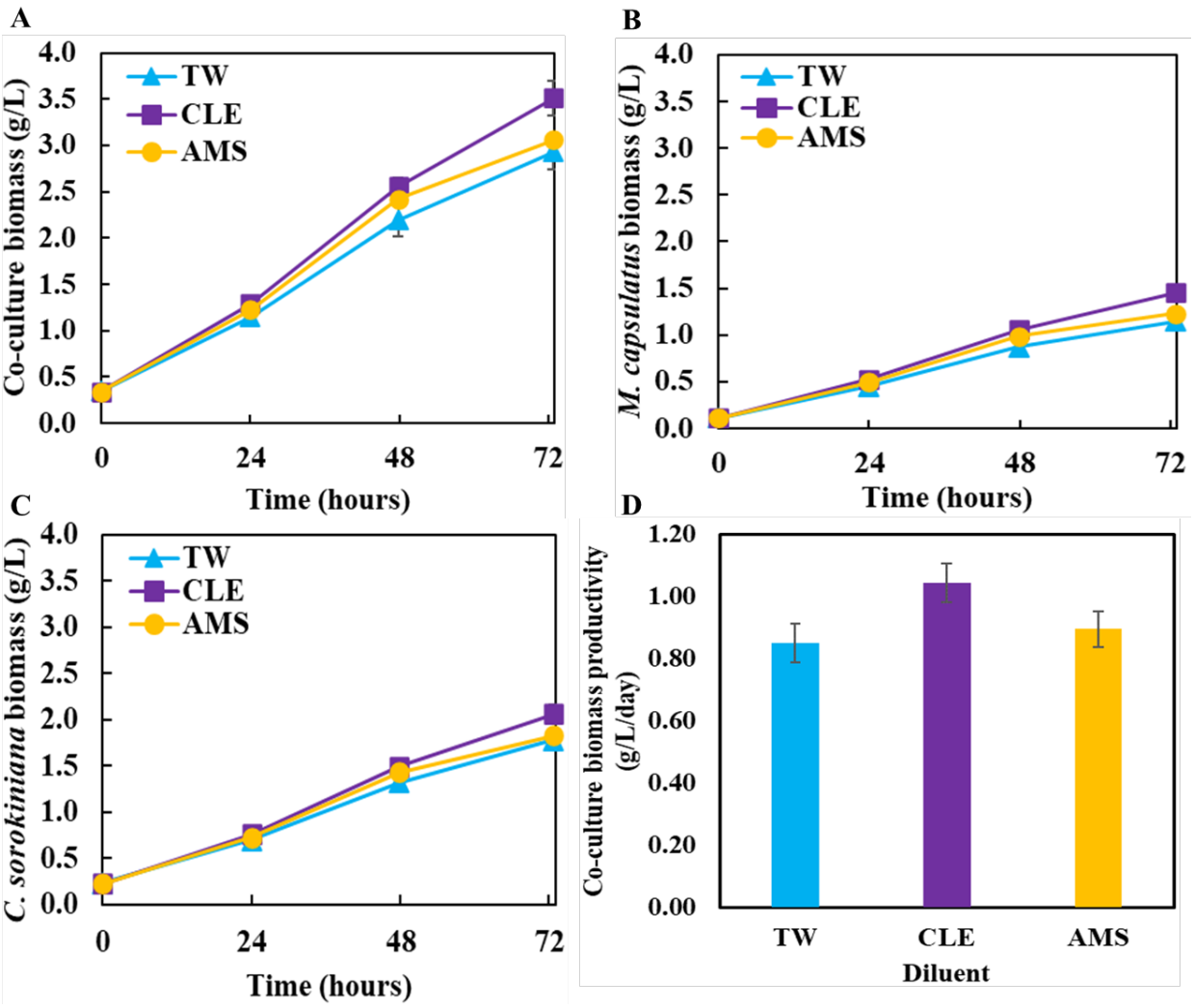
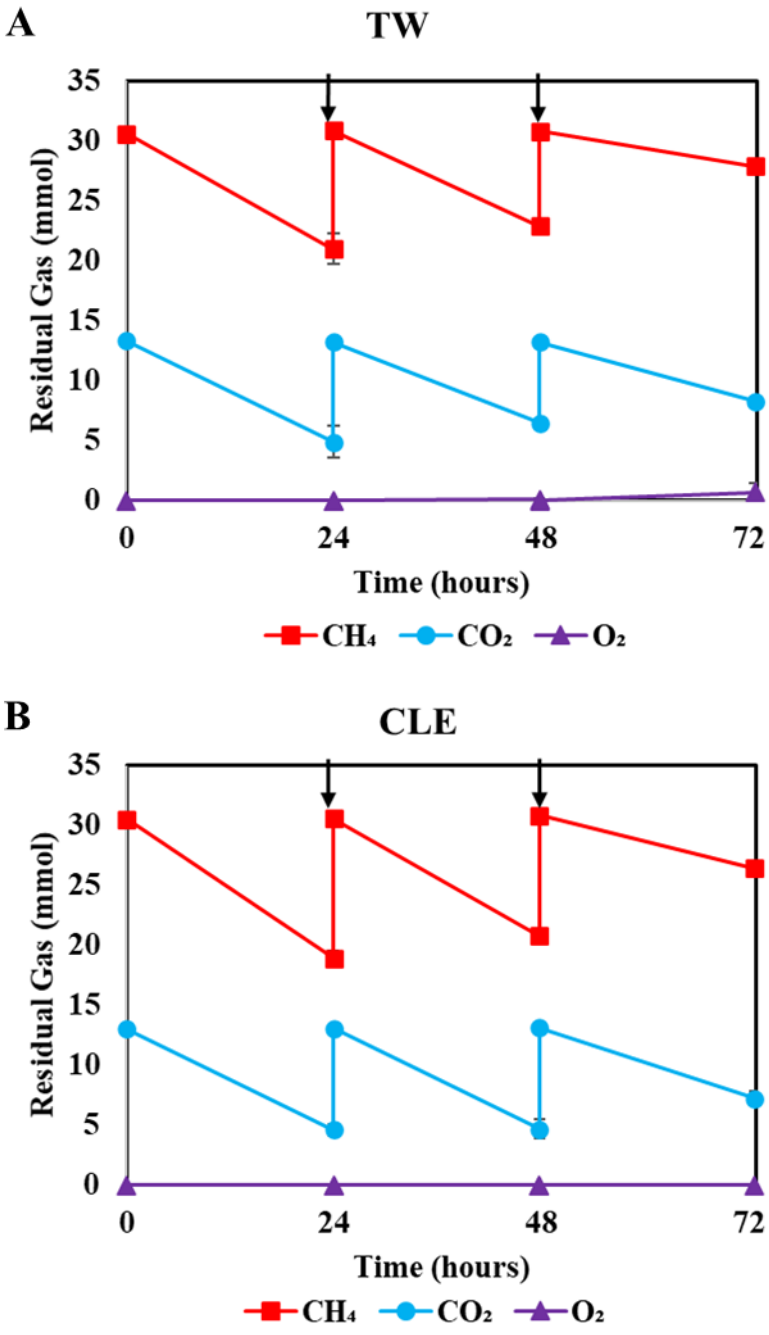


Figure 7: Growth profiles of co-culture cultivated on different AD mixtures. (A) Co-culture growth, (B) Individual strain growth of *M. capsulatus* and (C) *C. sorokiniana* in co-culture and (D) co-culture biomass productivity.

As shown in Figure 7B, *M. capsulatus* in the co-culture grew well on TW, CLE and AMS diluents. The highest methanotroph biomass concentration (1.45 g/L) in the co-culture was obtained on CLE and the methanotroph final biomass concentration was significantly different ($p<0.05$) from the

other AD mixtures (Figure 7B). *C. sorokiniana* had similar growth profiles on all AD mixtures in the co-cultures (Figure 7C) and *C. sorokiniana* growth was not significantly on among the different AD mixtures.

To further evaluate the co-culture performance and the recovery of carbon in biogas the gas phase composition in AD mixture was measured over time as plotted in Figure 8. The arrows indicate where the bottles were refed during the cultivation period. Figure 8 confirms that the coupling of methane oxidation with oxygenic photosynthesis enables continuous consumption of biogas without external oxygen supply. Both CH_4 and CO_2 were consumed consistently without O_2 accumulation, which eliminates the inhibition of excessive oxygen on microalgae growth and the risk of explosive gas. CH_4 assimilation in the co-culture grown on CLE (Figure 8B) was significantly different from CH_4 assimilation on TW (Figure 8A), and AMS (Figure 8C) at the end of the cultivation period as show in Table 2. As shown in Figure 8A, B and C, CO_2 biofixation by *C. sorokiniana* had a similar pattern among all diluents and the overall CO_2 assimilation at the end of the cultivation was not significantly different among the AD mixtures (Table 2).


A
TW
Residual Gas (mmol)
35
30
25
20
15
10
5
0
0
24
48
72
Time (hours)
CH4
CO2
O2
B
CLE
Residual Gas (mmol)
35
30
25
20
15
10
5
0
0
24
48
72
Time (hours)
CH4
CO2
O2

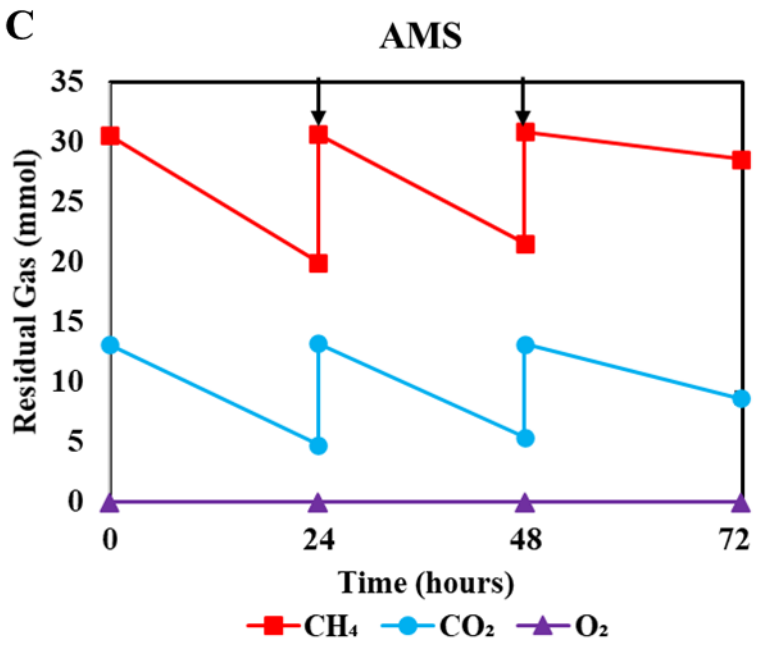


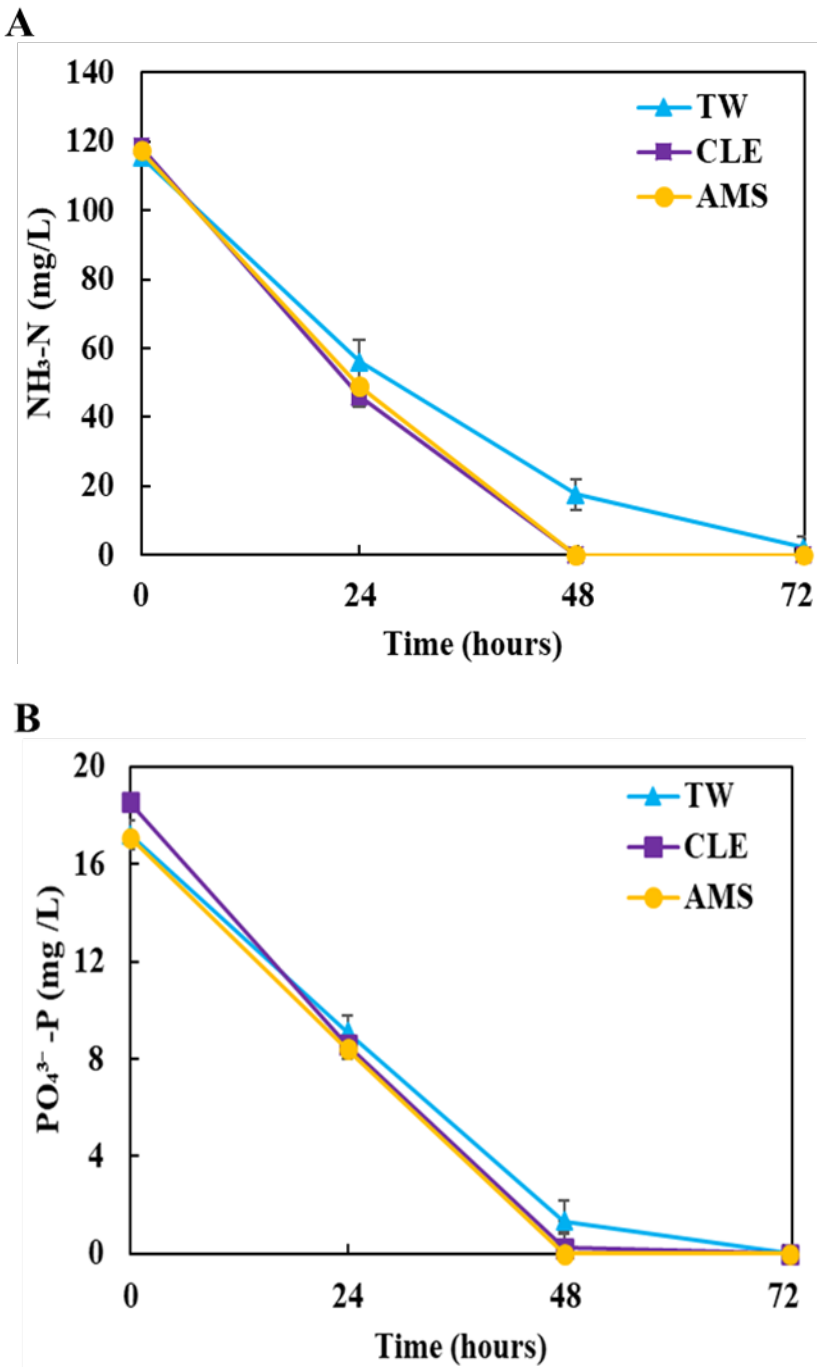
Figure 8: Time-course profiles of biogas composition in the headspace for co-cultures on different AD mixtures: AD effluent diluted with (A) tap water (AD-TW), (B) secondary clarifier effluent (AD-CLE), and (C) AMS medium (AD-AMS) (D) are plotted. Arrows indicate the points at which each bottle was refed with biogas.

Table 3: Overall gas substrate consumption by co-culture on different AD mixtures over 72 hour cultivation period.

Gas substrate	AD-TW	AD-CLE	AD-AMS
CH_4	2.76 ^{a} ±0.22	3.62 ^{b} ± 0.24	2.99 ^{c} ± 0.00
CO_2	4.83 ^{a} ± 0.23	5.53 ^{b} ± 0.29	5.02 ^{a} ± 0.07

Next, the nutrient recovery by the co-culture was assessed through inorganic nitrogen (NH_3-N) and phosphorus (PO_4^{3-}-P) measurements for all the different AD mixtures and the residual nutrient concentrations throughout the cultivation period are plotted in Figure 9. On CLE and AMS, NH_3-N and PO_4^{3-}-P was completed consumed within 48 hours which might account for the decrease in biogas consumption after 48 hours (Figure 8). The co-culture on TW had a slower nutrient recovery rate (Figure 9A and B) resulting in a small residual concentration (2.30 mg/L of

NH_3-N at the end of the cultivation period. This result further confirms the effectiveness of the co-culture in recovering the nutrients from wastewater, which shows near 100% recovery of ammonia nitrogen and orthophosphates. Nitrate and nitrite were not measure as they were negligible during initial tests of the wastewater.



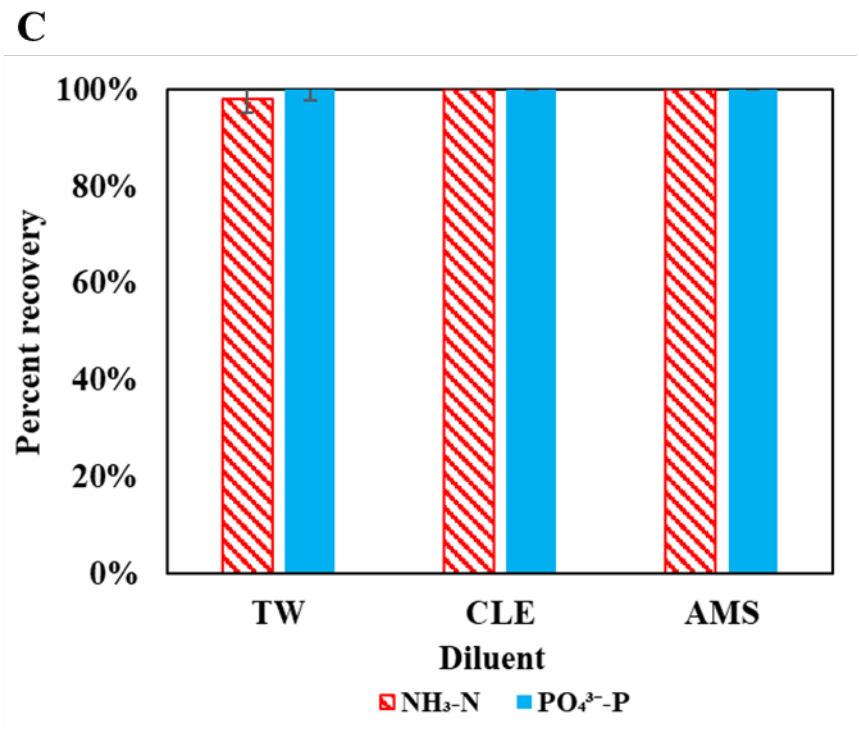


Figure 9: Residual inorganic (A) nitrogen, (B) phosphorus and (C) overall nitrogen and phosphorus recovery by the co-cultures on different AD mixtures over the cultivation period.

4.6.2 Co-culture performance on differently pretreated AD effluent diluted with CLE

For application of the co-culture technology for wastewater treatment, minimally pretreated or non-sterilized wastewater is necessary. However, most studies investigate microalgal-based technologies on sterilized (e.g. filtering or autoclaving) wastewater effluents. To further evaluate the potential of the co-culture platform to treat wastewater effluents, the growth of the co-culture on non-sterilized wastewater as compared to sterilized wastewater was examined. The pretreatments for this set of experiments were gravitationally settled (S), filtered (F) and filtered and autoclaved (A). For comparison purposes, the co-culture growth on sterilized, modified AMS medium was also compared.

In Figure 10 is plotted the biomass concentration profiles of the co-culture growth as well as the single culture within the co-culture. Interestingly, the highest co-culture biomass production was achieved on AD pretreated by gravitational settling, although there is no statistically significant difference among the different pretreatments (Figure 10A). Likewise, *C. sorokiniana* and *M. capsulatus* growth within the co-cultures demonstrated comparable biomass production among the different pretreatments; Figure 10B and C respectively. Similar results were also observed for the co-culture productivities (Figure 10D). This result clearly demonstrates the robustness of the co-culture, as its growth was not affected by other microorganisms present in the wastewater. In addition, there could be potential synergistic effects between the co-culture and microorganisms contained in the wastewater.

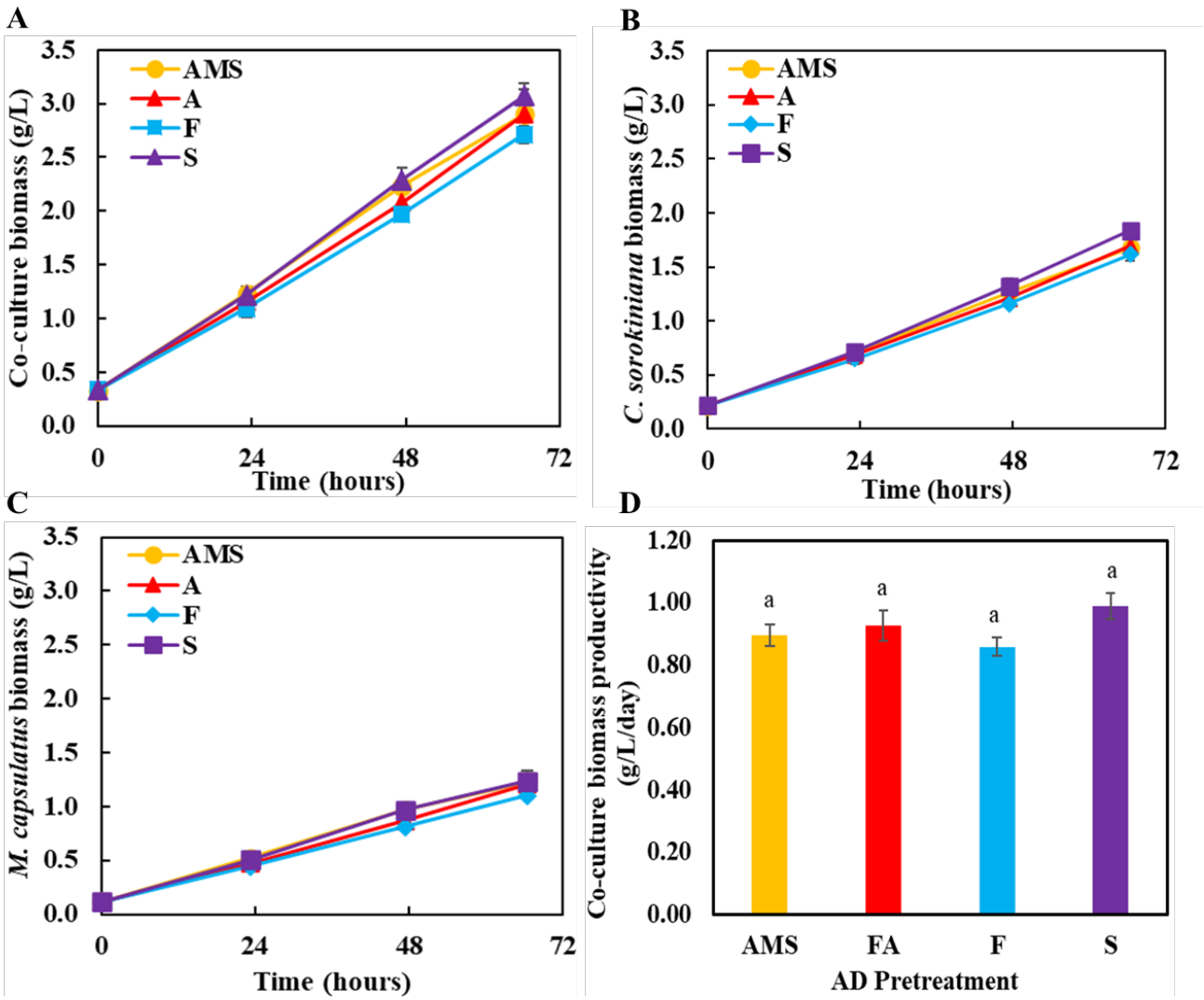


Figure 10: Time-course biomass concentration profiles of (A) co-culture, (B) microalga, and (C) methanotroph within the co-culture and (D) co-culture biomass productivity on AD pretreated by different methods.

Gas phase composition of co-culture on different pretreatments are plotted in Figure 11. The methane profiles among bottles share similar patterns which are consistent with the comparable methanotroph biomass production in Figure 10A. All the oxygen produced was completely assimilated by the methanotroph in all pretreatments; with a slight amount between the 48-66 hour period for the S pretreatment. The carbon dioxide gas phase profiles were also similar among

pretreatments and the control (Figure 11). Accordingly, comparable biogas co-utilization of CH_4 and CO_2 between S pretreatment and the sterilized forms (F, FA, AMS) demonstrate that the co-culture strains pretreated by S was not inhibited in spite of the presence of potential organic inhibitory compounds and the native microorganisms in the AD and CLE effluents.

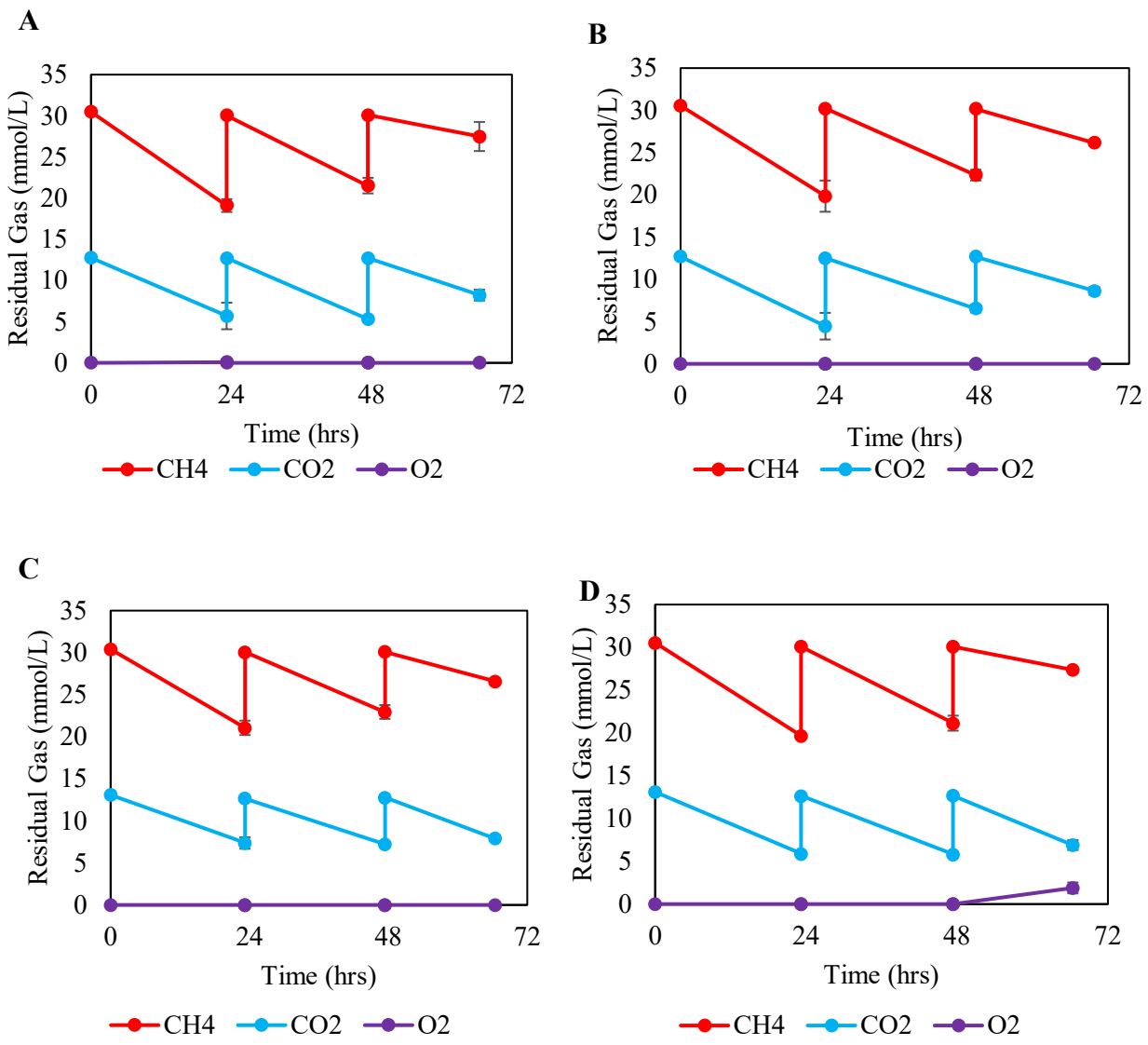


Figure 11: Residual biogas time-course profiles for co-cultures on (A) ammonium mineral salts (AMS) and AD pretreated by (B) microfiltration and autoclaving (FA), (C) microfiltration (F), and (D) gravitational settling (S).

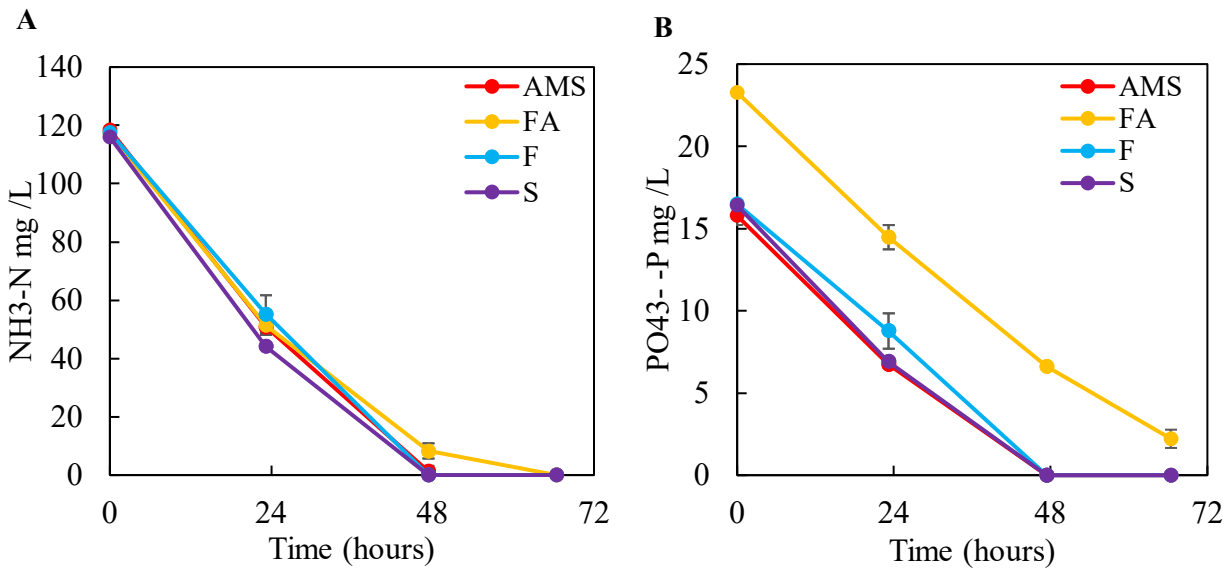


Figure 12: Nutrient profiles (A) NH_3-N and (B) PO43- -P of co-cultures on different AD pretreatments.

The residual nutrient content in each of the pretreatment conditions were measured during each sampling period to assess whether there were any differences in nutrient recovery. The time-course profiles are plotted in Figure 12. Still, the NH_3-N recovery profiles are similar as shown with the biomass production and gas substrate consumption. The NH_3-N on the gravitationally settled AD effluent is rapidly and completely assimilated within 48 hours when cultivated on an initial NH_3-N content of 120 mg/L (Figure 12A). The same is observed for the co-culture on AMS and F while the FA pretreatment completely recovers NH_3-N within the 72 hour cultivation period (Figure 12A). In Figure 12B, PO_4^{3-}-P is completely recovered during this period in the AMS, and F and S pretreatments within 48 hours while 6.61 mg/L PO_4^{3-}-P was present at this same time for the FA pretreatment. The FA pretreatment resulted in different initial NH_3-N concentration after autoclaving due to NH_3-N volatilization and consequently, the initial PO_4^{3-}-P concentration was increased by ~6.88 mg/L PO_4^{3-}-P as compared to the other bottles. This suggests that the phosphorus could have been completely recovered within 48 hours if the FA pretreatment has

started with similar PO_4^{3-}-P concentration as the other bottles. Nonetheless, the FA pretreatment continues to assimilate PO_4^{3-}-P with 2.21 mg/L PO_4^{3-}-P left at the end of the cultivation period.

4.6.3 Co-culture microalgal biomass to biocrude

Microalgal biomass have gained ground as feedstock for high-value biofuels due to their energy-rich biomolecules (carbohydrate, protein and lipids). However, nutrient cost coupled with the use of limited freshwater sources pose significant challenges for commercial application. The AD effluent and biogas produced at WRRFs is a convenient and sustainable source of essential nutrients (nitrogen, phosphorus and trace metals) and carbon substrate (CO_2) required for high growth rates and biomass production. The significant advantages offered through this approach are 1) reduction in environmental pollution by capturing the nutrients and 2) low cost biomass production which can be used for production bioenergy such as biofuels. However, the energy and cost-intensive downstream processing of microalgal biomass for biodiesel production is a major drawback for the economic feasibility of converting microalgae to biofuels. Producing biodiesel from lipids by the conventional transesterification process is energy intensive as the biomass has to be dried and the residual biomass containing proteins and carbohydrates are not utilized(Goswami et al., 2019). The traditional transesterification process also utilizes hazardous organic solvents (Cao et al., 2013) which can increase operating costs. Furthermore, acquiring high lipid content of microalgae is usually stimulated to nutrient depletion which in turn would affect microalgal growth rates and biomass productivity. In order to address these shortcomings, researchers are focusing on converting microalgal biomass to biofuels through hydrothermal liquefaction (HTL). HTL greatly reduces the energy input as wet biomass can be directly converted to biocrude and all components of the biomass can be converted to biocrude. HTL is more ecofriendly as it does not involve harmful solvents for oil extraction. Thus, integration of the

methanotroph-microalga co-culture for bioconversion of wastewater treatment to biocrude can yield significant economic and environmental benefits while addressing the shortfalls of the microbial biomass to biodiesel process. Firstly, the energy and carbon in biogas can be recovered through the conversion of biogas to microbial biomass resulting in valorization of an underutilized energy resource. Secondly, cultivation of the co-culture on AD and clarifier effluents reduces the production cost for microbial biomass production, in addition to mitigating potential environmental pollution caused by inefficient process of traditional wastewater treatment. Thirdly, the co-culture technology will be transforming an "energy-consuming" wastewater processes into a potentially "energy-positive" system by valorization of wastewater resources to biocrude. A more extensive discussion of other potential products for the co-culture biomass is found in Section 1.7.

4.7 Conclusions and future perspectives

The novel biological platform for wastewater treatment presented here has demonstrated simultaneously conversion of low value biogas into microbial biomass while removing nutrient from AD digestate. The biological platform is based on a methanotroph-microalga co-culture, where methane oxidation is coupled to oxygenic photosynthesis. Through an on-going collaboration with Columbus Water Works (a municipal WRRF in Georgia), it was demonstrated that the model co-culture delivered robust and stable growth on minimally treated AD digestate. The digestate was simply settled to remove solids then diluted with secondary clarifier effluent, and no sterilization was required. In addition, the co-culture demonstrated complete removal of inorganic nitrogen and phosphorus. While the co-culture demonstrated good growth on the unsterilized wastewater, further considerations need to be examined for commercial application. AD effluent can contain various pathogens such as viruses that could proliferate when the co-

culture is cultivated on unsterilized municipal wastewater. Due to this reason, it would be challenging to utilize municipal wastewater for food and feed. However, the co-culture may be used for feed (e.g. single cell protein) when grown on proper wastewater effluents (e.g. paper mill, winery) that do not pose a significant risk of transferring infectious.

Chapter 5: Comparisons between sequential single cultures and the co-culture for wastewater treatment

5.1 Introduction

Currently, the amount of excess fixed nitrogen in the world has caused increasing negative consequences to our ecosystems and the public health, including: worsening of the greenhouse effect, reduction of the protective ozone layer, adding to smog, contributing to acid rain, and contaminating drinking water (Driscoll et al., 2003; Galloway et al., 2004). Activated sludge is the most common biological treatment method to reduce carbon and nutrient content in wastewater, and anaerobic digestion (AD) is widely employed to reduce the amount of solid organic waste and the sludge produced from wastewater treatment. During the AD process, organic matter is converted to biogas and organic nitrogen is converted to ammonia. Anaerobic digester is a commercially proven technology, and arguably the most efficient solution for handling organic waste streams. AD offers many significant advantages: 1) AD provides containment of the GHGs (CH_4 and CO_2) as biogas, which not only reduces GHGs emission, but also provides a valuable fuel; 2) Macronutrients (e.g., N, P, K, etc.) are transformed but not lost to the environment. If these nutrients are recovered properly, AD can significantly reduce eutrophication and land pollution; 3) AD provides effective pathogen and odor mitigation(Topper et al., 2006; Nasir et al., 2012). It has been shown that AD can achieve 95% pathogen reduction (Angelidaki and Ellegaard, 2003), and thus, significantly reduces the public health risk. Because of these advantages, AD has been widely adopted for large-scale municipal wastewater treatment: 48% of total municipal wastewater flow in US is currently treated by AD (Qi et al., 2013), which corresponds to 1238 out of 14780 water resource recovery facilities (WRRFs) in the US.

In WRRFs with AD installed, the nutrient rich digestate is returned to a biological nutrient removal (BNR) unit for further treatment, and the energy rich biogas (CH_4) is cleaned up for

various applications. The most commonly used BNR for nitrogen removal is the so-called nitrification-denitrification process, where ammonia is oxidized to nitrate during nitrification and then nitrate is reduced to nitrogen gas during denitrification by activated sludge. Although widely applied, this technology does have limitations: the nitrification process requires large energy input to aerate the water, while the denitrification process requires supplement of an organic carbon source (such as methanol) to support nitrate reduction by activated sludge. In addition, although CH_4 in AD produced biogas is a valuable fuel, the value of biogas is low due to impurities (e.g., CO_2, H_2S, etc.) that are difficult to remove economically. Typically, several clean-up steps are required before the AD produced biogas can be used for electricity generation. As a result, among the 1238 WRRFs with AD installed, 15% (~184) of them flare the produced biogas, 64% (~791) burn the biogas for digester and building heating, while only 21% (~263) use biogas for power generation or driving machinery (Qi et al., 2013).

Due to the cost associated with digestate treatment and biogas utilization, the installation of AD has been limited to large scale WRRFs. If the low value biogas and nutrient-rich digestate could be converted to biofuels or other value-added products, AD would be significantly valorized to justify the installation of AD at mid to small scale WRRFs. The broad adoption of AD will not only bring significant environmental and societal benefits, but also generate enormous energy and economic potential.

In this work, a novel biological platform was proposed to achieve simultaneous biogas conversion and nutrient recovery using a methanotroph-microalga co-culture. Through the metabolic coupling of methane oxidation and oxygenic photosynthesis, zero GHG emissions can be achieved without external supply of oxygen, with complete removal of inorganic nitrogen and phosphorus.

5.2 Microalgae-based wastewater treatment and biogas upgrading

Due to the capability of photosynthesis and nutrient recovery, microalgae have been used in municipal wastewater treatment for over 50 years (Van Den Hende et al., 2014), and more recently for bioremediation of manure effluents (Abou-Shanab et al., 2013). Nutrient cost is a major limiting factor for algal biomass production; therefore, using alternative sources such as wastewater to support microalgal growth is highly attractive. It has been shown that supplementing CO_2 in the municipal wastewater can increase the algal biomass productivity by almost 3-fold (Abdel-Raouf et al., 2012). In addition, microalgae have been studied to upgrade biogas produced from AD of swine wastewater, and multiple studies have shown that microalgae or microalgal-bacterial cultures can remove >99% of H_2S in biogas (Muñoz et al., 2015). Therefore, using microalgae to remove CO_2 and H_2S is a promising method for biogas upgrading.

However, microalgae-based wastewater treatment and biogas upgrading has its limitations: (1) when O_2 is produced from photosynthesis and mixed with CH_4, the treated biogas becomes explosive and poses a serious safety risk; (2) the presence of excessive O_2 can inhibits the growth of microalgae and reduces its productivity (Ugwu et al., 2007); (3) due to the high cost of downstream processing that upgrades microalgae into biodiesel, most of the algae produced from wastewater treatment is fed back to AD to enhance biogas production, instead of producing higher-value products such as biodiesel.

5.3 Microalgae-methanotroph co-culture platform for simultaneous biogas conversion and nutrient recovery

Recent studies have demonstrated that natural microbial communities have developed a highly efficient way to recover the energy and capture carbon from both CH_4 and CO_2 through interspecies coupling of methane oxidation to oxygenic photosynthesis (Kip et al., 2010;

Raghoebarsing et al., 2005;). This coupling represents a major sink for both CH_4 and CO_2 in nature, where the methanotrophic activity is fueled by *in situ* photosynthetic production of O_2. From a systems engineering perspective, as shown in Fig. 1, such coupling offers several advantages for biogas conversion: (1) exchange of *in situ* produced O_2 and CO_2 dramatically reduces mass transfer resistance of the two gas substrates; (2) *in situ* O_2 consumption removes inhibition on microalgae and risk of explosion; (3) potential metabolic links could significantly enhance the growth of both strains in the co-culture; (4) compartmentalized configuration of the co-culture offers flexibility and more options for metabolic engineering.

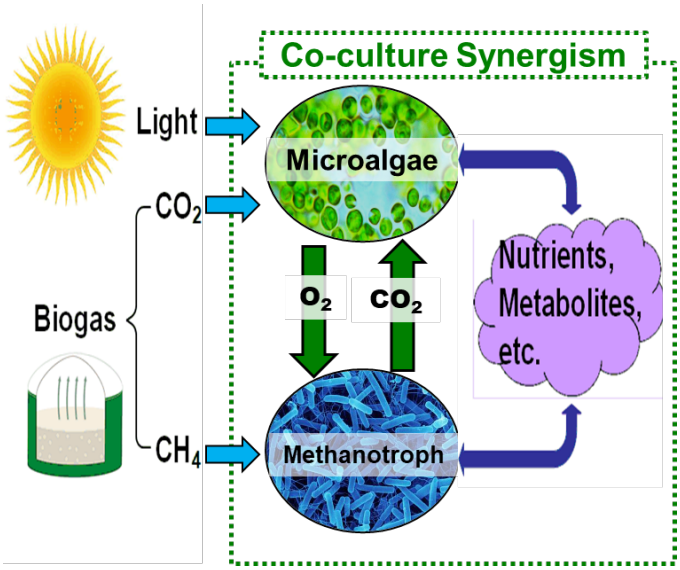


Figure 13: Potential interactions within microalgae-methanotroph co-culture.

Using the principles that drive the natural consortia, several synthetic methanotroph-microalga co-cultures for biogas conversion that exhibit stable growth under various substrate delivery and illumination regimes have been established (Roberts et al., 2018, 2017). However, in this work, the co-culture platform has been extended to simultaneous biogas conversion and nutrient recovery using *Methylococcus capsulatus* (Bath) and *Chlorella sorokiniana* as the model

co-culture. Specifically, instead of using synthetic media, nutrient-rich digestate is used as the culture medium to provide necessary nutrients for co-culture growth. Because the cost of nutrient is a major limiting factor for microbial biomass production, the proposed solution not only significantly reduces the cost associated with microbial biomass production, but also removes inorganic nitrogen and phosphorus from digestate; and thus, significantly reduces the cost associated with wastewater (digestate) treatment.

5.4 Materials and methods

5.4.1 Wastewater collection and growth media

Wastewater was collected from South Columbus Water Resources Facility in Columbus, GA. This facility treats wastewater that comes from homes, businesses and industries. Anaerobic digestate samples were collected in clean plastic containers from the mesophilic digester #2 through sampling ports. Clarifier effluent was also collected from the top of clarifier #2 (water before discharge into water bodies). These wastewater samples were then stored on ice for transportation to the lab where samples were frozen at -20°C. Before experiments wastewater samples are thawed and the solid fraction is allowed to settle over 24 hours. Sterile-filtered wastewater is prepared by filtering through a 0.2 μm filter (VWR, nylon). Cultures were grown on sterile-filtered AD effluent for which clarifier effluent was used to dilute the AD effluent to the desired nutrient level. Although the results in Chapter 4: demonstrate better growth performance on non-sterilized wastewater, filter-sterile wastewater was used for the following experiments to ensure the experimental observations were mainly due to the co-culture strains and were not influenced by native microorganisms in the unsterilized wastewater.

5.4.2 Methanotroph and microalga growth conditions

Cultures of *M. capsulatus* and C. *sorokiniana* were grown in 250 mL serum bottles sealed with a septum and aluminum cap. Pre-cultures of both strains were maintained on sterile-filtered and autoclaved anaerobic digestate diluted with the clarifier effluent to ensure sterile monocultures. For methanotrophic growth, methane was supplied to a final concentration of 70% (v/v) CH_4 and 30% (v/v) O_2 at 200 rpm and 37℃. C. sorokiniana was also grown on the same wastewater media, and CO2 was supplied to a final concentration of 30% (v/v) CO_2 and 70% (v/v) N_2 at 200 rpm, 37℃, and under continuous illumination at 180 μmol/m^2/s.

5.4.3 Analytical Measurements

5.4.3.1 Biomass, biogas and dissolved inorganic carbon measurements

Biomass was measured via a UV/Vis spectrophotometer (Beckman Coulter DU® 730) to obtain the optical density (OD) at 750 nm. The biomass concentration was then obtained through OD_{750} to biomass calibration curves. Biomass yield was calculated as the difference in biomass between the initial and final biomass. 2.5 mL gas samples are withdrawn with a gas-tight syringe and measured using GC (Agilent 7890B customized with FID, TCD, Unibeads IS 60/80 mesh and MolSieve 5A 60/80 SST columns). Gas calibrations were performed by injecting gas mixtures of CH_4, CO_2 and O_2 of known compositions and calibration curves generated through regression analysis. Samples were first centrifuged to remove cell biomass. The supernatant is then filtered through a 0.2 μm filter. The total inorganic carbon (TIC) of the liquid phase is measured via a Shimadzu TOC-VCSN analyzer.

Nutrient analyses

Total nitrogen, ammonia-nitrogen, total phosphorus and orthophosphate were all measured using Hach kits. Samples were centrifuged at 12,000 rpm for 4 mins then filtered (0.2 μm) and

frozen for analysis within a week. Analysis of the freezing protocol shows percent error of 2-4% 14 days after freezing, indicating that only small amounts of ammonia may have been volatized.

5.5 Case study rationale

Currently, microalgae-based technology is extensively studied for upgrading biogas. Through microalgal biofixation of CO_2 in biogas, the CH_4 content can be increased to greater than 90% thereby improving the fuel properties of biogas (i.e. energy content). The upgraded biogas can be injected into pipeline but stringent regulations on CH_4 and O_2 content pose challenges for this application. In addition, using the biogas is not economical for small-scale wastewater facilities so the upgraded biogas may be used mainly for heating and driving machinery. Thus, the co-culture technology is proposed as an alternative technology for upgrading biogas to microbial co-culture biomass. To assess this potential, biogas and nutrient recovery by the co-culture was compared to the sequential singles cultures (i.e. the microalga then the methanotroph) through different case studies.

5.6 Experimental design

For cases 1 and 2, each 250 mL serum bottle started with 100 mL of the filtered AD effluent diluted 5 times with CLE. The feed gas composition of the co-culture was 70% CH_4, 30% CO_2 while the single cultures feed gas compositions were 70% N_2, 30% CO_2 for *C. sorokiniana* and 70% CH_4, 30% N_2 for *M. capsulatus*. Every 24 hours, the total amount of O_2 produced by the single cultures of *C. sorokiniana* was determined and injected into each vial of *M. capsulatus* single culture. As a result, the inoculation of *M. capsulatus* vials occurred 24 hours after the *C. sorokiniana* vials. The initial inoculum concentrations for each strain in the co-culture were the same as that for each single culture; OD_{750} 0.2 for *M. capsulatus* and OD_{750} 0.6

for *C. sorokiniana*. 48 hours after inoculation, 20 mL of undiluted, filtered AD effluent was added to the bottle to prevent nutrient limitation. After inoculation, both liquid and gas samples were taken once per day to measure total OD_{750}, gas composition and individual biomass concentration. The experimental design for Case 3 was similar to cases 1 and 2 except all cultures were sparged with biogas daily. Case study 4 had a similar experimental design to case 3 except for two modifications: 1) the nutrient between the single cultures were split such that the summation of the nutrients in the single cultures would equal that of the co-culture. This split was determined based on the amount of nutrients consumed in case 3; 2) the light intensity for the experiment was increased to 550 μmol/m^2/s

5.7 Experimental results and discussion

5.7.1 Case study 1: Sequential single cultures and co-culture comparison under nutrient replete but carbon limited condition

The following experiments were performed to assess the potential of the co-culture for complete carbon recovery from biogas when unlimited nutrients were available. In addition, the co-culture performance was compared with sequential single culture, i.e., *C. sorokiniana* followed by *M. capsulatus*.

The biomass profiles of the single cultures within the co-culture and the sequential single culture are plotted in Figure 14. *C. sorokiniana* in the co-culture and the sequential single culture grew similarly until 72 hours; however, the microalgal growth in the co-culture continued to stationary phase at ~120 hours (Figure 14A). *M. capsulatus* demonstrated significantly higher growth in the co-culture grew well in the co-culture achieving final biomass that was more than 3.49 times that of the single culture (Figure 14B). Table 4 reports a comparison of the biomass produced in the single culture and the co-culture. Clearly, there is a large percent increase of

methanotrophic biomass within the co-culture as compared to the single culture which suggests the methanotroph might benefit more in the co-culture.

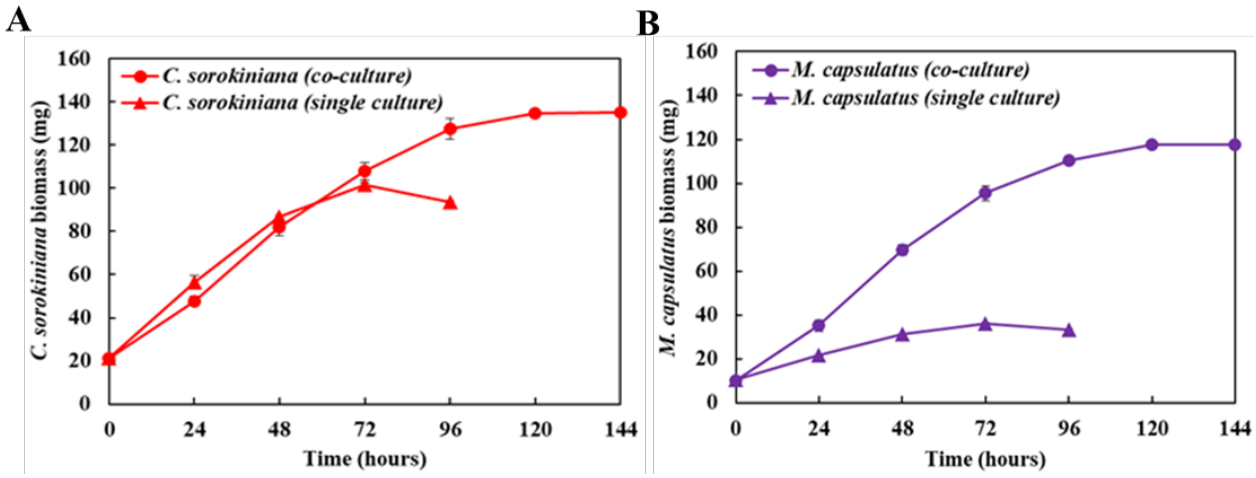


Figure 14: Biomass profiles of (A) *C. sorokiniana* and (B) *M. capsulatus* in co-culture compared with the sequential single cultures.

Table 4: Biomass production comparison between the single cultures and individual strain within the co-culture.

Biomass produced	***C. sorokiniana***	***M. capsulatus***
Single culture (mg)	72.4	22.8
In co-culture (mg)	113.8	107.3
% increase of individual strain biomass within the co-culture	57%	371%

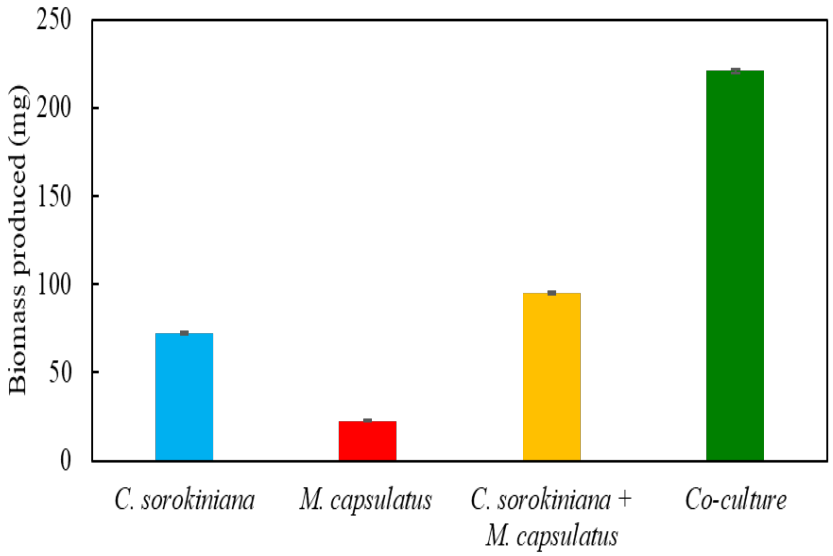
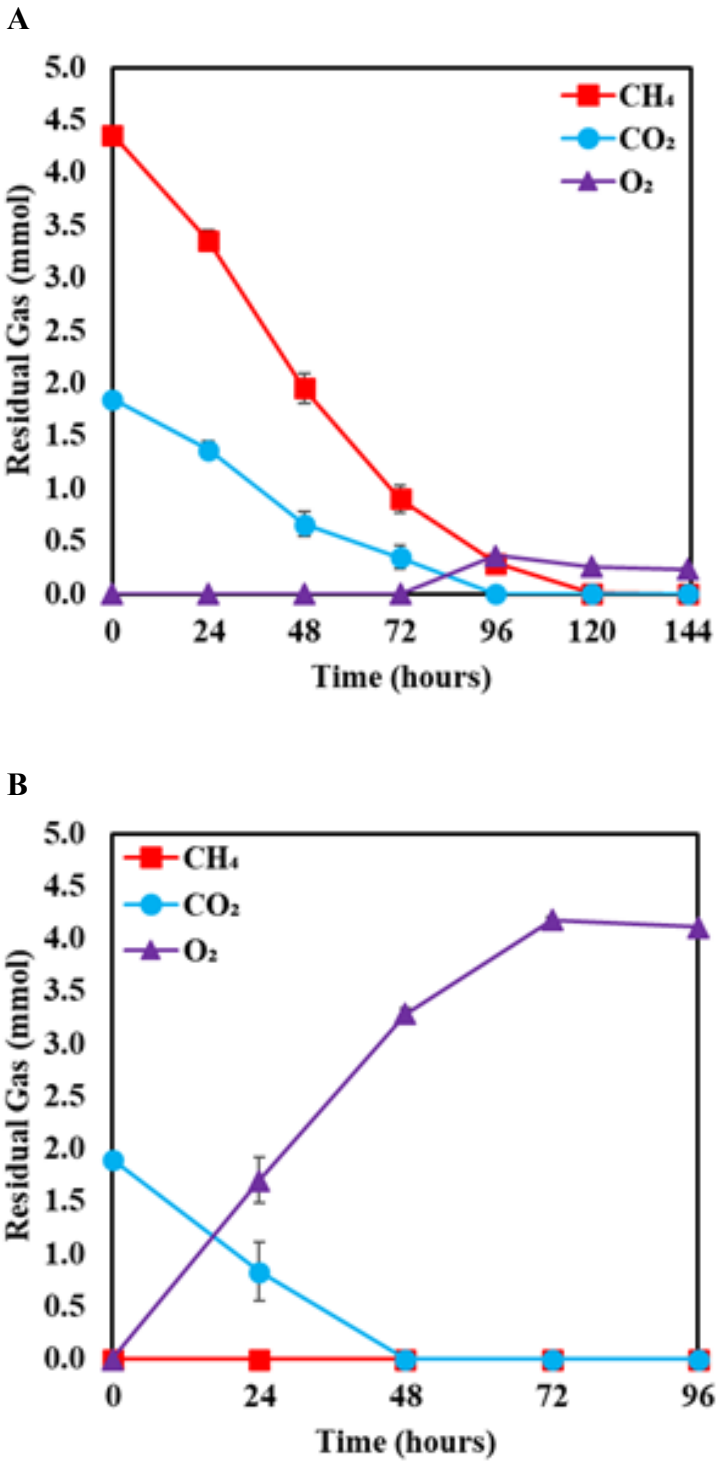


Figure 15: Comparison of biomass production between sequential single cultures (*C. sorokiniana* – first column, *M. capsulatus* – second column, total biomass or summation of the first two columns – third column) and the co-culture (last column).

To help explain what contributes to the higher biomass production in the co-culture, as well as to examine whether the biogas fed to the bottles was fully consumed, the gas phase profiles of the single and co-culture was measured over time, and plotted in Figure 16. In the co-culture, methane is continually oxidized by *M. capsulatus* until methane is completely consumed within 120 hours (Figure 16A). As compared to the *C. sorokiniana* single culture, the rate of photosynthetic CO_2 assimilation from the headspace of the co-culture was reduced as *M. capsulatus* provided additional CO_2 after CH_4 oxidation. CO_2 generated by *M. capsulatus* can then be further utilized for photosynthesis to produce more O_2. As a result, this carbon recycling and *in situ* exchange of O_2 and CO_2 allowed continuous CH_4 oxidation for complete recovery of CH_4. The *C. sorokiniana* single culture headspace profile (Figure 16B) shows that growth stopped with 72 hours due to gas substrate limitation. Within 48 hours, the CO_2 in the headspace was completely consumed and residual CO_2 in the liquid phase enabled an additional day of oxygen production up to ~72 hours after which there was no further oxygen production. In the *M. capsulatus* single

culture (Figure 16C), CH_4 was oxidized in the until there was no more O_2 produced by the *C. sorokiniana* single culture. After 72 hours, there was about 27% of CH_4 left in the headspace and residual CO_2 produced by methanotrophic CH_4 oxidation.



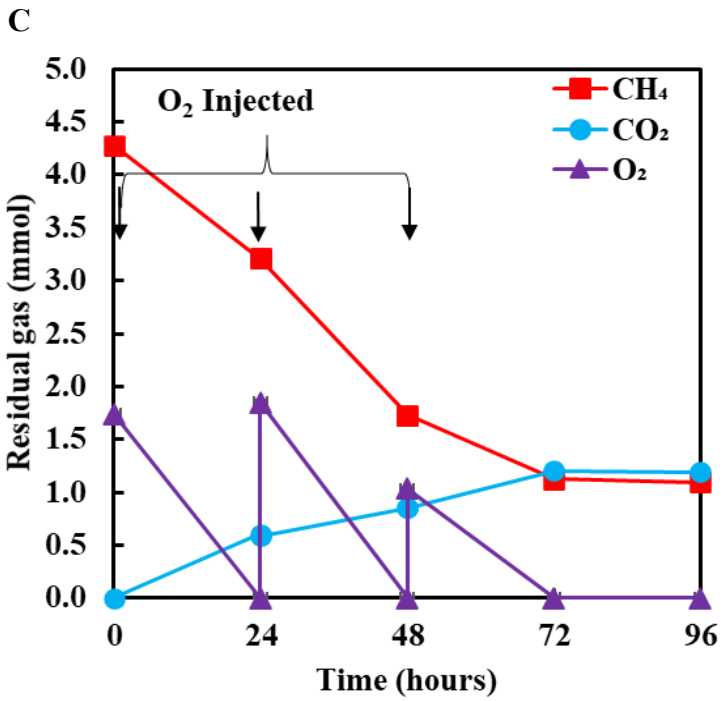


Figure 16: Gas phase profiles for (A) co-culture, sequential single culture of (B) *C. sorokiniana* and (C) *M. capsulatus*.

Figure 17A and Figure 17B show the residual NH_3-N and PO_4^{3-}-P concentrations among the *M. capsulatus* and *C. sorokiniana* single cultures as compared to the co-culture. 48 hours after inoculation 20 mL of undiluted AD effluent was added to each culture to ensure that the nutrient would be replete. This is shown by the vertical lines at the 48 hour period. NH_3-N is recovered from the AD effluent by all cultures during the cultivation period. *M. capsulatus* single culture exhibits the slowest nutrient recovery while the co-culture assimilation rate is highest recovery among the cultures within the first 48 hour period. After 72 hours, nutrient recovery in the microalga culture was limited due to CO_2 limitation. Methanotroph assimilation of NH_3-N was limited as there was no more oxygen produced by the microalga in the same period. The co-culture continued to recover both NH_3-N and PO_4^{3-}-P until both carbon substrate in biogas (CH_4 and CO_2) was completed consumed.

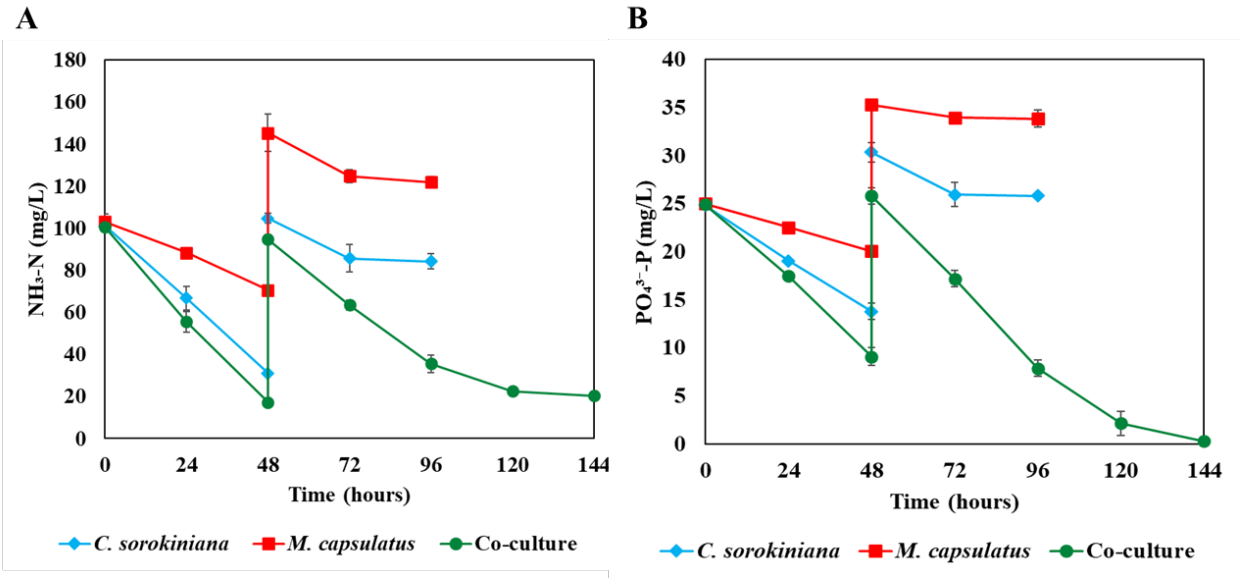


Figure 17: Time-course profiles inorganic nitrogen (A) and inorganic phosphorus (B) during the single cultures and the co-culture growth.

5.7.2 Case study 2: Single cultures and co-culture comparison under carbon-and nutrient-limited condition

For this experiment, the same amount of carbon substrate and nutrient was supplied to the single culture and the co-culture. The cultures were not refed biogas neither was any nutrients added after the initial inoculation. As a result, the growth would be considered carbon and nutrient limited. In Figure 18, the biomass time-course profiles for the single cultures and the co-culture are plotted. After the initial 24 hour period, the biomass concentration exhibits a clear separation among the single cultures and the co-culture. This is further evident by the large differences in the maximum biomass concentration between the single cultures and the co-culture. Table 5 below shows a comparison of the biomass productivity and increase among the *C. sorokiniana* and *M. capsulatus* single cultures and the co-culture. These results clearly show a more significant biomass productivity in the co-culture.

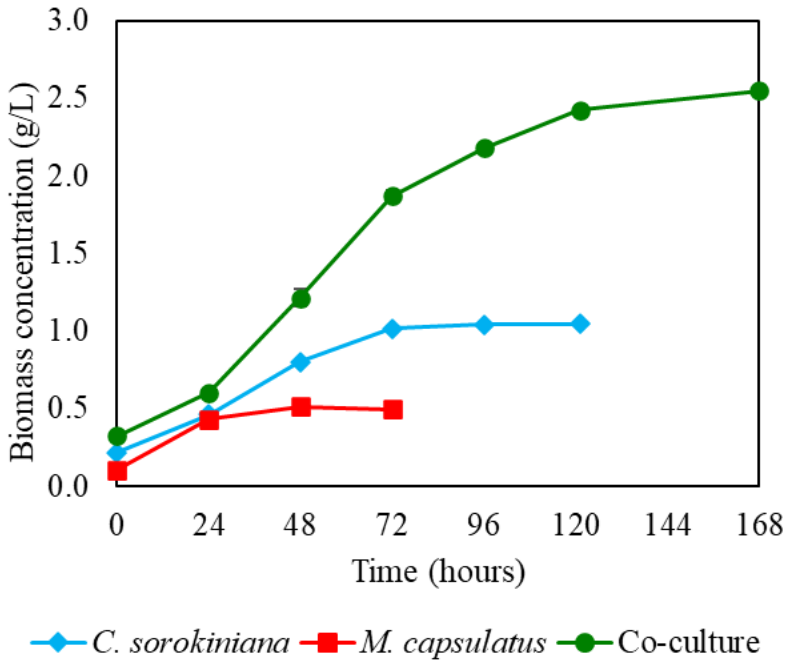


Figure 18: Biomass growth for *C. sorokiniana* single culture, *M. capsulatus* single and the co-culture.

Table 5: Biomass concentrations and productivity comparison for single culture and the co-culture.

	M. capsulatus	***C. sorokiniana***	**Co-culture**
Initial concentration	0.106 ± 0.000	0.219 ± 0.000	.326 ± 0.000
Final maximum concentration	0.513 ± 0.008	1.043 ± 0.002	2.550 ± 0.010
Biomass increase from initial concentration	4.83 times	4.76 times	7.82 times
Biomass productivity [a] (g/L/day)	0.204 ± 0.004	0.266 ± 0.005	0.416 ± 001

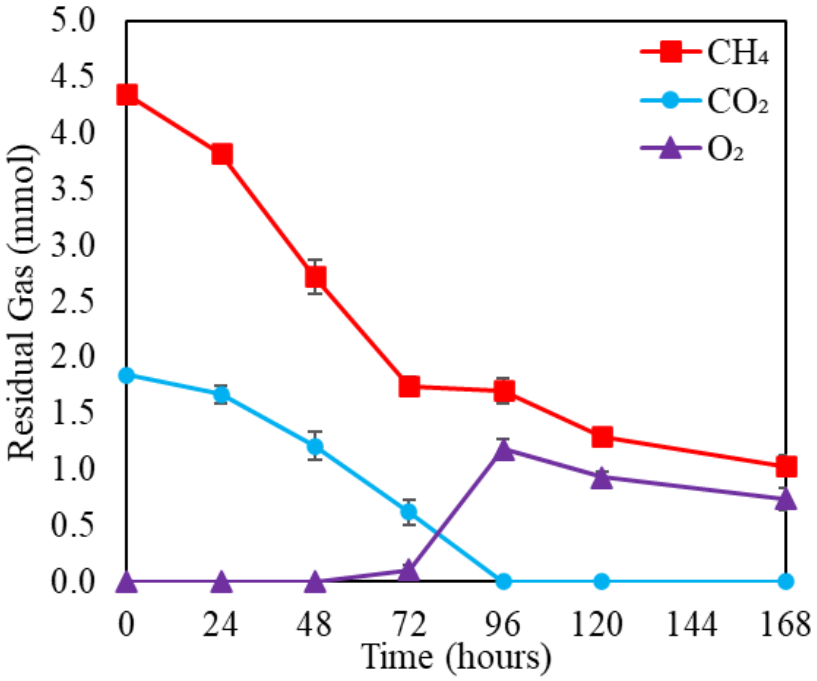
[a] Biomass productivity is calculated for the biomass produced before stationary phase is reached; i.e. 0-48 hours for *M. capsulatus*; 0-72 hours for *C. sorokiniana* and 0-121 hours for the co-culture

To further examine the reason for the differences in biomass concentration, the gas phase composition was monitored throughout the cultivation period. Like case study 1, the *in situ* recycling of carbon in the co-culture enabled significantly higher biomass production at the end of

the cultivation period (Figure 19A). Between 72 and 96 hours when headspace CO_2 was completely assimilated, O_2 was produced in the headspace was quite significant as compared to the previous growth periods. This resulted due to pH increase beyond the pH growth range (pH:6.6 – 7.5) of the methanotroph. Microalgae cultures experience increasing pH especially at low CO_2 concentrations due to lower carbonic acid resulting from rapid CO_2 assimilation. In addition, hydroxide ions are also released in the microalgal cultures when grown photosynthetically (Besson and Guiraud, 2013; Kim et al., 2013; Solimeno et al., 2015). Subsequent to pH adjustment of the co-culture, methane assimilation was resumed. Interestingly, methane was remained in the co-culture despite the presence of residual oxygen.

The residual gas composition of the *C. sorokiniana* and *M. capsulatus* single cultures are plotted in Figure 19B and Figure 19C. Steady assimilation of CO_2 and production of O_2 within the 72 hour period indicates the photosynthetic growth of the microalga (Figure 19B). After 72 hours, the small biomass increase and O_2 production results from small residual CO_2 in the culture broth. CH_4 was not consumed in the microalga culture and show small fluctuations due to measurement error. *M. capsulatus* single culture (Figure 19C) oxidized methane until there was no oxygen left after 48 hours. Residual CO_2 production by the methanotroph was also observed at the end of the cultivation period. Accordingly, the lower biomass concentrations and smaller biomass productivity in the microalga and methanotroph single cultures are due to substrate limitation.

A


Residual Gas (mmol)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
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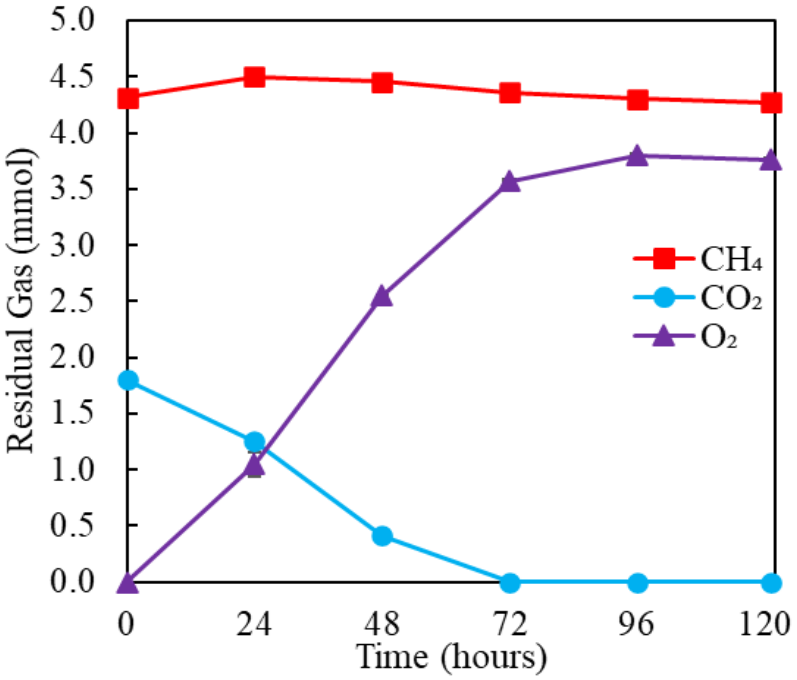
0
24
48
72
96
120
144
168
Time (hours)
CH4
CO2
O2

B


Residual Gas (mmol)
5.0
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Time (hours)
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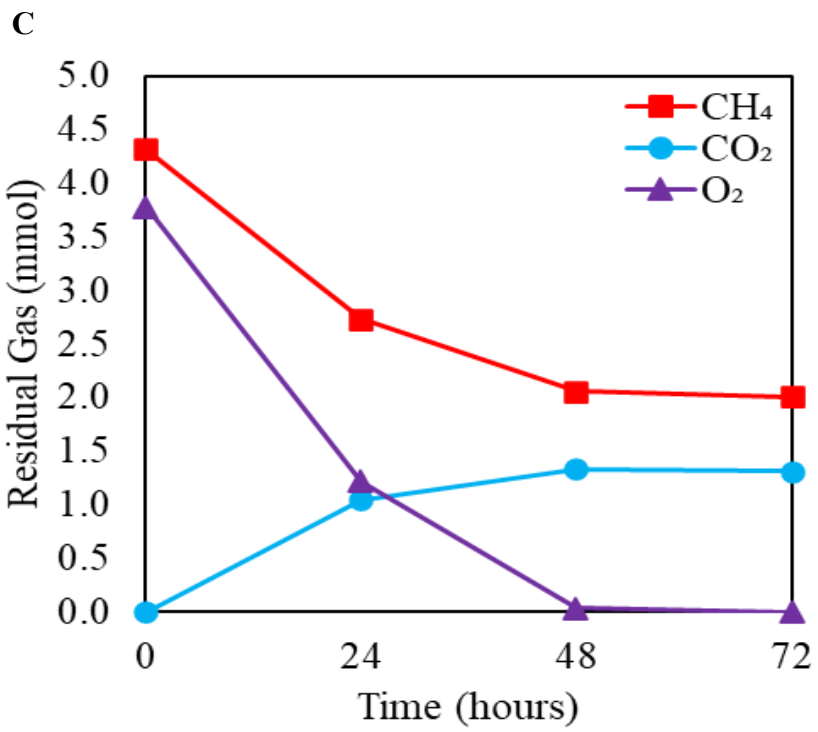


Figure 19: Residual gas composition of (A) co-culture (B) *C. sorokiniana* single culture and (C) *M. capsulatus* single culture.

Next, whether the co-culture offers improvement in nutrient recovery compared to the *C. sorokiniana* and *M. capsulatus* single cultures was examined. Figure 20 compares the nutrient removal by the single cultures and the co-culture, with Figure 20A for total nitrogen (TN), Figure 20B for NH_3-N, Figure 20C for total phosphorus and Figure 20D for orthophosphate. These plots clearly show that the single cultures did not completely removed nutrients (Figure 20A-D), as the cell growth stopped when carbon source became depleted. The co-culture was able to recover both TN and TP at a much faster rate than the single cultures; however, there remained both TN and TP in all the cultures at the end of the cultivation period. On the other hand, the co-culture was able to completely remove both inorganic nutrients (NH_3-N and PO_4^{3-}-P) at a much faster rate, mainly due to the enhanced growth enabled by the *in-situ* exchange of CO_2 and O_2. Further, the complete recovery of NH_3-N and PO_4^{3-}-P while there is residual TN and TP suggests the co-culture is not

capable of recovering the inorganic nutrients. Initial measurements of nitrate and nitrite concentrations in the AD were negligible, thus there were not measured.

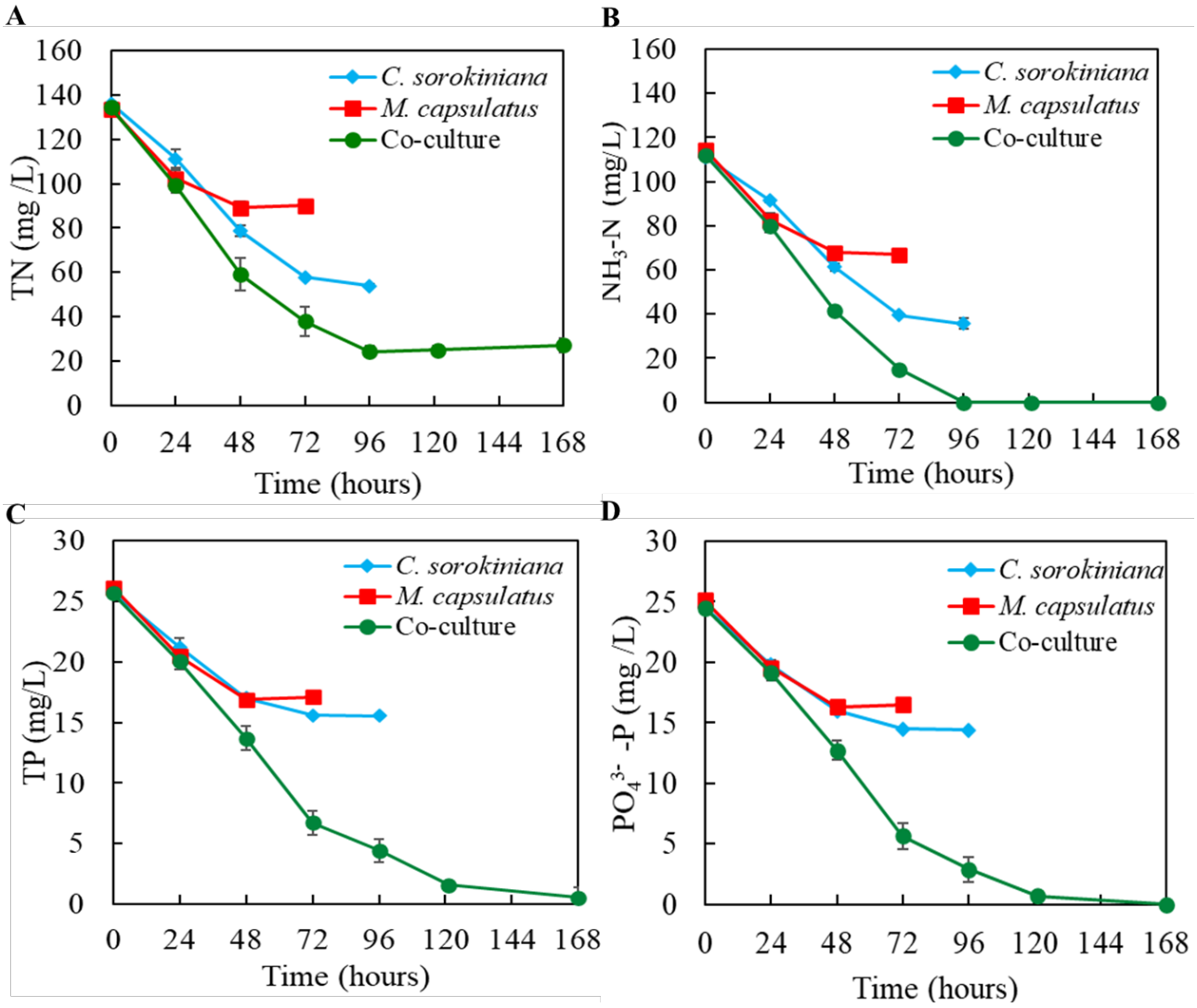


Figure 20: Nutrient profiles for (A) total nitrogen (TN), (B) NH_3-N, (C) total phosphorus (TP), and (D) PO_4^{3-}-P of the single cultures and by the co-culture.

Finally Figure 21 compares the nutrient recovery performance of both the *C. sorokiniana* and *M. capsulatus* single cultures together with that of the co-culture which clearly demonstrates an improvement provided by the co-culture over sequential single cultures for nutrient removal. To determine if the enhanced nutrient removal by the co-culture was due to the enhanced growth,

the amount of biomass produced vs. the amount of N and P removed was plotted, as shown in Figure 22. For NH_3-N removal, Figure 22A shows that the co-culture appears to recover more N per unit biomass produced than both single cultures at the beginning of the batch culture, while the rate decrease as more biomass was produced. This was likely due the reduced N supply from liquid medium. For PO_4^{3-}-P removal (Figure 22B), the co-culture and both single cultures show little differences at the beginning of the batch culture, and the recovery rate reduces as more biomass was produced. This result suggests that the enhanced nutrient recovery by the co-culture was mainly due to the enhanced co-culture growth compared to single cultures.

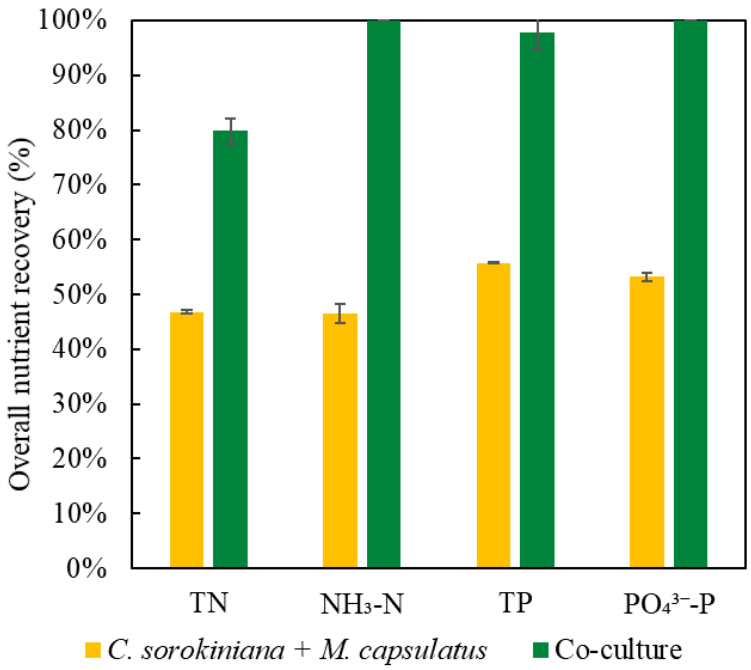


Figure 21: Percent nutrient recovery by summation of the single cultures and the co-culture for total nitrogen (TN), ammonia nitrogen (NH_3-N), total phosphorus (TP), and orthophosphate (PO_4^{3-}-P).

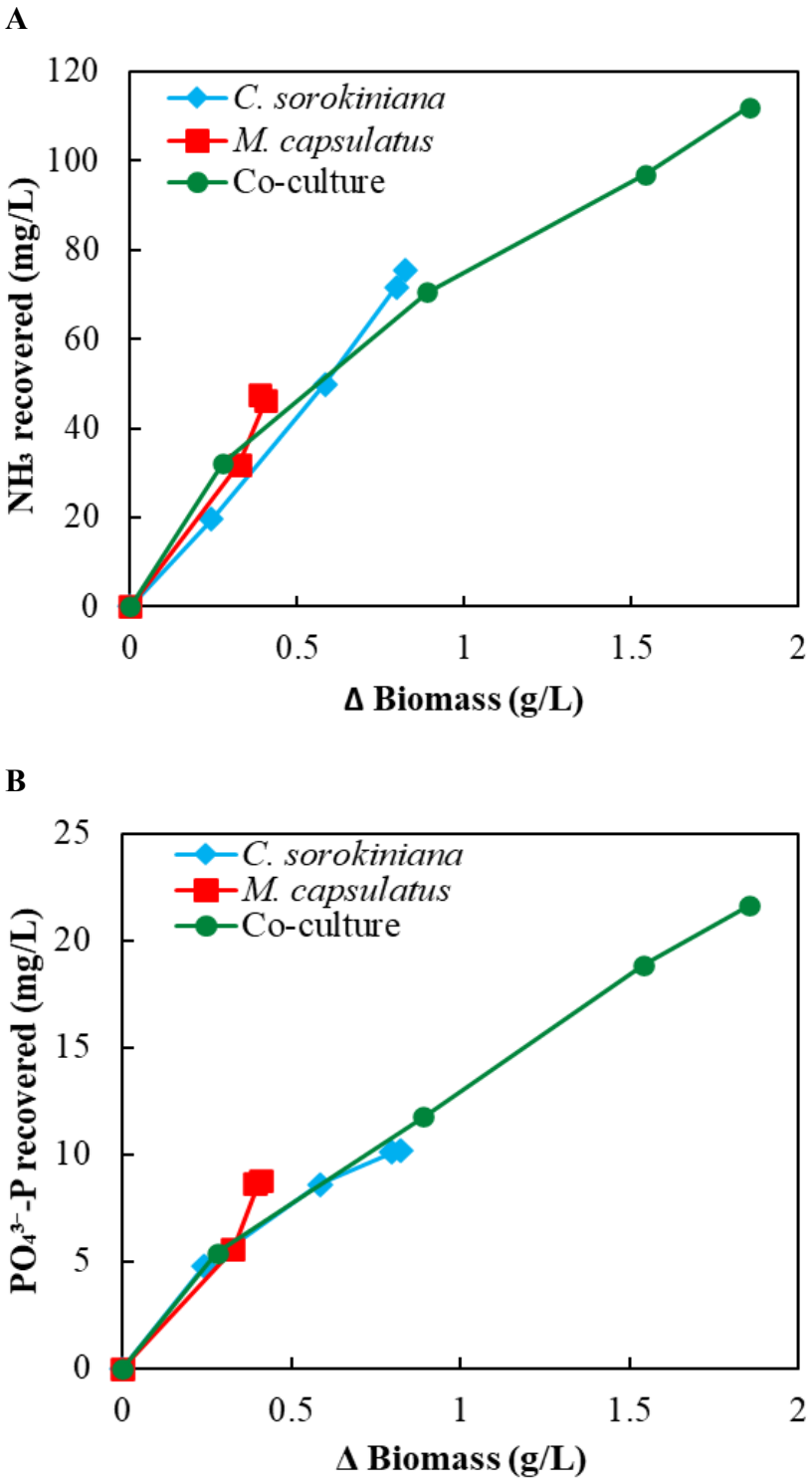


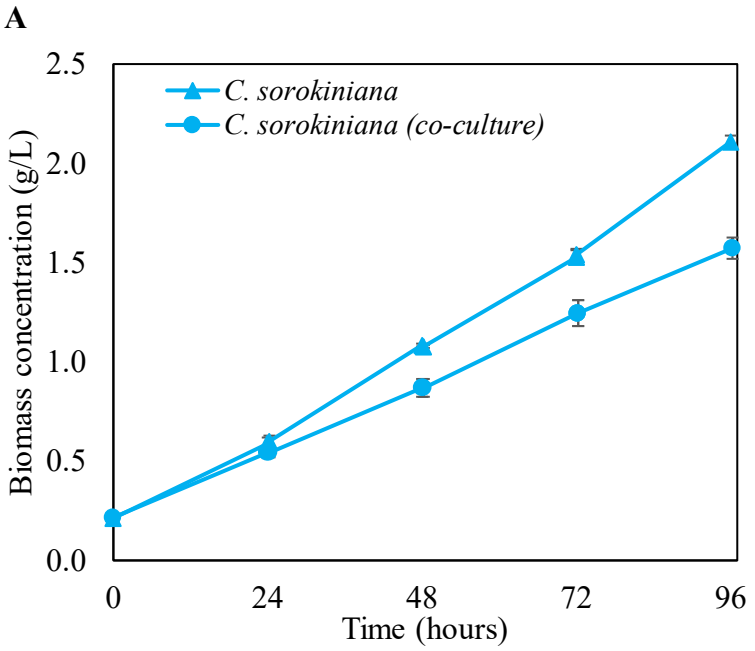
Figure 22: Correlation of the biomass produced with the recovery of (A) NH_3-N and (B) PO_4^{3-}P.

5.7.3 Case study 3 and 4: Sequential ingle cultures and co-culture comparison under carbon-replete but nutrient limited condition

From the above cases, the co-culture has demonstrated significant benefits over the sequential single cultures for biogas and nutrient recovery when the biogas substrate was limited. However, case studies 3 and 4 investigate whether the co-culture still maintains these benefits when biogas was refed into both the sequential single cultures and the co-culture; i.e. carbon replete condition.

Case study 3

In case 3, *C. sorokiniana* single culture and *C. sorokiniana* growth in the co-culture are plotted in Figure 23A. In both cases, the microalga shows a linear increase with time. Within the first 24 hours, both the microalga single culture and the microalga growth in the co-culture reached similar biomass concentrations of 0.600 ± 0.007 and 0.544 ± 0.045 g/L, respectively. Thereafter, there is a split in the cultures where *C. sorokiniana* single culture reached the highest biomass concentration within 96 hours as compared to *C. sorokiniana* growth in the co-culture.



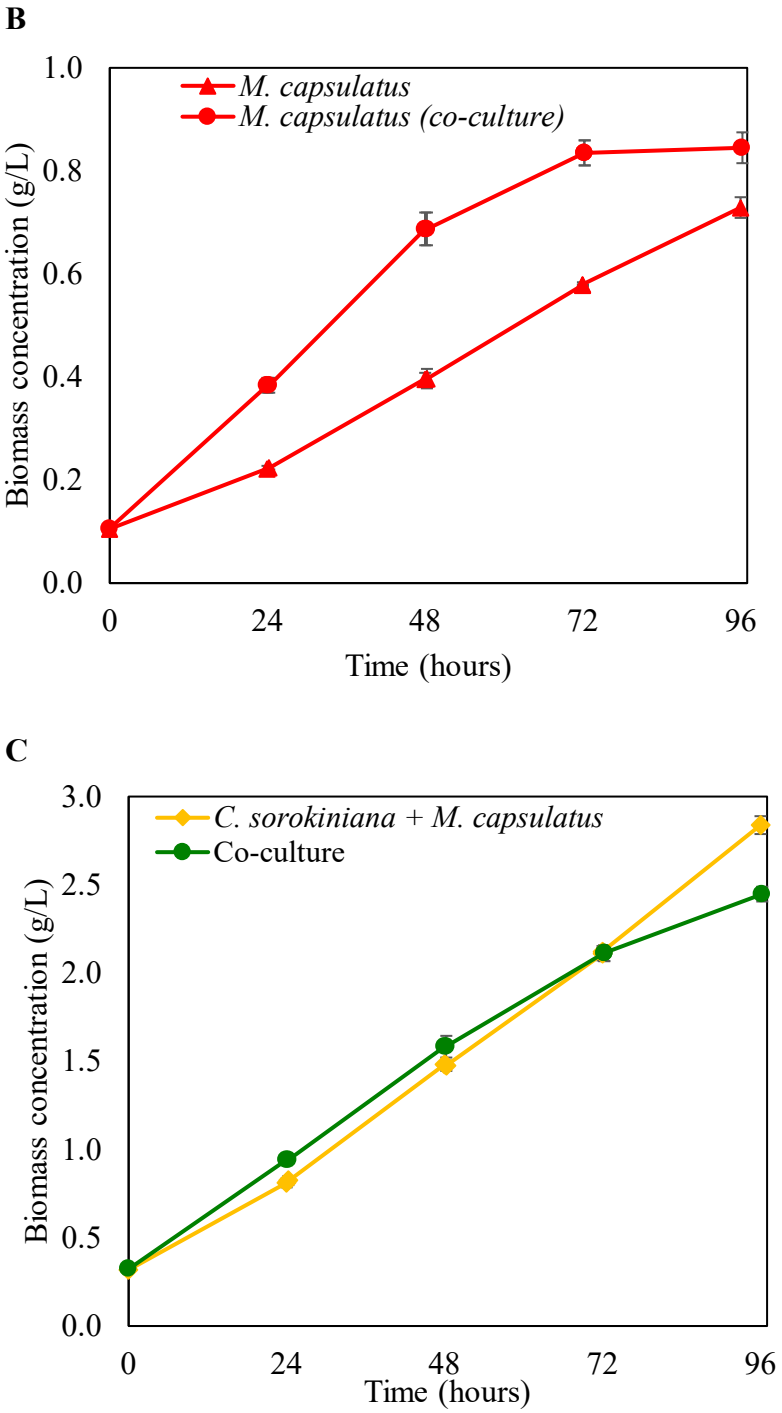
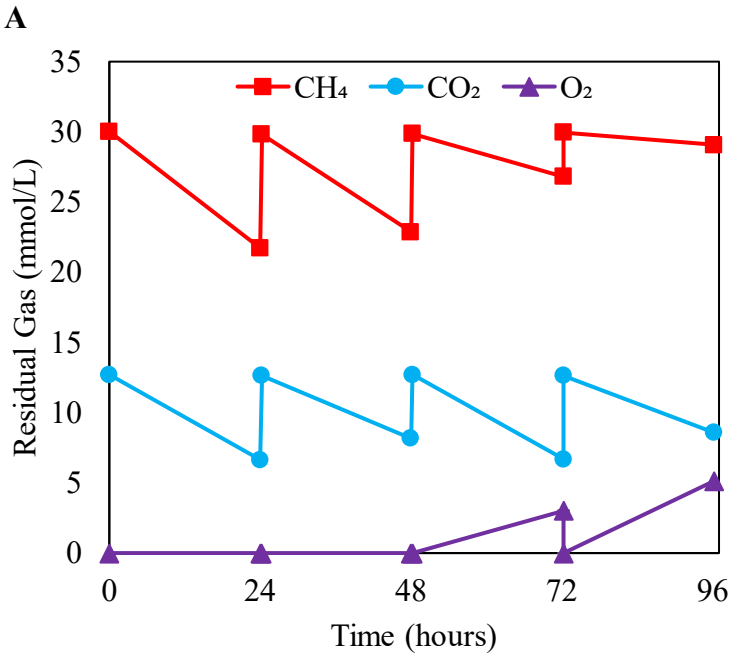


Figure 23: Biomass comparison between (A) *C. sorokiniana* single culture and *C. sorokiniana* growth in the co-culture; (B) *M capsulatus* single culture and *M. capsulatus* in the co-culture and (C) the overall co-culture and summation of the single culture biomass.

In Figure 23B, *M. capsulatus* single culture growth is slower as compared to *M. capsulatus* growth within the co-culture. These results are similar to that of case 1 where the methanotroph in the co-culture exhibited higher biomass production and productivity than the methanotroph single culture. While the *in situ* produced O_2 might provide an advantage in the co-culture it appears there could be other metabolic links that enable more enhanced growth of *M. capsulatus* within the co-culture. Figure 23C shows the biomass produced by the single cultures in each sampling period is summed and compared to the co-culture growth. In this comparison, the biomass production is quite similar up to 72 hours of growth after which the co-culture growth declined at 96 hours. When the co-culture gas profiles (Figure 24A) were examined, residual oxygen was present in the headspace indicating that the methanotroph strain growth declined in the co-culture which contributed to the overall decline in the co-culture biomass production.



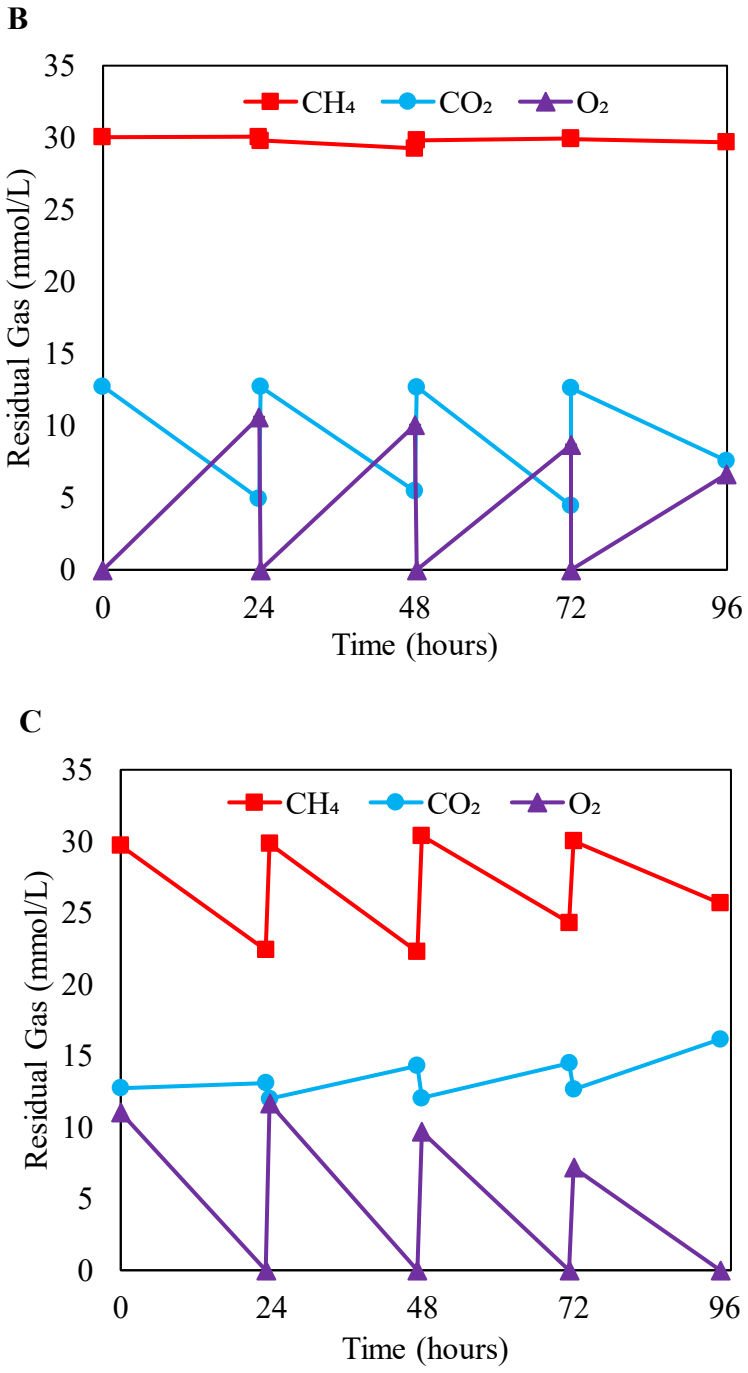


Figure 24: Residual profiles of (A) the co-culture, and the (B) *C. sorokiniana* and (C) M. capsulatus single cultures.

The inorganic nutrient recovery plotted in Figure 25A and Figure 25B, demonstrate steady decline in both NH_3-N and PO_4^{3-}P, respectively. The NH_3-N recovery (Figure 25A) in the *C. sorokiniana* single culture exhibits a steady at the same rate until all the NH_3-N is recovered at 96 hours. Within the co-culture, NH_3-N rate was initially faster than *C. sorokiniana* single culture; however, the recovery rate decline after the 48 hour period was possibly due to the reduced methanotroph growth in the co-culture. Figure 25B shows that PO_4^{3-}-P is steadily assimilated in all culture and there was residual PO_4^{3-}-P in the *C. sorokiniana* and *M. capsulatus* single cultures at 96 hours while complete consumption of PO_4^{3-}-P is reached in the co-culture within 72 hours. As a result, rapid depletion of PO_4^{3-}-P might be the reason for the decline in the co-culture (and methanotroph) growth after at 48 hours.

To explain, the rate of PO_4^{3-}-P consumption between 24 and 48 hours was 0.35 mg/L/h. and the residual PO_4^{3-}-P concentration at 48 hours was (2.40 mg/l). If the co-culture recovered PO_4^{3-}-P at the same rate—as between 24 - 48 hours—the PO_4^{3-}-P would be completely assimilated at 55 hours. It is well known that phosphorus is an essential nutrient for cellular metabolism of both microalgae and methanotrophs and its limitation can have adverse effects on cell growth. In microalgae, P limitation has been reported to: lower cell growth rate, reduce photosynthesis, reduce protein production as well as decreased ADP and ATP levels (Procházková et al., 2013; Sundstrom and Criddle, 2015). Phosphorus is also important for methanotroph growth as the intracellular membrane of methanotrophs are constructed of phospholipids. Increasing levels of orthophosphate have also been reported to increase methane oxidation (Veraart et al., 2015). Accordingly, P deprivation could account for some of the observation within the co-culture after 48 hours: 1) the reduced growth of the co-culture (especially for the methanotroph) after 48 hours and the more pronounced decline between 72 and 96 hours;

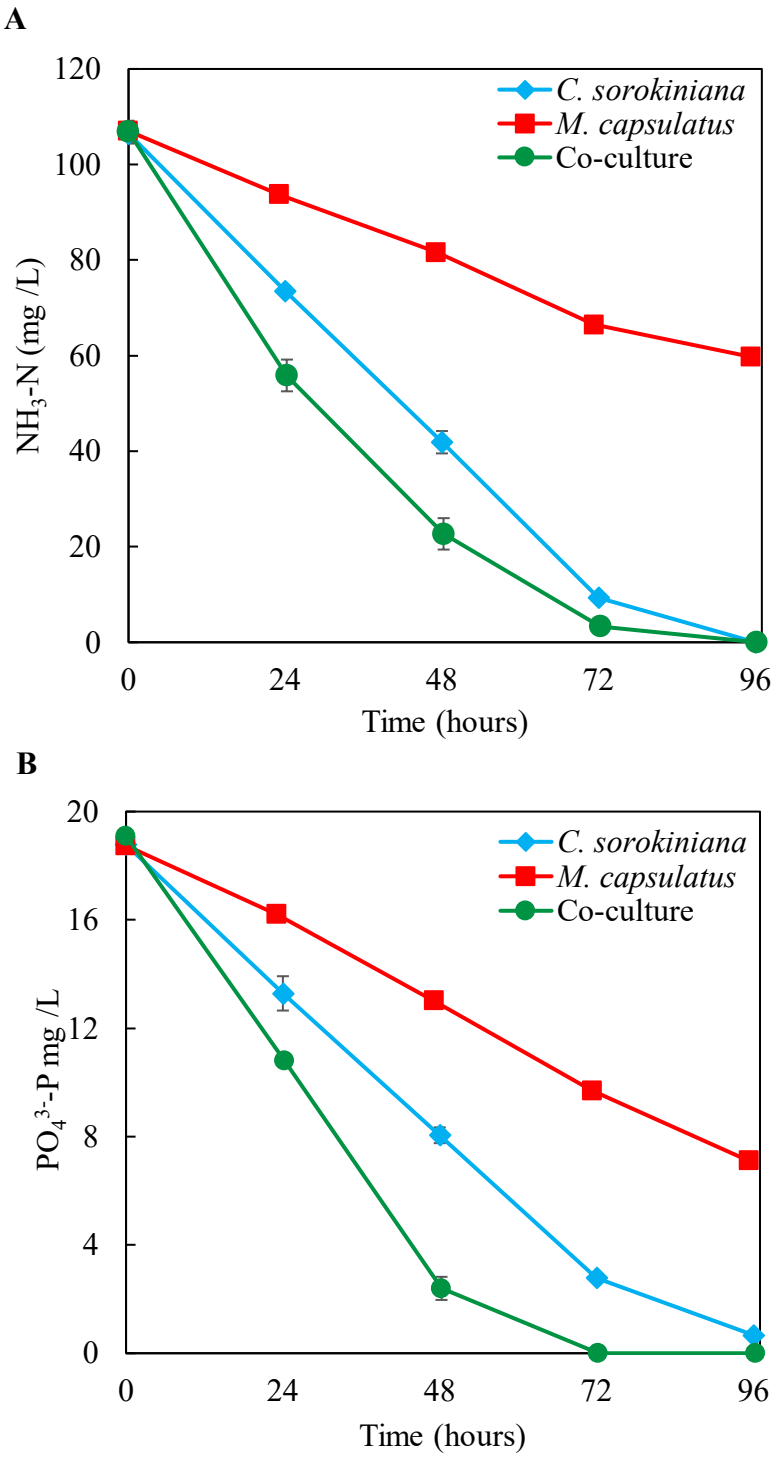


Figure 25: NH_3-N (A) and PO_4^{3-}-P (B) recovery comparison among culture of *C. sorokiniana*, *M. capsulatus* and the co-culture.

2) the reduced *M. capsulatus* growth and increase in residual O_2 in the headspace; and 3) the reduced NH_3-N recovery rate in the co-culture after 48 hours. In contrast to the case 1 and 2, the fast growth of the co-culture due to gas feeding every 24 hours may results in faster depletion of micronutrients which are especially essential for the growth of both *C. sorokiniana* and *M. capsulatus*. The residual TN and TP (Appendix A: Figure 38A and B) also confirm that the co-culture is unable to recover the organic nitrogen and phosphorus.

In Figure 26 is plotted the comparison of the biomass produced and the nutrient recovered. Figure 26A shows that the NH_3-N recovery is similar between the co-culture and the sequential single cultures. Recovery of NH_3-N by the co-culture decreases after the 24 hours of growth presumably due to decrease in nutrients. For PO_4^{3-}-P, the co-culture and *C. sorokiniana* have similar recovery rates; however, Figure 26B indicates that *M. capsulatus* utilized more orthophosphate during growth.

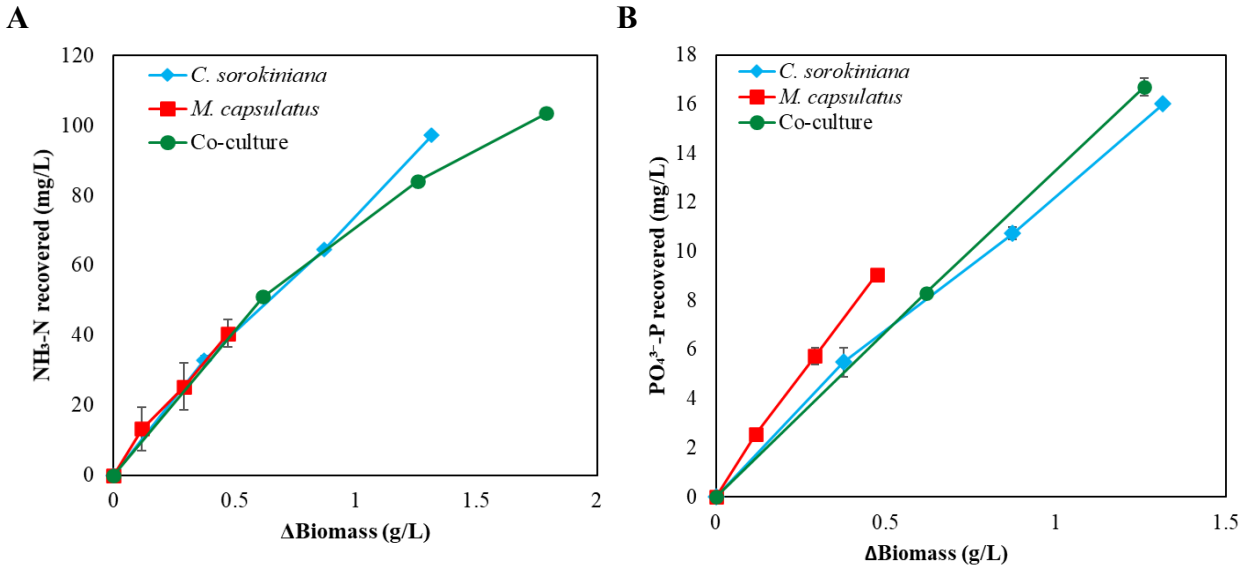


Figure 26: Correlation of the biomass produced with the recovery of (A) NH_3-N and (B) PO_4^{3-}-P under carbon unlimited growth.

Case study 4

As discussed in case study 3, under carbon-unlimited growth, *C. sorokiniana* growth within the co-culture was reduced as compared to *C. sorokiniana* growth in the single culture (Figure 23A). However, in case study 1, C. sorokiniana growth in the co-culture demonstrated comparable growth to *C. sorokiniana* in single culture under carbon-limited condition before CO_2 was completely assimilated. For this reason, case study 4 was designed to determine the reason for these observations. It was postulated that the light intensity is potentially limiting as the biomass concentration increases. Photosynthetic growth of the microalga will increase linearly until the irradiance provided is saturating. However, the self-shading effect can cause reduced growth of the microalga. The light penetration through the bottles decrease as the culture gets dense, resulting in less light availability to each cell (Cheirsilp and Torpee, 2012). In extremely dense cultures, dark regions can also be created, further limiting photosynthetic growth.

This experiment was similar to case 3 except that the light intensity was increased from 180 to 550 μmol/m^2/s. The biomass comparison in case 4 exhibits higher microalgal concentration in the single culture as shown in case 3. This might indicate that the light intensity is not high enough to penetrate the high biomass concentration. There are a few reasons that this might be the case. Firstly, the self-shading effect might also be enhanced by the presence of the methanotroph. Microalgal cells in the middle of the bottle will be obtain reduced light due to the methanotroph in addition to other microalgal cells. Secondly, the light intensity of 550 μmol/m^2/s may not be completely saturating as preliminary light intensity tests (Figure 39) show *C. sorokiniana* single culture can have higher biomass growth when the light intensity was increased beyond 550 μmol/m^2/s . Lastly, red light wavelength is strongly absorbed by the microalga chlorophyll pigment. As a result, it has been reported that light penetration in the culture is significantly

reduced as compared to white light. It is possible that faster growth in the co-culture is still limited by light penetration and can be remedied by incorporating internal light source in bottles.

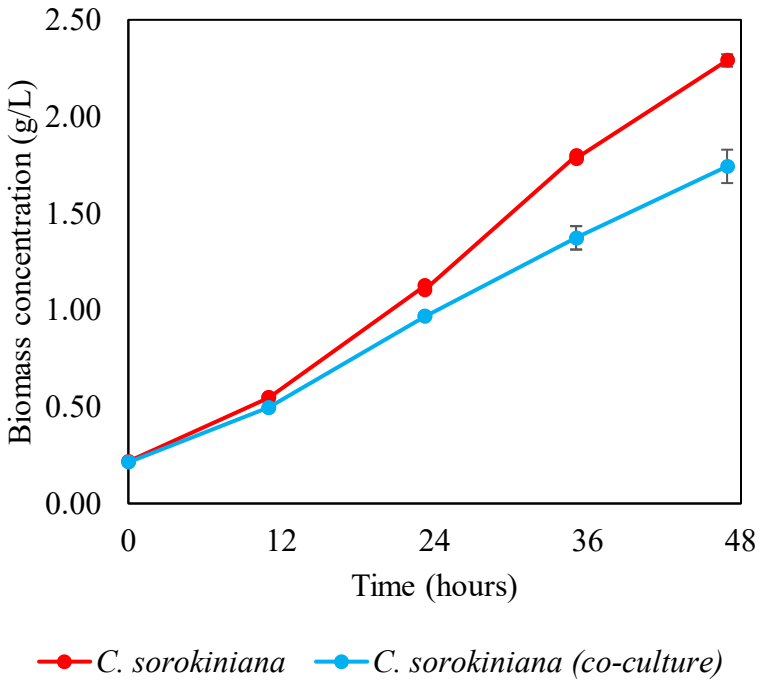


Figure 27: Case 4: *C. sorokiniana* biomass comparison.

5.8 Conclusions

The metabolic coupling of both stains in the co-culture can provide significant advantages for co-utilization of CH_4 and CO_2 with simultaneous nutrient recovery and increased biomass production. Various case studies have showed that the co-culture has a greater advantage for biogas utilization and nutrient recovery when carbon substrate is limiting. When the co-culture was grown with under unlimited carbon substrate, the co-culture biomass production was similar to summation of *M. capsulatus* and *C. sorokiniana* single cultures. The results also demonstrate that the co-culture has the potential for zero emissions; i.e. it can completely recover both methane and

carbon dioxide in biogas. Thus, the co-culture technology could serve as a more economical technology for bioconversion of biogas to microbial biomass as well as reducing GHG emissions. Further, the co-culture approach is still a safe route for upgrading biogas without external oxygen supplementation.

Chapter 6: Simultaneous Biogas and Nutrient Recovery by the Co-culture under Chemostat operation

6.1 Introduction

Greenhouse gas (GHG) emissions from water resource recovery facilities are projected to increase by 45% from 1990 to 2020 with wastewater CH_4 emissions accounting for a large fraction of the waste sector (Paolini et al., 2018). At municipal WRRFs, biogas derived from anaerobic digestion of sludge is a renewable energy source; however, removing impurities in biogas present technical and economic barriers for biogas utilization for combined heat and power and upgrading to biomethane. Consequently, biogas is being flared or used for heating digesters with mainly large-scale facilities employing CHP technology.

As a result, biological approaches are gaining more attention as they are considered to be more efficient and economically feasible methods for recovering carbon substrate from biogas. Biofixation of CO_2 by microalgae has been extensively investigated for upgrading biogas to produce a gas stream with a high methane content similar to that of natural gas thereby increasing the caloric value of the gas. One major process challenge is the release of oxygen into the upgraded CH_4 gas stream. Oxygen content above 1% is not acceptable for most biomethane standards and oxygen content in upgraded biogas can create explosive mixtures. Another approach for utilizing methane is to utilize methanotrophic bacteria as a methane sink through microbial conversion of CH_4 into microbial biomass. Methanotrophic biomass can potentially be utilized for a diverse set of products such as single cell protein, ectoine, biopolymers (polyhydroxyalkanoates) and other value-added products (Khosravi-Darani et al., 2013; Strong et al., 2015; Strong et al., 2016).

In previous chapters of this study, batch experiments of the methanotroph-microalga co-culture demonstrated good potential for bioremediation of the wastewater effluents and complete recovery of both CH_4 and CO_2. Biogas is converted to microbial biomass with potential use for

upgrading the biomass to biofuels through methods such as hydrothermal liquefaction. Furthermore, co-culture growth on the untreated AD effluent as growth medium would significantly reduce production and operating costs. However, for significant environmental and economic impact on human society, biogas recovery by the co-culture must be performed at large scale to properly assess the biogas utilization capabilities of the co-culture technology. Scaled-up cultivations usually show reduced productivities due to the differences in factors governing biomass performance (Lopes da Silva and Reis, 2015). Two essential factors limiting co-culture productivity are: 1) the light received by each microalgal cell and 2) the low solubility of methane. Critical parameters that affect the light per cell are reactor surface-to-volume (S/V) ratio, geometry, orientation, thickness of reactor wall. On the other hand, the mass transfer of methane will be affected by the reactor configuration and residence time. Other factors such as mixing times and mixing speeds from in large scale photobioreactors (PBRs) can dramatically affect mass transfer, mixing of biomass for exposure to light and minimizing hydrodynamic (shear) stress.

In this chapter, an initial study was conducted to investigate the performance of the co-culture technology in a scaled-up 3L photobioreactor under chemostat cultivation with *C. sorokiniana* and *M. capsulatus* as the model co-culture pair. The recovery of both CH_4 and CO_2 in the synthetic biogas mixture was examined during steady state chemostat operation and the results show the *C. sorokiniana*-*M. capsulatus* co-culture can convert both CH_4 and CO_2 in biogas into microbial biomass without an external oxygen supply while continuously recovering the nutrients in non-sterilized AD effluent.

6.2 Material and methods

6.2.1 Preculture and wastewater medium

The methanotroph and microalga strains used for this co-culture study were *M. capsulatus* Bath and *C. sorokiniana*, respectively. Pre-cultures of both strains were maintained on sterile-filtered and autoclaved anaerobic digestate diluted with the clarifier effluent to a final NH_3-N concentration of 150 m/L NH_3-N. Filtered and autoclaved AD effluent was used to ensure sterile monocultures for the chemostat experiment. For methanotrophic growth, methane was supplied to a final concentration of 70% (v/v) CH_4 and 30% (v/v) O_2 at 200 rpm and 37℃. *C. sorokiniana* was also grown on the same wastewater media, and CO_2 was supplied to a final concentration of 30% (v/v) CO_2 and 65% (v/v) N_2 at 200 rpm, 37℃, and under continuous illumination at 400 µmol m-2 s-1 using DayWhite LED lights over a 48 hour period before inoculation. The precultures were fed twice daily to maintain high growth rate and prevent carbon substrate limitation over the 48-hour preculture period on the high light intensity. After cultivation on day 1, the monocultures were diluted with AD mixture to supply additional nutrients and prevent nutrient limitation. On the second day of *C. sorokiniana* preculture, CO_2 in the was increased to 35% balanced with 65% N_2 in order to have the cells growing under an environment with higher CO_2 concentrations in preparation for continuous CO_2 sparging in the PBR.

6.2.2 Analytical methods

5 mL gas samples are withdrawn from the gas sampling port and measured using GC (Agilent 7890B customized with FID, TCD, Unibeads IS 60/80 mesh and MolSieve 5A 60/80 SST columns). Gas calibrations were performed by injecting gas mixtures of CH_4, CO_2 and O_2 of known compositions and calibration curves generated through regression analysis in Microsoft Excel. Total carbon and inorganic carbon measurements were performed by first centrifuging

samples to remove cell biomass and the supernatant is then filtered through a 0.2 μm filter. For IC measurements, 0.05-0.1 mL of 1M NaOH was added to a centrifuge tube before adding 2 mL of cell suspension in order to raise the pH and convert the aqueous CO_2 into carbonate and hydrogen carbonate ions. Then, the total inorganic carbon (TIC) of the liquid phase is measured via a Shimadzu TOC-VCSH analyzer. Nutrient analyses such as ammonia-nitrogen and orthophosphate were all measured using Hach kits. Samples were centrifuged at 12,000 rpm for 4 minutes, filtered (0.2 μm, nylon) and measured and in a Hach DR1900 spectrophotometer. The optical density of the co-culture was measured by a UV-Vis spectrophotometer (Beckman Coulter) at 750 nm after appropriate dilution of 2-15 to maintain measurements in the linear absorbance range.

6.2.3 Biomass estimation

The individual strain biomass estimation was performed using the method described in Appendix C. Based on previous batch experiments, the co-culture reached a stable mass ratio of *C. sorokiniana*:*M. capsulatus* of 1.42 (2.2:1 based on OD_{750}) at the end of the growth period. Due to the metabolic coupling of the co-culture strains, where methanotroph growth is dependent on microalgal production of O_2, it was assumed that the co-culture maintained the same stable ratio in the PBR during batch and chemostat operation in the PBR and the individual strain concentration was calculated accordingly.

The volumetric biomass productivity (P) was calculated according to the following equations:

$$\text{Batch operation: } P_{batch} = \frac{X_i - X_o}{t_i - t_o} \quad (16)$$

$$\text{Chemostat operation: } P_{chemostat} = DX$$

where X_o and X_i are the biomass concentrations at $t_o and\ t_i$, D is the dilution rate and X is the steady state biomass concentration.

6.2.4 Considerations for recovery of NH_3-N and PO_4^{3-}-P in the PBR

Ammonia can exist as two forms in aqueous solutions: unionized (free) ammonia (NH_3-N) and ionized ammonia (NH_4^+). Continuous sparging of the AD mixture with biogas can cause NH_3 volatilization when there is a high fraction of free ammonia in the AD mixture which could lead to significant overestimation of the NH_3-N recovery by the co-culture. Although, the concentration of each ammonia species in the AD effluent is dependent on several factors, temperature and pH are the most important. Using equations by Emerson et al. , the fraction of free ammonia in the wastewater was estimated at the PBR temperature and pH to assess the potential volatilization of NH_3-N.

First, the pKa of the ammonium ion is calculated at the PBR temperature using equation (17) below:

$$\text{pKa} = 0.09018 + \frac{2727.92}{\text{T}} \quad (17)$$

where T is the temperature in Kelvin (K) in the range of 273-323 K

Then the fraction (α) of free ammonia can be estimated using equation (18):

$$\alpha = \frac{1}{10^{pKa-pH} + 1} \tag{18}$$

Based on these two equations, the fraction of free ammonia was estimated to be 1.28% in the AD mixture at 37 ℃ and pH 7.

Next, the potential of orthophosphate loss through abiotic means is evaluated. The induction point for phosphorus precipitation was reported when pH was near or above pH 8 (Ferguson et al., 1973). As the pH of the PBR was maintain at pH 7 throughout the study, phosphorus precipitation was considered to be negligible. NH_3-N and PO_4^{3-}-P recovery equations can be found in Appendix E.

6.2.5 Photobioreactor development and setup

One essential cultivation parameter for co-culture investigation is the irradiance supplied for photosynthetic growth of the microalga in order that oxygen is then supplied for methane oxidation by the methanotroph. Light-limited growth of the microalga will cause O_2-limited growth of the methanotroph. Consequently, biomass productivity, biogas utilization and nutrient recovery can be modulated by the irradiance input. To achieve sufficiently high light intensities, a modified light system was developed for investigations of the co-culture in a bench-top (3L) continuous bioreactor. While flat panel type photobioreactors have been utilized to reduce the light path for increased light penetration, this initial co-culture study in a bench-scale PBR was conducted using a modified cylindrical bioreactor (New Brunswick Co., Eppendorf BioFlo 110). Two major modifications are discussed below.

1. Development of the light panel: In order to supply light around the circumference of the bioreactor, two hemispherical boards made of polycarbonate were used to affix the lights

around the reactor. Instead of LED strips or individual bulbs, a LED light sheet (AspectLED) was used such that 288 LED bulbs are illuminating the surface of the photobioreactor. Velcro strips were attached to the light panel for tight installation around the PBR (Figure 28). With this setup, the maximum irradiance that can be achieved is 1100 $\frac{\mu mol}{m^2.s}$. To investigate the co-culture performance under different irradiances, a dimmer was installed for controlling the light intensity by limiting the voltage. As no light meter was setup inside the PBR, the light intensity was correlated to the voltage output of the dimmer through linear regression.

2. Temperature control: Temperature is another important growth condition for maintaining high biomass productivity and consequently biogas and nutrient recovery rates. The BioFlo 110 unit maintains constant temperature of the bioreactor through a heating blanket that wraps around the surface of the reactor. However, if the heating blanket were used, the microalgal cell inside the PBR would not be illuminated. As a result, a heat system was designed to maintain constant temperature. A flexible heating pad (Omega^{TM} , USA) was installed at the bottom of the reactor to be able to provide heating without blocking the light (Figure 28). The control unit developed with the heating pad was able to maintain the temperature within 0.5-1 ℃ of the setpoint without the internal cooling coil and within 0.5 ℃ with the cooling coil. This test was performed without the light system being installed to independently test the operation of the heating pad. Multiple tests indicated there was no localized overheating at surface where the heating pad was installed.

6.2.6 Photobioreactor (PBR) considerations:

While the incidental irradiance (at the surface of the PBR) is important, the transmitted irradiance (inside of the reactor) is most vital for the co-culture bioconversion of CO2 and CH_4 in biogas and nutrient uptake from the AD effluent. The rate of microalgal photosynthetic assimilation of CO_2 and methane oxidation by methanotroph is dependent on the effective light penetration and as a result, the percent of cells in the PBR receiving illumination (Wang et al., 2015). For continued biomass accumulation, the light intensity received by the microalga has to be greater than the light compensation point for the microalga such that increases in microalgal cells due to photosynthesis is greater than the loss of cells due to respiration (Wang et al., 2015). In suspended systems such as PBRs, the light penetration depth decreases significantly with increases in biomass. Even more, in the co-culture the *M. capsulatus* cells may further decrease light penetration to *C. sorokiniana*. Thus, a static test was performed to investigate the light intensity at the inner glass vessel surface and the geometric center of the PBR at different co-culture cell densities at two different incidental light intensities. Co-cultures grown in vials with AD mixture were used to perform the static test in the bench-scale PBR.

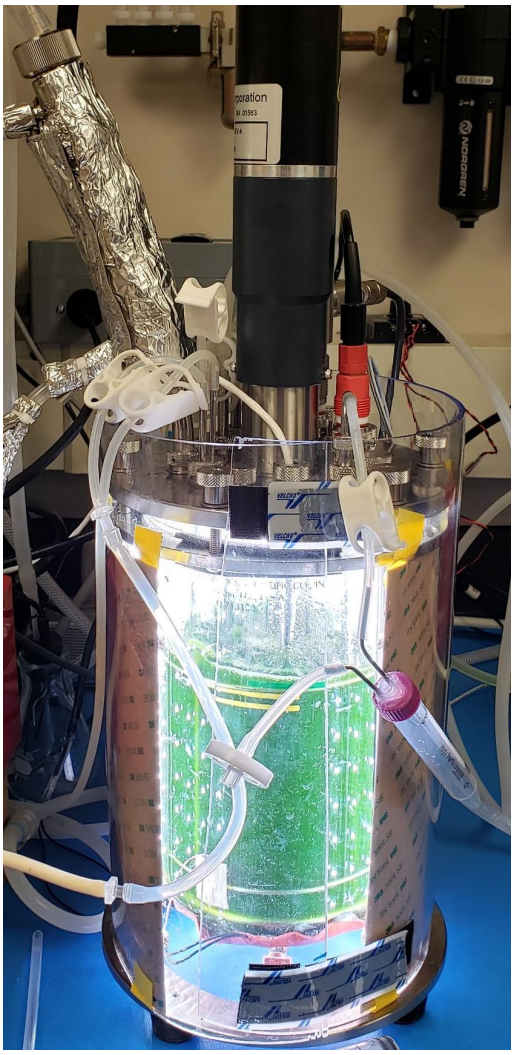
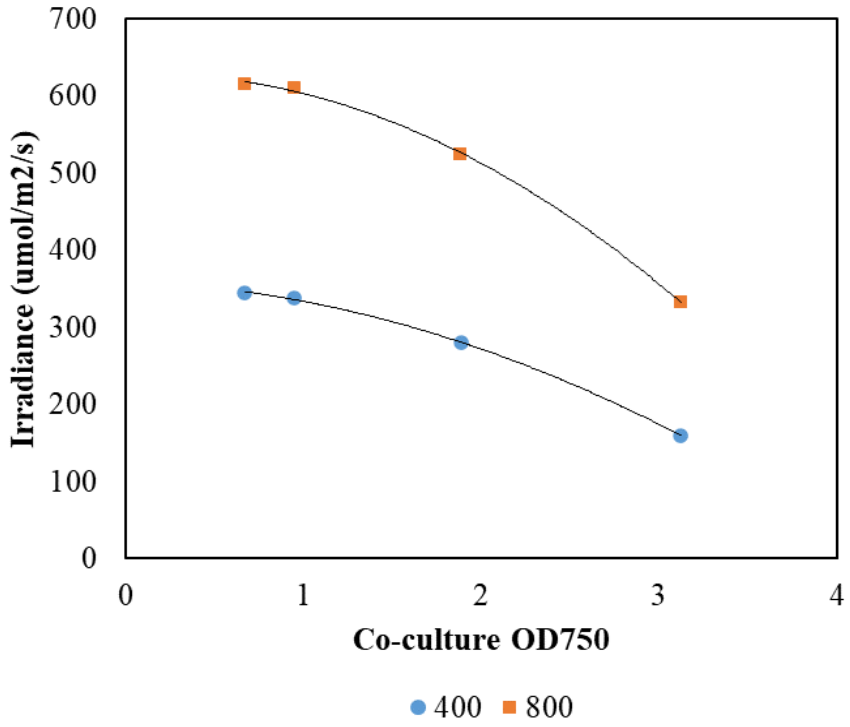


Figure 28: Photobioreactor: Modified BioFlo 110 with light panel and flexible heating pad for bench-scale co-culture investigations.

After starting at an initial OD_{750} of 3.12, the co-culture cells were serially diluted and the light intensity in the cell broth was measured with a HydroFarm Quantum Par Light meter. Due to the curvature of the glass vessel there is *ca.* 0.5 mm of co-culture cell suspension between the light meter and the glass vessel and the inner diameter of the glass vessel is *ca*. 2.5". Figure 29A shows the light attenuation at the inner wall of the glass vessel and Figure 29B illustrates the light

intensity at the geometric center of the PBR for light intensities of 400 and 800 $\frac{\mu mol}{m^2 s}$. Overall, the trends at both light intensities indicate the light intensity at the inner wall of the PBR decreases by more than 50% as the co-culture OD_{750} increases (Figure 29A and Figure 29B). There is more a pronounced attenuation at the center of the PBR due to less light penetration caused by the self-shading effect. At the lowest OD_{750} of 0.672, the irradiance at the center of the PBR is significantly reduced to ~150 $\frac{\mu mol}{m^2 s}$ when the incidental irradiance is 800 $\frac{\mu mol}{m^2 s}$ (Figure 29B). At an OD_{750} of 3.12, the irradiance at the geometric center is reduced to ~21 $\frac{\mu mol}{m^2 s}$. Similar results have been reported by Wang et al and Tredici (Tredici, 2010; Wang et al., 2015).

A



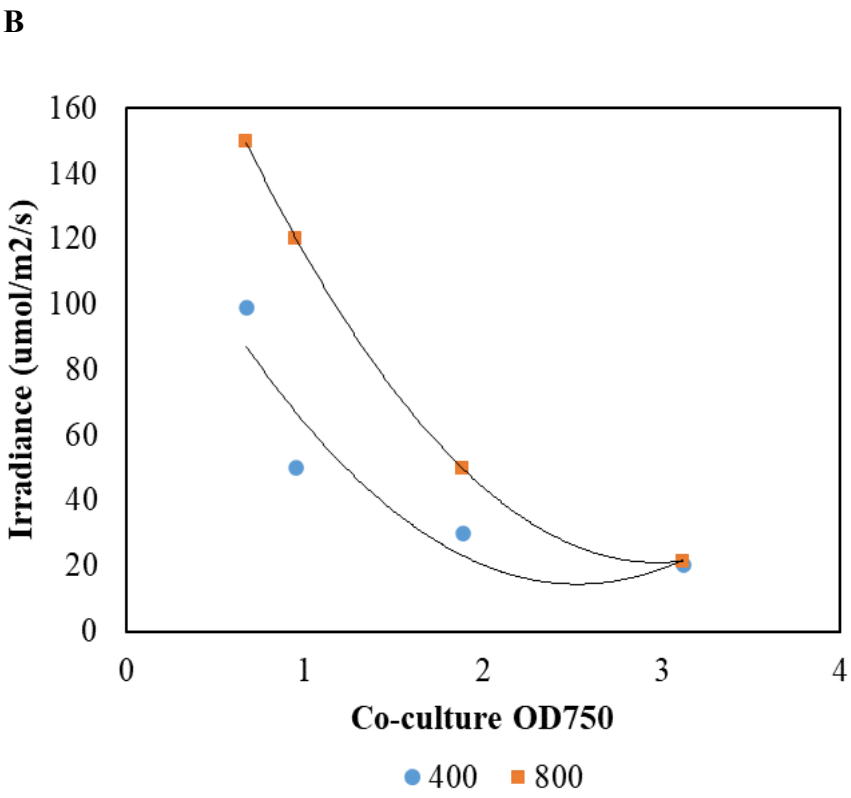


Figure 29: Light attenuation inside the PBR caused by self-shading effect of co-culture cells. (A) The light intensity measured at the inner wall of the glass vessel and (B) geometric center of the PBR at different co-culture optical densities. Incidental irradiances tested were 400 and 800 µmol/m^2/s.

6.2.7 Objectives and experimental procedure

Towards commercial application of the co-culture for treating wastewater effluents, the co-culture platform performance was evaluated in a benchtop PBR under batch and chemostat (continuous) operation. Specifically, the chemostat experiment was designed to (1) evaluate biogas conversion into microbial biomass with simultaneous nutrient recovery; 2) evaluate the areal biomass productivity at steady state conditions and 3) demonstrate the metabolic coupling and process safety of the co-culture technology at steady-state.

Co-culture was grown in the photobioreactor of 3 L with a working culture volume of 1.7 L. The irradiance at the surface of the glass vessel was provided by the light panel based on the desired light intensity and the temperature was maintained at 37 ℃ using the flexible heater at the bottom of the glass vessel (Figure 28). Initial test of the light system showed that only at high light intensity (800 – 1000 μmol/m^2/s) there was the potential for overheating by the light system without the flexible heater being installed. At the stated light intensities, temperatures in the photobioreactor reached up to 41 ℃ when left overnight due to heat generated by the LEDs. Any overheating due to combination of the light panel and the heating pad was controlled by the BioFlo 110 unit using an internal cooling coil. The agitation as set at 400 rpm to minimize over-agitation and shear stress on the microalgal cells. Biogas was supplied by mixing CH_4 and CO_2 using an in-house gas mixing system and was introduced at the bottom of the photobioreactor at a flowrate of 300 $\frac{sml}{min}$ using a gas sparger. Calibration of the dissolved oxygen (DO) probe was performed by sparging 21% O_2 and 79% He through the initial AD mixture at a flow rate of 300 sml/min after the DO reading on the instrument was stable for at least 10 minutes. The pH set point was 7.0 which was an average of the defined medium pH for *C. sorokiniana* (pH 7.2) and *M. capsulatus* (pH 6.8). Gas sparging of 30% CO_2 into the AD effluent decreases the pH due to the formation of carbonic acid; however, the pH was maintained through automatic addition of 2M sodium hydroxide (NaOH). The exhaust gas temperature was continuously measured by a surface temperature sensor and recorded via the Vernier Logger Lite software. To minimize water vapor in outlet gas and loss of water from the cell broth, the outlet gas was passed through a condenser maintained at ~5 ℃ by circulating water from a cold-water reservoir through the condenser. During chemostat operation, AD effluent was fed to the reactor photobioreactor through Watson-

Marlow 120 peristaltic pumps (Watson-Marlow, USA) and the cell broth was removed in the same manner.

The first condition in this study as performed in fed-batch mode. Gravitationally settled (non-sterilized) AD effluent diluted with clarifier effluent to a final concentration of 150 mg/L NH_3-N was initially introduced into the reactor. The individual co-culture strains were added to obtain individual OD_{750} of 0.2 for *M. capsulatus* and 0.6 for *C. sorokiniana* to achieve a final OD_{750} of 0.8 in 1.7 L of the AD mixture. Synthetic biogas (65% CH_4, 30% CO_2 and 5% He) was continuously sparged at 300 $\frac{sml}{min}$ through the AD effluent during fed-batch operation. Helium was used as an internal tracer to calculate the exhaust gas slow rate as described by Stone et al. (Stone et al., 2019). The photobioreactor was continuously illuminated with an incidental irradiance of 400 $\frac{\mu mol}{m^2 s}$. By starting in batch operation, the co-culture can be accumulated to high cell densities for higher biogas conversion and nutrient recovery. Fed-batch operation was maintained for less than 36 hours to prevent limitation of NH_3-N and PO_4^{3-}-P as well as minimizing depletion of micronutrients to maintain good health of co-culture.

When the limiting nutrient (NH_3-N) concentration reached within 15% of the initial concentration, the photobioreactor operation mode was switched to chemostat or continuous operation where AD effluent was fed into the reactor and cell broth was removed at a constant dilution rate of 0.0157 hr^{-1}. This conservative dilution rate corresponds a hydraulic residence time of 63.7 hours (2.65 days) which would allow high cell density of the co-culture for high-throughput of biogas and nutrient recovery. The irradiance was increased to 1000 $\frac{\mu mol}{m^2 s}$ in order to increase the light penetration and reduce self-shading effects at high cell density. Biogas was still sparged at 300 $\frac{sml}{min}$ and other conditions as temperature and pH were the same as the fed-batch operation. To

maintain low nutrient concentrations in the reactor, the nutrient feed rate into the reactor was controlled by matching the consumption rate of the limiting nutrient (NH_3-N or PO_4^{3-}-P). This will be achieved by varying the nutrient concentration of the AD effluent through dilutions with clarifier effluent while keeping the dilution the same.

Table 6: Summary of photobioreactor experimental conditions.

Parameter	**Condition 1 (batch)**	**Condition 2**
Operation mode	Fed-batch	Chemostat (continuous)
Dilution (hr^{-1})	-	0.0157
HRT (hrs)	-	63.7
Irradiance (µmol/m^2/s)	400	1000
AD NH_3-N concentration (mg/L)	150 mg/L NH_3-N	Varied
Gas composition	65% CH_4, 30% CO_2 and 5% He	
Agitation, temperature, total working volume, Cs: Mc inoculum OD_{750} ratio	400 rpm, 37 °C, 1700 ml, 2:1	

6.3 Results and Discussions

6.3.1 Co-culture biomass production

During fed-batch operation, the co-culture biomass increased linearly with reasonably high growth rates during the fed-batch growth after which the PBR was switched to chemostat operation and steady-state growth was kept for 3 days (Figure 30). The co-culture achieved a total biomass concentration of 1.74 g/L before the photobioreactor was switched to continuous mode as shown in Figure 31. At 56 hours, the co-culture reached and was maintained an average steady state biomass concentration and productivity of 2.17 ± 0.030 g/L and 0.818 ± 0.011 g/L/day at a dilution

rate of 0.157 hr^{-1} (Figure 30). The three days of steady-state are denoted as A, B and C in Figure 31. During steady-state cultivation, *M. capsulatus* was held at 0.898 ± 0.013 g/L with a biomass productivity of 0.338 ± g/L/day while the *C. sorokiniana* maintained a steady-state biomass concentration and productivity of 1.274 ± 0.018 g/L and 0.481 ± 0.008 g/L/day, respectively. The co-culture biomass productivity was 13% higher than co-culture productivities observed in the batch cultures (0.72 g/L/day) that were gas fed every 24 hours (Section 5.7.3) presumably due to constant supply of biogas for growth.

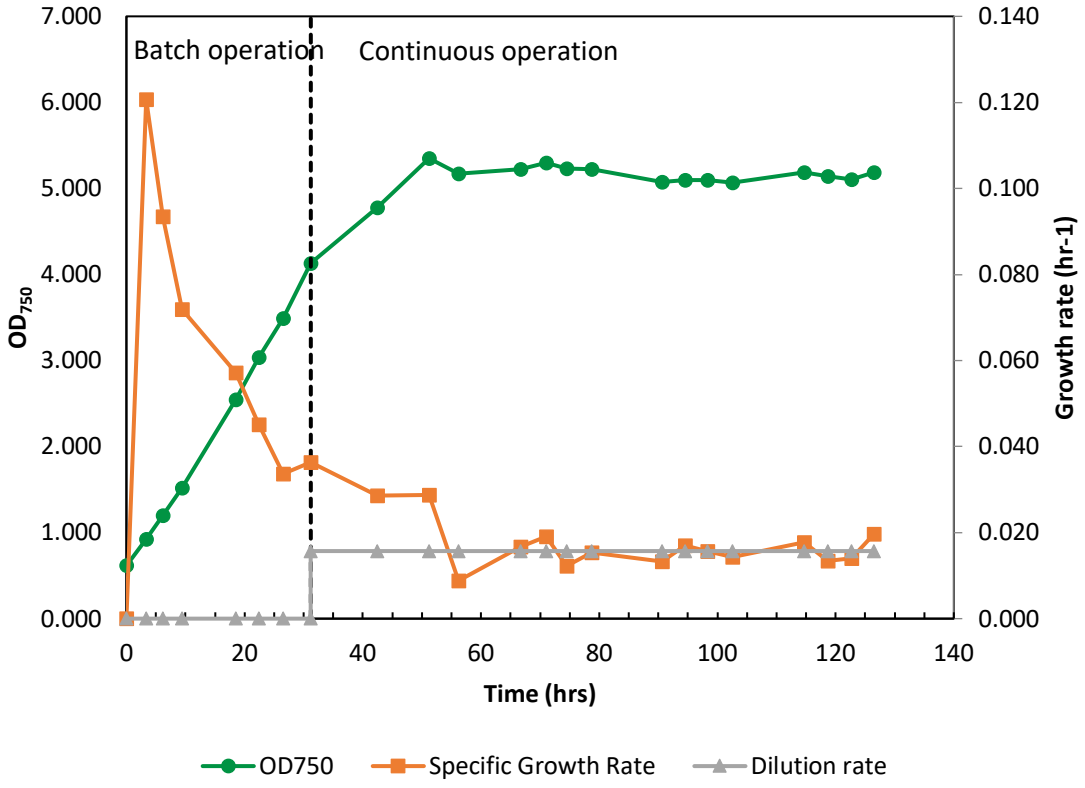


Figure 30: Dynamics of co-culture growth during batch and chemostat operation of the photobioreactor.

C. sorokiniana and *M. capsulatus* individual strain estimation within the co-culture is plotted in Figure 31. During fed-batch growth both the microalga and the methanotroph exhibit linear growth profiles similar to that of the co-culture. *C. sorokiniana* growth is most-likely limited by the transmitted irradiance as the co-culture density increases. As demonstrated in Section 6.2.6, until microalgal cells are near of the surface of the glass vessel the transmitted irradiance will be significantly reduced. *M. capsulatus* cultivation within the co-culture is metabolically coupled with oxygenic photosynthesis for methane oxidation. This would indicate *M. capsulatus* was limited by the rate of photosynthetic O_2 production by *C. sorokiniana*. The dissolved oxygen concentration in the control volume was between 0.12-0.18 µM during both fed-batch and chemostat operation and was considered to be negligible.

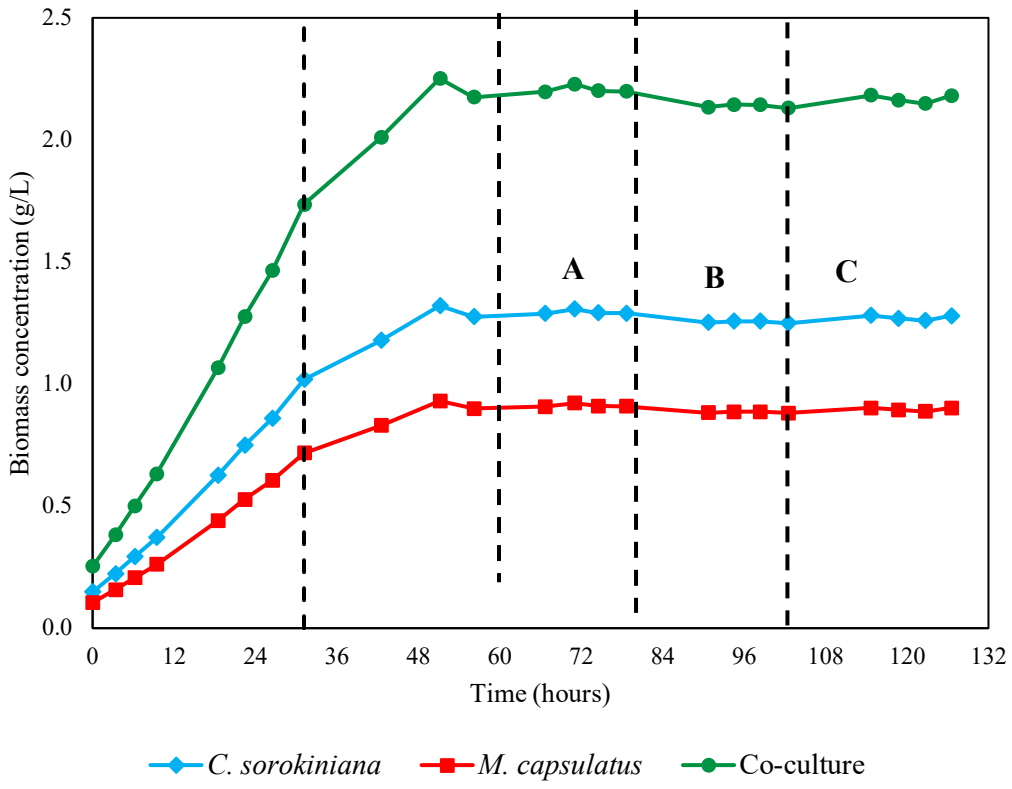


Figure 31: Biomass plots of C. *sorokiniana*, M. *capsulatus* and the co-culture.

Table 7: Steady-state chemostat biomass productivity of *C. sorokiniana*, *M. capsulatus* and the overall co-culture.

Species	**Biomass productivity (g/L/day)**
C. sorokiniana (in co-culture	0.481 ± 0.008
M. capsulatus (in co-culture)	0.339 ± 0.008
Co-culture	0.818 ± 0.011

Next the illuminated areal productivity of the co-culture during this initial bench-scale PBR investigation was estimated. The illuminated areal productivity is an important factor assessing the biomass production on microalgal technology requiring solar/LED light input as the areal productivity will affect operating costs and consequently the minimum biomass selling price. The areal productivity target set by the U.S. Department of Energy (DoE) (Davis et al., 2016) is an annual average of 25 $g/m^2/day$. The biomass productivity of the co-culture reached under chemostat steady-state operation was 22.82 ± 0.32 $g/m^2/day$ (Figure 32). While this relatively high co-culture productivity was lower than the DoE target, the PBR design, irradiance input and operating conditions were not optimized in this study. As indicated above, the microalgal cells were significantly light-limited as the transmitted light was more than 90% reduced towards the center of the reactor. Improvements to microalgal production will result in methanotroph biomass production due to the metabolic coupling through oxygenic photosynthesis. The areal productivity estimated above only considers the light (LED) energy input. However, CH_4 in biogas is also a significant source of energy. As a result, when considering the biomass production as a result of the total energy input, CH_4 as an energy source should be considered.

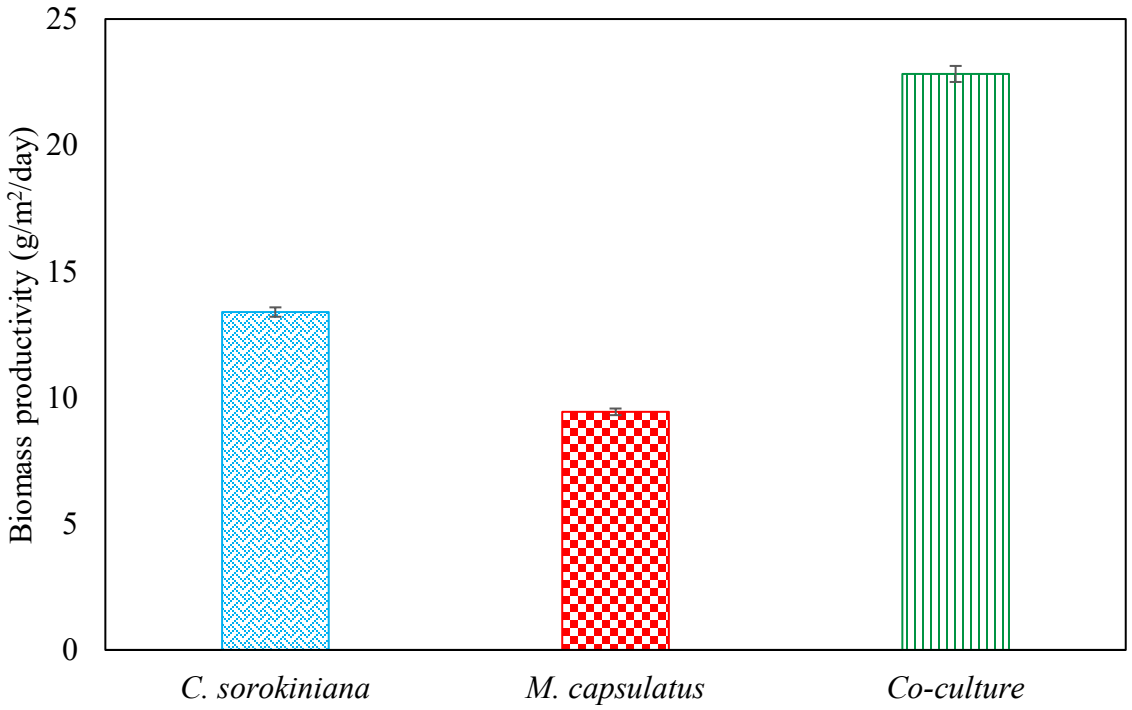


Figure 32: Co-culture illuminated areal productivity of co-culture during chemostat mode.

6.3.2 Biogas conversion into microbial biomass

The minimum specific CO_2 and CH_4 uptake rate by *C. sorokiniana* and *M. capsulatus* were determined to be 0.658 ± 0.016 mmol CO_2/g/h and 0.634 ± 0.015 mmol CH_4/g/h, respectively and was calculated using the yield coefficient of CO_2 and CH_4 conversion into biomass $\left(Y_{\frac{CO_2}{X}}; Y_{\frac{CH_4}{X}}\right)$. This CO_2 uptake rate was similar to a recent co-culture study by Hill et al. although the methane uptake rate in their study was lower; presumably due to the lower O_2 production rate (Hill et al., 2017). In Figure 33 is plotted the residual gas concentrations in the outlet of the PBR under steady-state chemostat operation. The residual CH_4 and CO_2 concentrations were similar to the inlet concentrations resulting in the estimated CH_4 and CO_2 conversion being less than 2%. As shown in Figure 33, there was no residual oxygen in the outlet gas measurements indicating that the net

rate of oxygen production by photosynthesis was equal to the rate of consumption by *M. capsulatus*. The AD mixture also contained the native bacteria which might compete with *M. capsulatus* for oxygen. However, batch culture studies of microalga single culture indicate that the oxygen consumption by AD and clarifier bacteria was not significant. To improve biogas conversion, the operating conditions would have to be optimized for better co-culture growth. Recently, Chui et al. demonstrated 16% CO_2 reduction could be achieved by a microalga culture fed 20% CO_2. This might be due to their smaller PBR diameter (1.8 times) which enabled better light penetration as compared to the larger diameter vessel used in this study.

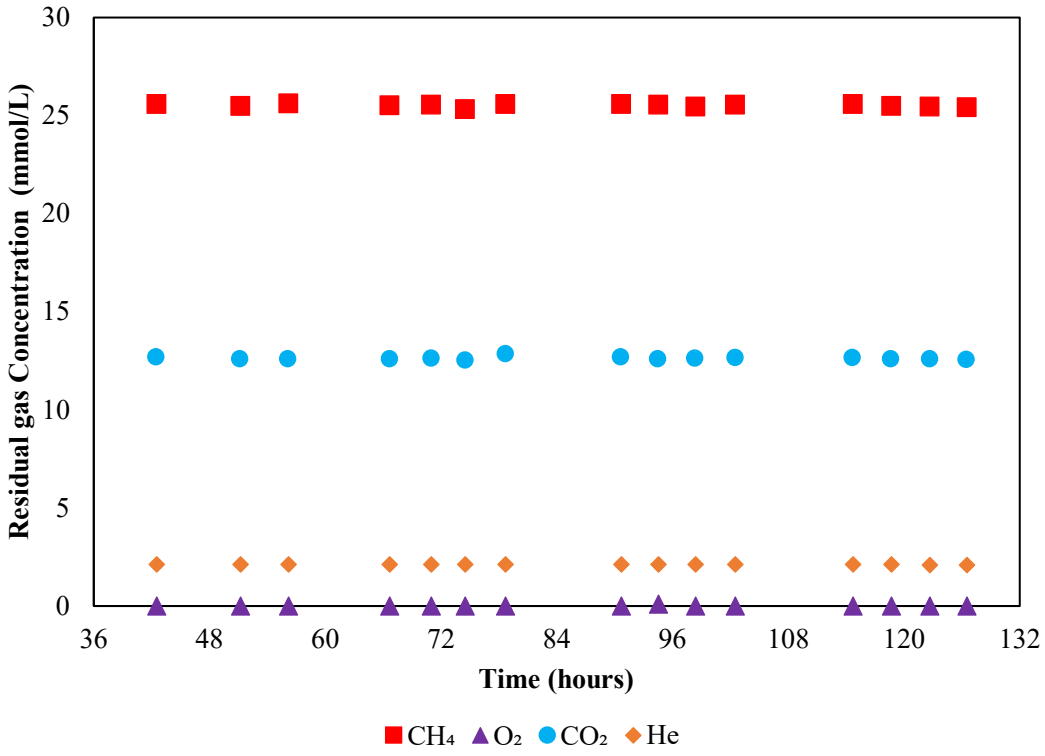


Figure 33: Residual gas profiles of co-culture during chemostat operation.

6.3.3 Nutrient recovery in bench-scale chemostat

For application of the co-culture technology to treat AD effluent, one of the critical parameters to be evaluated is the potential of the co-culture to reduce nutrient pollutants down to permissible discharge levels. In this study, the inorganic nutrient concentrations in the co-culture were monitored and controlled to maintain low nutrient residual concentrations in the bioreactor during chemostat growth in order to maintain good co-culture growth and biogas conversion. In Figure 34, the NH_3-N and PO_4^{3-}-P concentrations throughout the co-culture cultivation period were plotted. During batch cultivation, both NH_3-N and PO_4^{3-}-P concentrations decreased steadily with biomass increase. After switching to chemostat operation, there was a slight accumulation in both NH_3-N and PO_4^{3-}-P due to higher nutrient feed rate than the co-culture nutrient consumption rate. When the nutrient feed rate was matched with the consumption rate the NH_3-N residual concentration in the photobioreactor decreased to a low concentration of 0.078 mg/L of NH_3-N. However, the phosphorus content was only reduced to ~4.8 mg/L PO_4^{3-}-P at 70.9 hours and was maintained throughout the day to 78.6 hours. To ensure the co-culture was not cultivated under an extremely nitrogen-limited condition, the nitrogen feed rate was increased to maintain a residual NH_3-N concentration of ~1.8 mg/L throughout the last day of the chemostat cultivation period. The PO_4^{3-}-P concentration in the photobioreactor was maintained at ~3.2 mg/L during the last day of steady state cultivation. The chemostat outflow nutrient concentration were then compared to the final chlorinated effluent data from South Columbus Water Resource Recovery facility (obtained through personal communication). The final effluent data examined had an NH_3-N concentration ranging between 0.3 and 1.9 mg/L while the orthophosphate concentration ranged between 0.99 and 4.82 mg/L. This suggests that the co-culture technology has the potential to augment current aerobic ponds which are energy intensive. Conversely, while the residual outflow

PO_4^{3-}-P concentration is low, it still does not meet the permissible discharge limit set forth by the Clean Water Act of 1mg/L of phosphorus (USEPA) and Department of Energy target of 0.3 mg/L.

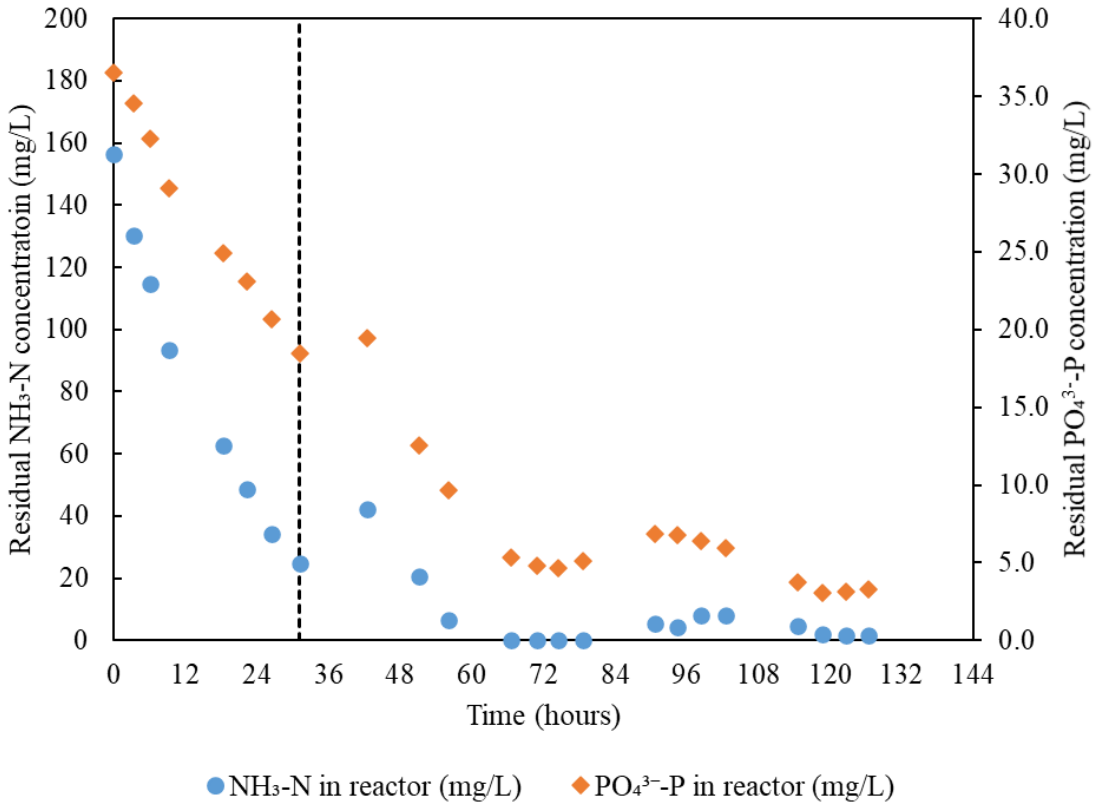


Figure 34: Residual nutrient concentration in photobioreactor.

Before switching from to chemostat operation from fed-batch mode, the co-culture recovered 84% and 50% of NH_3-N and PO_4^{3-}-P, respectively. On average, during the three days of chemostat operation, up to 99%, of NH_3-N was recovered while up to 44% of PO_4^{3-}-P was recovered. The small outflow concentration of PO_4^{3-}-P (3.2 mg/L) would suggest a higher recovery of phosphorus; however, the low estimated PO_4^{3-}-P recovery % is due to the residual amount in the 1.7L of the reactor volume. During fed-batch growth, the averaged NH_3-N recovery rate for NH_3-N was 164.9 mg/day while PO_4^{3-}-P was 25.5 ± 8.5 mg/day. Under

chemostat cultivation, the average NH_3-N recovery rate ranged between 103 and 167 mg/day and PO_4^{3-}-P was recovered up to 40.4 mg/day.

In the vial experiments performed in Chapter 5, the co-culture was able to completely recovery orthophosphate (PO_4^{3-}-P) while there was residual orthophosphate left under chemostat operation. To understand the reason for the residual PO_4^{3-}-P during chemostat operation, the inorganic nutrient (NH_3-N, PO_4^{3-}-P) uptake by the co-culture under the different operation conditions was examined. Complete removal of both N (NH_3-N) and P (PO_4^{3-}-P) requires the municipal wastewater to have a similar N:P ratio as uptake N:P ratio of the co-culture. In the case where the municipal wastewater N:P ratio > optimal N:P ratio for complete N & P recovery by the co-culture, the wastewater is considered to be P-limited. On the other hand, when the wastewater N:P ratio < optimal N:P ratio for the co-culture, the wastewater is N-limited. Figure 35 shows the N:P recovery ratio by the co-culture during fed-batch and chemostat operation. The average ratio was 14:1 for the co-culture during batch operation which was similar to the 16:1 ratio for phytoplankton asserted by Redfield (Geider and La Roche, 2002). This N:P recovery ratio agreed well with vial experiments (Appendix A: Figure 37) indicating that even after 24 hours of inoculation in the bioreactor the cells were still recovering nutrient in a similar manner as the vial precultures. In contrast, the N:P recovery ratio decreased to *ca*. 10:1 when the co-culture was cultivated under chemostat mode over a period of 3 days. This suggests that there was an increase in cellular P uptake relative to N during chemostat growth is based on the biochemical variation of organic macromolecules and accumulation of energy or nutrient reserves (Geider and La Roche, 2002) when photobioreactor operation is switched from fed-batch to chemostat operation. Consequently, operating at different dilution rates in chemostat mode might be an effective

approach for recovering higher amounts of phosphorus. Despite the increased PO_4^{3-}-P recovery under chemostat operation, the co-culture was unable to completely recover all the PO_4^{3-}-P.

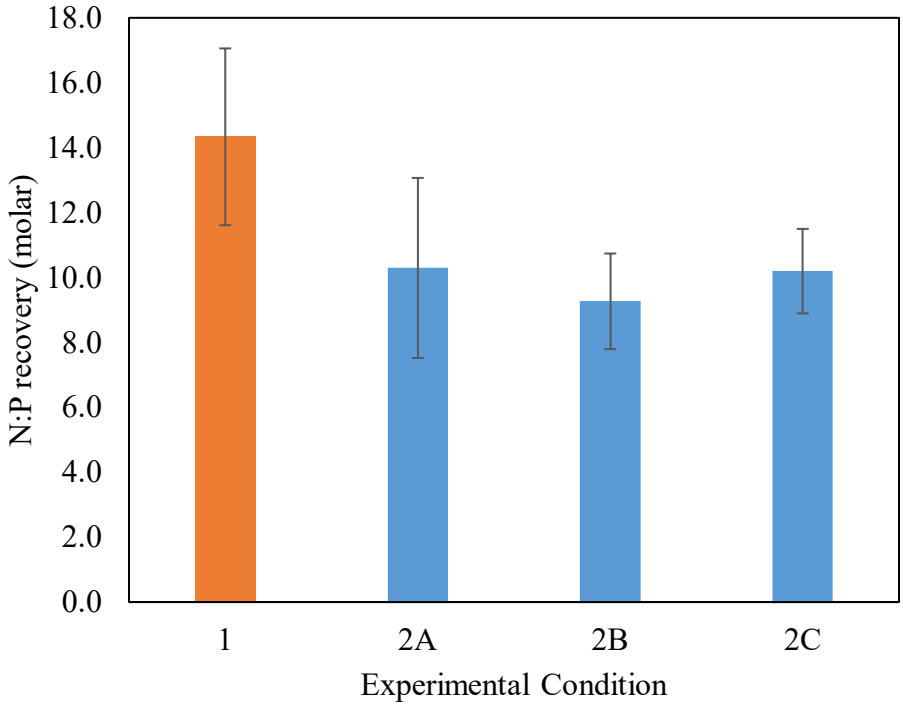


Figure 35: N:P recovered by co-culture during batch and chemostat operation.

To further evaluate the reason for incomplete recovery of the PO_4^{3-}-P, molar N:P uptake of the co-culture was compared to the N:P ratio of the residual N and P in the PBR (Figure 36). The dotted line shows the N:P ratio (molar) of the AD mixtures fed to the PBR. These results indicate that N:P recovery by the co-culture is independent of the N:P ratio of the nutrients in the photobioreactor. Under the P-replete growth, the co-culture N:P recovery ratio was consistently higher than the N:P ratio of the AD mixture in the photobioreactor. Although the initial N:P ratio of the AD mixture was 9.55, the co-culture recovered a higher amount of N relative to P (14:1) under batch operation. Due to more N being recovered, the AD mixture started becoming N

limited, thus decreasing the N:P ratio in the PBR below 5:1. However, or complete and simultaneous recovery of both N and P, the N:P ratio of the AD mixture has to be follow the green arrow (Figure 36).

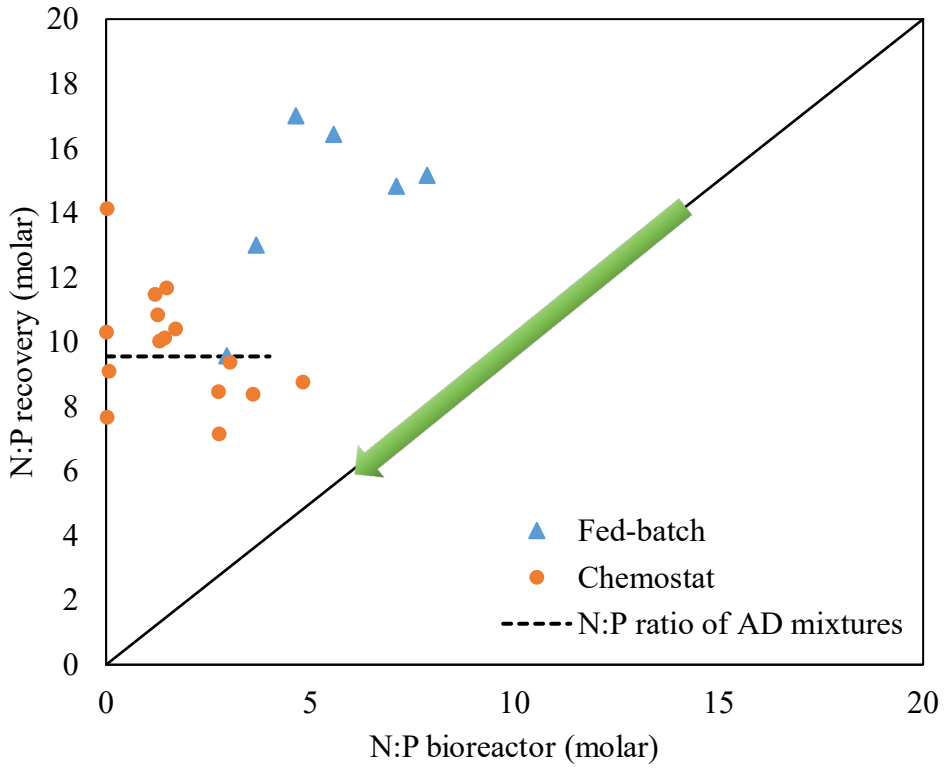


Figure 36: Dynamics of molar N:P recovery (uptake) ratio of the co-culture during batch and chemostat operation as compared to the molar N:P ratio of the residual nutrients in the photobioreactor. For complete recovery of both N and P in the bioreactor both the N:P recovery ratio of the co-culture and the N:P ratio in the bioreactor must follow the green arrow.

The recovery rate of nutrient removal is linked to the growth rate and the nutrient demands. Consequently, a faster growing co-culture may have lower N:P uptake ratio due to higher P demands (Hillebrand et al., 2013). Screening for co-culture pairs that have low N:P uptake ratio as well as higher dilution rates in the chemostat can be investigated for the potential of recovering more P. Other experimental conditions such as the temperature and light intensity can also be

investigated for luxury uptake (Powell et al., 2008) of phosphorus by the co-culture. Lastly, the outflow from the bioreactor can also be recycled back to the PBR for further removal of P.

6.4 Conclusions and future perspectives

The co-culture technology presented in this chapter demonstrated good potential as a sustainable and scalable platform for simultaneous bioconversion of biogas (CH_4 and CO_2) into microbial biomass. Biogas was converted into microbial biomass without an external oxygen supply as methane oxidation was metabolically coupled with oxygenic photosynthesis. Satisfactory illuminated areal productivity of the co-culture was obtained on minimally treated (gravitational settling and dilution) AD effluent which suggests that with optimization of PBR design and process conditions, the co-culture can further developed as a feasible wastewater treatment technology for bioremediation of AD-derived biogas and liquid digestate effluent at municipal WRRFs. While the co-culture was able to achieve complete recovery of NH_3-N during steady state, complete phosphorus recovery was hindered by high N:P uptake ratios during fed-batch growth with resulted in N-limited AD mixtures when switched to chemostat operation.

While promising results of the co-culture technology was obtained in the bench-scale PBR, further investigations modifications should be performed for improving biogas conversion into microbial biomass on wastewater effluents. To improve biomass production enhancing the available light per microalgal cell in the PBR is critical. Using the current PBR, waterproof LEDs can be installed inside the bench-scale photobioreactor. Additionally, a smaller diameter PBR (5-9 cm diameter) or flat-panel reactors can be implemented as they allow a high illumination surface area to volume ratio (Huang et al., 2017). Biofilm reactors have also gained recent interest as a noteworthy bioreactor configuration because they have advantages of smaller areal footprint as compared to suspended bioreactors, better light penetration (Wang et al., 2015) and higher biomass

productivities (Ercan and Demirci, 2015). Biofilm reactors also address the challenges of harvesting biomass in suspended culture systems.

As CO_2 biofixation and CH_4 oxidation are initiated by photosynthesis, biogas recovery can be improved with better light penetration. Another consideration is further optimization of the transmitted irradiance by increasing the light intensity as solar irradiance can be reached up to 2400 μmol/m^2/s during summer months. For commercial application of the technology, hybrid solar/LED- lighting is necessary and the operational cost of artificial illumination would have to be balanced with the operational cost. One process modification to improve biogas conversion is to implement a gas recycle stream (Yun and Park, 1997) as single pass systems are known to be inefficient. The low residence times in the bench scale reactors does not allow sufficient contact time for uptake of gaseous substrates. Furthermore, CH_4 is a sparingly soluble gas and recycling is necessary to increase contact time with methanotroph cells. Sequential bioreactors for microalgal production have demonstrated high CO_2 conversion rates as a result of longer residence times (Jacob-Lopes et al., 2009).

By increasing the biomass productivity, it is expected that there will also be increased nutrient recovery as well. Alternatively, operating conditions such as temperature and growth rate can be investigated for recovering the residual phosphorus content. Although a conservative dilution rate was used in this chemostat bench-scale demonstration, higher dilution rates (i.e. growth rate of co-culture aet steady state) are shown to increase phosphorus uptake (lower N:P ratios) (Hillebrand et al., 2013).

Due to the high inlet gas concentrations of CH_4 and rate of CH_4 oxidation, highly accurate calculation of the CH_4 consumed was difficult even with slight (<0.4%) variation in gas measurements at steady-state conditions. To acquire better CH_4 measurements, the outlet gas can

be diluted 2-3 times with nitrogen gas in a buffer tank to lower the concentration. Alternatively, online gas measurements will also enable more accurate quantification of CH_4 consumption. Quantify the co-culture biochemical composition (protein, carbohydrate, lipids and mineral) content should be evaluated is also necessary to evaluate a suitable product. Initial estimates of the biochemical composition indicate that the co-culture have good protein content (Appendix C).

Chapter 7: Overview of conclusions and Future work

7.1 Summary of work and objectives completed

The biological co-culture technology is a promising technology for upgrading underutilized biogas streams to value-added products. One significant advantage of the platform is fueled by the metabolic coupling of the two strains which allows simultaneous co-utilization of both CH_4 and CO_2. In this study, the integration of the co-culture technology for biogas upgrading and nutrient recovery was assessed in vial (batch) and bench-scale photobioreactors. Specifically, the objectives were to 1) investigate the performance of the co-culture on raw municipal wastewater to assess the potential for commercial application; 2) determine whether the methanotroph-microalga co-culture technology has any advantage over current microalgal-based technology; 3) demonstrate the application of the co-culture for biogas upgrading and nutrient recovery in a photobioreactor system under chemostat cultivation.

To achieve these objectives a mathematical model had to be developed in order to decompose the total biomass production into individual strain concentration as the optical density obtained during experiments represents the change for the overall co-culture biomass. Due to CH_4 being the unique carbon substrate for *M. capsulatus*, the consumption of methane was used for determining the biomass concentration based on the yield ratio of biomass produced per amount of substrate consumed. Then, the microalga biomass concentration can be independently determined through yield coefficients using carbon dioxide consumed or oxygen produced by photosynthesis.

In general, the co-culture demonstrated robust growth on AD digestate and secondary clarifier effluent that was minimally pretreated by gravitational settling, thereby eliminating the use of fresh water for dilution. Additionally, the co-culture cultivation on AD digestate did not

require addition of supplementary minerals as it showed comparable growth on defined medium and no apparent inhibition. Under carbon-limited growth, the *in situ* carbon recycling in the co-culture enables improved biomass production and nutrient recovery as compared to the single cultures; however, when all cultures were supplied with unlimited carbon, the biomass concentrations of the summed single cultures and the co-culture were comparable.

The bench-scale investigations of the co-culture demonstrated the potential of the co-culture as a more economical wastewater treatment technology. Co-utilization of CH_4 and CO_2 was achieved without external oxygen and there was no residual oxygen in the outlet gas stream, underscoring the process safety aspects of the co-culture for biogas utilization as CH_4 and O_2 were not mixed. Microbial conversion of biogas into biomass resulted in good illuminated areal productivity. Furthermore, inorganic nutrient recovery by the co-culture was considered more energy efficient than conventional activated sludge processes as no oxygen had to be supplied for aeration.

7.2 Recommendations for future work and closing statement

Further improvement and fundamental understanding of the co-culture technology performance on raw AD liquid digestate and biogas is necessary. While the strains used in this study demonstrated stable growth under the conditions tested, strain selection can be conducted to find more suitable microalgal and especially a methanotrophic strain that is tolerant to the harsh conditions in wastewater as high organic and ammonia content can inhibit cellular processes of both strains. If the individual strains are robust, the co-culture will have the potential to be used for bioremediation of different types of wastewater—industrial, domestic, agricultural. Use of recently isolated microalgal and methanotroph strains found wastewater effluents may reveal a pair that can tolerate harsh conditions. The co-culture performance should be further evaluated on

minimally diluted AD digestate and raw biogas instead of synthetic biogas should be used for a more comprehensive analysis of the commercial application as VOCs may have an adverse effect on co-culture growth. Furthermore, biogas derived from different AD substrates (sorghum, corn stover, sludge co-digested with FOGs etc.) should be investigated as the impurities in the biogas derived from different feedstock vary (Henard et al., 2018). When considering the performance of the species selected, microalgae and/or methanotroph with a relatively high N and P biomass content may improve nutrient recovery as more N and P will be stored in their biomass. For recovery of organic nitrogen, small fractions of activated sludge or ammonification bacteria can be added to co-culture for improving organic nutrient recovery. At high light intensities in vial experiments, the methanotroph growth was potentially limited by mineral depletion. Wastewater at other locations in the municipal WRRF may contain the necessary mineral and this stream can be mixed with the AD effluent. Additionally, at high light intensities investigations can be performed with a reduced microalga: methanotroph ratio to increase the abundance of the methanotroph and limit the residual oxygen in the headspace of the co-cultures.

On the process side, two essential considerations are maximizing the use of natural light for the co-culture to reduce electricity cost and increasing the liquid contact time with methane for improving CH_4 uptake. Due to diurnal cycles, a mixture of natural light and artificial light can be used to maintain high productivities and efficiencies depending on the process requirements. A photobioreactor design that maximize the harvestable light by microalgae has to be implemented; especially since light conversion efficiency for microalgae is generally less than 10%. Energy saving light sources must also be implemented. Secondly, as mass transfer of methane of methane will be a challenge at commercial application, a reactor design that improves methane-liquid contact time for reduction of mass transfer limitations or use of a novel methanotroph with a high

affinity for methane will increase conversion of biogas should be considered. One option for limiting mass transfer limitations as well as high cost of harvesting suspended cultures can be decreased by utilization of a biofilm reactor for growing the co-culture; however, further research must be conducted to understand attachment of the co-culture to the substratum, nutrient recovery rates, and biogas conversion rates and energy costs. The complete biomass composition of the co-culture should be analyzed to determine a suitable energy product for the co-culture biomass. Lastly, to validate the claims of reduced operating costs due to the advantages provided by the co-culture a techno-economic analysis of the process is crucial.

: N:P uptake ratio under batch cultivation in vial experiments

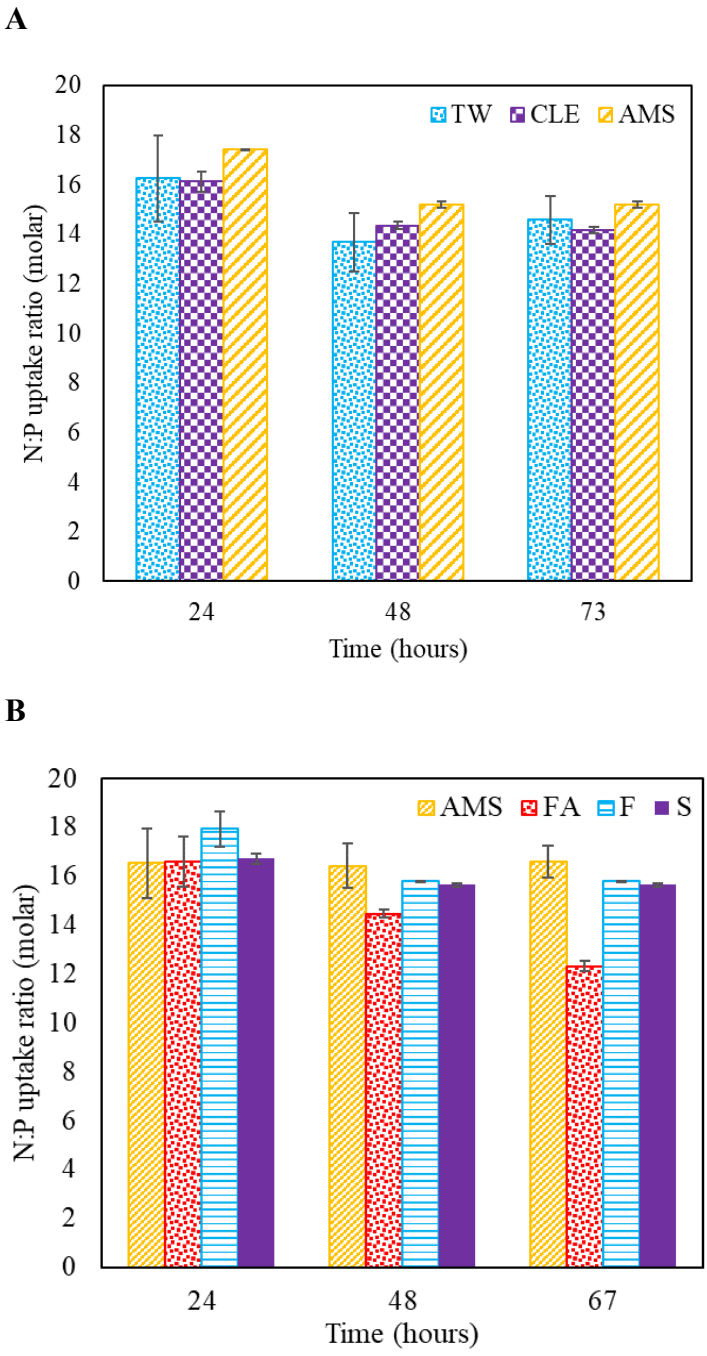


Figure 37: Nutrient uptake ratio of co-culture in vial experiments of (A) differently AD mixtures and (B) differently pretreated AD effluent diluted with CLE.

▌: Additional plots for Case 3

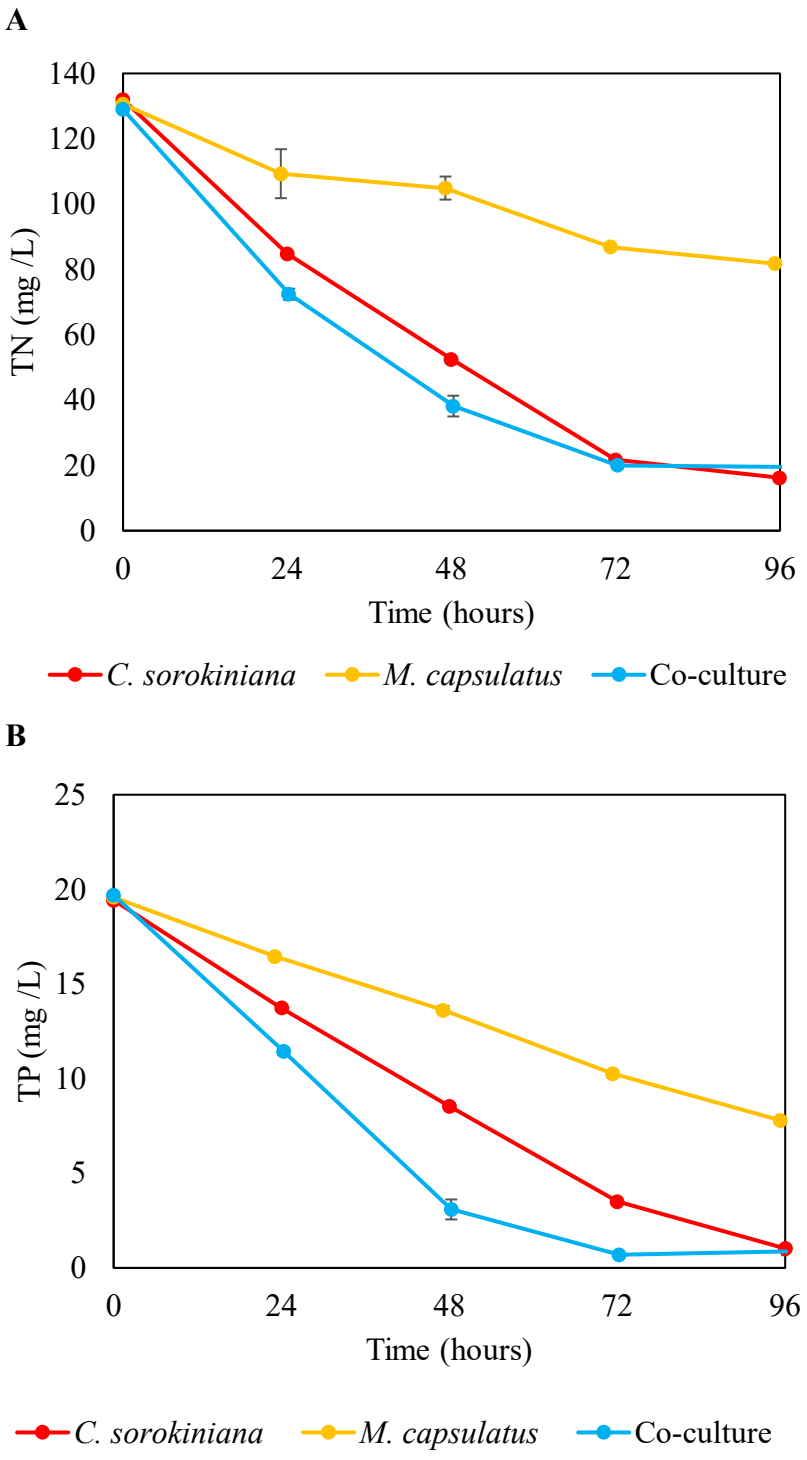


Figure 38: Case 3: TN (A) and TP (B) plots for *C. sorokiniana*, *M. capsulatus* and the co-culture.

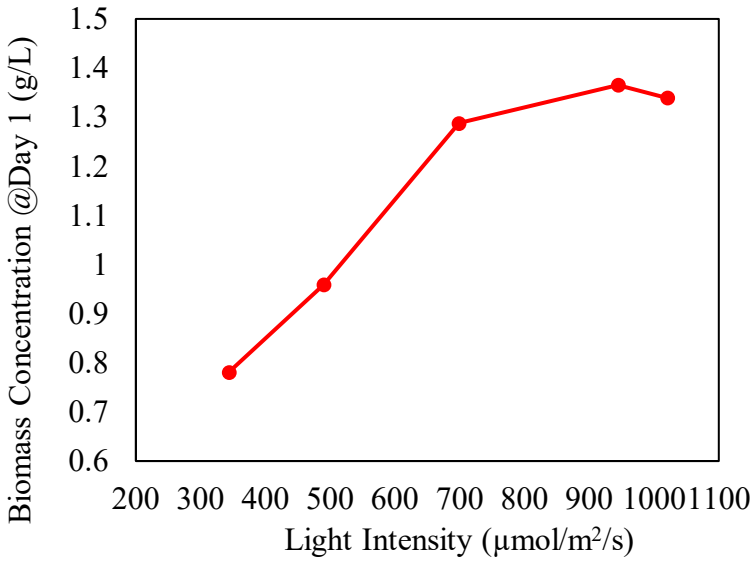


Figure 39: Biomass concentration of *C. sorokiniana* on day 1 when grown under different light intensities.

: Initial evaluation of co-culture protein and carbohydrate content

To determine a suitable product for the microbial biomass produced at the end of wastewater treatment, the biomass biochemical composition needs to be evaluated. Under nutrient replete growth, the individual strain biomass is expected to maintain the relative fraction of their biochemical molecules (proteins, carbohydrates, lipids) until a macro- or micronutrient is either limiting or depleted. Changes in the biomass composition can also be cause by other stressor such as pH and temp. However, the co-culture biomass can show differences when cultured together. In first attempts at determining the biomass composition and validating the protocol, a static mixture of the methanotroph and microalga were performed, and the carbohydrate and protein content analyzed.

The carbohydrate content was analyzed according to Wychene and Laurens (Wychen and Laurens, 2013) while the protein content was evaluated according to (Higgins et al., 2015). Table 7 shows an initial comparison of the protein and carbohydrate content between the methanotroph and microalga single cultures and a statically mixed co-culture. Briefly all cultures were washed twice with DI water to remove any residual dissolved nitrogen sources. Then 1.5 ml of *C. sorokiniana* and *M. capsulatus* cell suspensions at predetermined concentrations were oven dried. A statically mixed co-culture was produced by mixing *C. sorokiniana* and *M. capsulatus* cells to obtain the same final concentration as the individual strains in the final volume and the same volume of cells as the single cultures were oven dried at the same time. As indicated in Table 7, the microalga has a good protein content ranging between 32% and 40% for different trials and carbohydrate content being 9.8% but were slightly lower than that observed in literature (Rasouli et al., 2018). On the other hand, the methanotrophs have a much higher protein content of 67% and lower carbohydrate content of ~6% which is similar to the UniBio process (72% protein and

4% carbohydrates) utilizing the same methanotroph for SCP (UniBio, 2016). The remaining balance of the biomass compositional analysis may comprise of lipids and ash content; neither of these two components were measured.

Table 8: Total protein and total carbohydrate content comparison between the single cultures and the co-culture.

Sample	**Protein content (%w/w)**	**Carbohydrate content (%w/w)**
Chlorella sorokiniana	32.07 – 40.02	9.80
Methylococcus capsulatus	67.07	6.02
Co-culture	48.57	10.07

: Individual co-culture species estimation and biomass development in photobioreactor

Individual species estimation:

For the methanotroph:

$$\mathrm{OD_m} = \alpha_m + \beta_m X_m \tag{19}$$

For the microalga:

$$\mathrm{OD}_{alg} = \alpha_{alg} + \beta_{alg} X_{alg} \tag{20}$$

Total co-culture:

$$\mathrm{OD_{total}} = OD_a + OD_m \tag{21}$$

Assuming stable strain ratio (based on mass) of *C. sorokiniana*:*M. capsulatus*

$$\mathrm{X}_{alg} = \kappa\, \mathrm{X_m} \tag{22}$$

$$\mathrm{OD}_{total\ (measured)} = \alpha_m + \beta_m X_m + \alpha_{alg} + \beta_{alg} \kappa X_m \tag{23}$$

Notation	Definition	Value
m	Methanotroph	-
alg	Microalga	-
X_m	Biomass concentration of methanotroph (g/L)	-
X_{alg}	Biomass concentration of microalga (g/L)	-
α_m	Constant	0.00898
α_{alg}	Constant	0.00831
β_m	Methanotroph coefficient	1.796
β_{alga}	Microalga coefficient	2.77
κ	Stable ratio constant	1.42

Biomass development equations

Co-culture specific growth rate

$$\frac{dX}{dt} = \left(\mu - \frac{F}{V}\right) X$$

$$\frac{dX}{dt} = (\mu - D)X$$

Integrate:

$$\int_{X_1}^{X_2} \frac{1}{X}\, dX = (\mu - D) \int_{t_1}^{t_2} dt$$

$$\mu = \frac{\ln\left(\frac{X_2}{X_1}\right)}{t_2 - t_1} + D$$

Illuminated biomass productivity

$$P_i = \frac{24XDV}{A_i}$$

Notation:	**Description**
X_2	Biomass concentration at t_2 (g/L)
X_1	Biomass concentration at t_1 (g/L)
μ	Specific growth rate (hr^{-1})
F	Wastewater flow rate into the reactor (L/hr)
V	Liquid volume in photobioreactor (L)
D	Dilution rate (hr^{-1})
P_i	Illuminated biomass productivity ($g/m^2/day$)
A_i	Illuminated area of the photobioreactor(m^2)

: Co-culture nutrient recovery in the PBR

Percent nutrient recovery (R) during chemostat operation

The fraction of NH_3-N or PO_4^{3-}P recovered is calculated by

$$R(\%) = \frac{Z_{cons}}{Z_{in} + Z_{rxtor,\,t_1}} .100\%$$

The percent nutrient recovery is calculated by the change of nutrient between two sampling points (t_2 and t_1)

Overall reactor mass balance:

$$\Delta Z_{accum} = \Delta Z_{in} - \Delta Z_{out} + \Delta Z_{prod} - \Delta Z_{cons}$$

For NH_3-N and PO_4^{3-}P, the mass balance is

$$\Delta Z_{cons} = \Delta Z_{in} - \Delta Z_{out} - \Delta Z_{accum}$$

$$\Delta Z_{in} = F_{in} C_{in} (t_2 - t_1)$$

$$\Delta Z_{out} = F_{out} \left[\left(\frac{C_2 + C_1}{2} \right) (t_2 - t_1) \right]_{out}$$

$$\Delta Z_{accum} = C_2 V_2 - C_1 V_1$$

Combing all equations:

$$\Delta Z_{cons} = F_{in} C_{in} (t_2 - t_1) + F_{out} \left[\left(\frac{C_2 + C_1}{2} \right) (t_2 - t_1) \right]_{out} + C_2 V_2 - C_1 V_1$$

The N:P ratio is calculated by:

$$N{:}P\ recovery\ (molar) = \frac{\frac{N_{cons}}{MW_N}}{\frac{P_{cons}}{MW_P}} = \frac{\frac{N_{cons}}{14.0067}}{\frac{P_{cons}}{30.9737}}$$

$$N{:}P\ bioreactor\ (molar) = \frac{\frac{C_{N,\ rxtor}}{MW_N}}{\frac{C_{P,\ rxtor}}{MW_P}} = \frac{\frac{C_{N,\ rxtor}}{14.0067}}{\frac{C_{P,\ rxtor}}{30.9737}}$$

Notation:	**Description**
Z	Mass of wastewater nutrient in PBR (NH_3-N or PO_4^{3-}-P)
C	Mass concentration of wastewater nutrient in PBR (NH_3-N or PO_4^{3-}-P)
N_{cons}	Mass of NH_3-N (mg/L)
P_{cons}	Mass of PO_4^{3-}-P (mg/L)
MW_N	Molecular weight of nitrogen
MW_P	Molecular weight of phosphorus

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