METHANE PRODUCTION FROM ANAEROBIC CO-DIGESTION OF

CHLORELLA VULGARIS AND WASTEWATER SLUDGE

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DEDICATION

This writing is dedicated to the betterment of science and the quest for sustainable energy generation. May future generations be inspired to continue protecting public and environmental health.

I also dedicate this writing to my parents, Donna and Richard Williams. This research would not have been possible without them.
ABSTRACT OF THE THESIS

Methane Production from Anaerobic Co-Digestion of *Chlorella vulgaris* and Wastewater Sludge

by

Carissa Lee Williams

Master of Science in Civil Engineering with a Concentration in Environmental Engineering
San Diego State University, 2012

Increasing demand for energy coupled with concerns over limited fossil fuel reserves and apprehensions over their contributions to greenhouse gas emissions have made the search for low carbon energy sources a high priority. Algal biomass could serve as an alternative source of renewable biofuels. Research efforts to date have primarily focused on the production of algal biofuels through lipid extraction, which involves high temperature and high pressure, resulting in an energy intensive process. In this research, the use of algal biomass as a supplementary feedstock to anaerobic digesters for the production of methane gas is evaluated. To test the potential of algal biomass as a supplementary feedstock, lab-scale anaerobic digesters are set-up. The methane gas production of various combinations of thickened waste activated sludge (TWAS) and algal biomass is investigated.

*Chlorella vulgaris* (*C. vulgaris*) is used as representative microalgae. In addition, the effects of operational parameters, such as biomass loading, temperature and alkalinity, on biogas production are investigated.

The results show that the biogas production for all biomass loading combinations of *C. vulgaris* and TWAS ranged from 0.47-0.57 mL per mg volatile solids (VS) digested. On average, VS and chemical oxygen demand (COD) were reduced 48 and 38%, respectively, at 35°C. Average total coliform (TC) and fecal coliform (FC) concentrations of 6.3x10⁴ and 1.0x10⁴ CFU per gram of total solids (TS), respectively, were measured in the digested waste at 35°C. Thus, the residual meets the USEPA requirements for pathogen reduction (FC < 2x10⁶ CFU per g TS) and vector attraction reduction (> 38% reduction in VS) for land application. The total nitrogen and phosphorus content of the residual was determined to be in the range of 9-17% as N and 3-7% as P (7-16% as P₂O₅), respectively, revealing its potential value as a fertilizer. It was also observed that decreased digestion temperatures resulted in lower biogas yields, while initial alkalinity in digesters did not appear to affect biogas production.

From the results of the research, it can be inferred that algae can be co-digested with wastewater sludge, or by itself, to produce methane gas at wastewater treatment plants (WWTPs). This suggests that algae can be utilized as an energy source through anaerobic co-digestion with wastewater sludge. This is significant because algae can be grown with the nutrient and CO₂ contained in waste streams at WWTPS, thereby minimizing the release of nutrients and effluent water to the environment. This reduced nutrient load results in treatment cost savings, while the reduction in effluent discharge decreases environmental pollution.
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CHAPTER 1

INTRODUCTION

Increasing demand for energy coupled with concerns over limited fossil fuel reserves and apprehensions over their contributions to greenhouse gas emissions have made the search for low carbon energy sources a high priority. Biomass (e.g. from algae) could be a key resource for future power supplies. Unlike other renewable sources, it does not suffer from intermittency of supply and it is carbon-neutral. Consequently, an appreciable number of research efforts have been directed towards algal biomass as a potential source of renewable biofuel. These efforts have primarily focused on the production of biofuels through lipid extraction (Amin 2009), which involves high temperature and high pressure, resulting in an energy intensive process. This research explores the potential of algal methane gas via anaerobic digestion (AD) as a carbon-neutral energy source. In particular, the research investigates methane production from the anaerobic co-digestion of the microalgae, \textit{Chlorella vulgaris} (C. vulgaris), with wastewater sludge.

1.1 ANAEROBIC DIGESTION AND ENERGY PRODUCTION

AD is a naturally occurring biological process in which large numbers of anaerobic bacteria convert organic matter into methane (CH$_4$) and carbon dioxide (CO$_2$), a mixture called biogas, in the absence of oxygen (U.S.EPA 2006). It is a widely used biological process for treating wastewater sludge. Municipal wastewater treatment plants (WWTPs) generate sludge as a by-product of the physical, chemical and biological processes used in the treatment of wastewater. In order to dispose of this sludge, the harmful pathogens must be stabilized and the volume of sludge should be reduced.

AD has been used for the purpose of wastewater sludge stabilization for over a century (Ahring 2002). This process stabilizes the organic matter in wastewater sludge, reduces pathogens and odors, and reduces the total solids (TS) by converting part of the volatile solids (VS) fraction to biogas (U.S.EPA 2006). The result of AD is a product that contains stabilized solids, as well as some available forms of nutrients such as ammonia-
nitrogen (U.S.EPA 2006), and is commonly used as fertilizer or soil amendment (Ahring 2002; Vergara-Fernández et al. 2008). Although many sludge stabilization methods exist, AD is unique for its ability to produce biogas, mostly methane, which can be used to generate energy in the forms of electricity, vehicle fuel, cooking fuel, and heat. The energy is renewable and carbon-neutral.

The process of AD can be divided into three separate steps: hydrolysis, fermentation and methanogenesis (U.S.EPA 2006). In hydrolysis, the complex compounds are broken down into soluble sugars (Singh and Olsen 2011). Then, fermentative bacteria convert these into alcohols, acetic acid, volatile fatty acids (VFAs), and a gas containing H₂ and CO₂, which is metabolized during methanogenesis into primarily CH₄ (60–70%) and CO₂ (30–40%) by methanogenic bacteria (Cantrell et al. 2008). The efficiency of each step is influenced by the temperature and the amount of time the process is allowed to react (U.S.EPA 2006). Alkalinity also affects process efficiency by providing a buffering capacity for change in pH.

Methane is converted to electricity by burning it as a fuel in a gas turbine or steam boiler. Compared to other hydrocarbon fuels, burning methane produces less carbon dioxide for each unit of heat released (Oilgae 2012). At about 891 kilojoules per mole, methane's combustion heat is lower than any other hydrocarbon; however, as the simplest hydrocarbon, it produces more heat per unit mass than the more complex hydrocarbons (Oilgae 2012). In many cities, methane is piped into homes for domestic heating and cooking purposes. In this context it is usually known as natural gas, and is considered to have an energy content of 1,000 BTU per standard cubic foot (Oilgae 2012). Methane in the form of compressed natural gas is used as a vehicle fuel, and produces less harmful air emissions than fossil fuels such as gasoline and diesel.

There are multiple wastewater treatment facilities through the U.S., but many do not have anaerobic digesters. For example, the State of California has 311 sewage wastewater treatment facilities (CEC 2005), 137 with anaerobic digesters (EBMUD 2008), as well as 2,300 dairy operations and 3,000 food processing establishments (CEC 2005). Currently, about 50% of sewage sludge, 2% of dairy manure, and less than 1% of food processing wastes/wastewater generated in the state are used to produce biogas (CEC 2005). Converting these wastes into energy can help operating facilities offset the purchase of electricity and provide environmental benefits by reducing air and groundwater pollutants.
While wastewater sludge is the common substrate for anaerobic digestion, various other substrates as well as co-substrates have been explored (Rui et al. 2009; Samson and Leduy 1982; Saxena, Tandon, and Singh 1984; Sialve, Bernet, and Bernard 2009; Hansson 1983; Labatut, Angenent, and Scott 2011; Gunaseelan 1997; Owens and Chynoweth 1993; Shanmugam and Horan 2009). These include landfill waste (Owens and Chynoweth 1993; Shanmugam and Horan 2009; Gunaseelan 1997; Mata-Alvarez et al. 1990; Mata-Alvarez, Macé and Llabrés 2000) manure (Cantrell et al. 2008; R. Chen et al. 2012; Gonzalez-Fernandez, Molinuevo-Salces, and García-Gonzalez 2011; Labatut, Angenent, and Scott 2011; Nielsen and Heiske 2011; Rao et al. 2010; Vardon et al. 2011; L. Wang et al. 2010), and fats, oils and grease (FOG) (Park and Li 2012), among others. Over the past few years, an increasing number of research efforts have been directed towards microalgae as a potential source of renewable fuel to sustainably replace fossil-based fuels. However, little corresponding work has been done on the use of algae as a supplementary feed to wastewater anaerobic digesters to produce methane gas.

1.2 Algae as Energy Source

Algae are photosynthetic organisms that convert sunlight, water, nutrients and CO₂ to biomass (Chisti 2008), which is comprised of proteins, carbohydrates, and lipids in varying amounts depending on the species and growing conditions (Sialve, Bernet, and Bernard 2009). The high lipid content makes algae an excellent source of renewable biofuel compared to other energy crops, such as soybean and palm oil, which produce biofuel in amounts less than 5% of their total biomass (Chisti 2008). Algae are the highest yielding feedstock for biodiesel (Hossain 2008). On average, algae produce about 15,000 gallons per acre per year, while the best energy crop, palm oil, yields 635 gallons per acre per year (Cooke and Fang 2008). Table 1.1, adapted from Chisti (Chisti 2007), shows the land area needed to produce enough oil to cover 50% of the U.S. transportation fuel needs from microalgae compared to other biofuel crops. It shows that U.S. crop area would need to be increased 846% to generate the requisite oil from corn; while, on the other hand, microalgae that is 30% oil by weight would only require 2.5% of the current crop area to produce the same amount of oil. Not only is this important for biodiesel production for transportation, but high lipid content also translates into greater methane production during anaerobic digestion. Theoretically,
Table 1.1. Comparison of Sources of Biodiesel

<table>
<thead>
<tr>
<th>Crop</th>
<th>Oil yield (L/ha)</th>
<th>Land area needed (M ha)a</th>
<th>Percent of existing US cropping area that would be required to meet 50% of U.S. transportation fuel needs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>172</td>
<td>1540</td>
<td>846</td>
</tr>
<tr>
<td>Soybean</td>
<td>446</td>
<td>594</td>
<td>326</td>
</tr>
<tr>
<td>Canola</td>
<td>1190</td>
<td>223</td>
<td>122</td>
</tr>
<tr>
<td>Jatropha</td>
<td>1892</td>
<td>140</td>
<td>77</td>
</tr>
<tr>
<td>Coconut</td>
<td>2689</td>
<td>99</td>
<td>54</td>
</tr>
<tr>
<td>Oil palm</td>
<td>5950</td>
<td>45</td>
<td>24</td>
</tr>
<tr>
<td>Microalgaeb</td>
<td>136,900</td>
<td>2</td>
<td>1.1</td>
</tr>
<tr>
<td>Microalgaec</td>
<td>58,700</td>
<td>4.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

a For meeting 50% of all transport fuel needs of the United States
b 70% oil (by wt) in biomass
c 30% oil (by wt) in biomass


Methane can be produced from any of the three constituents of algae – carbohydrates, proteins and lipids (Oilgae 2012), but lipid content has the greatest influence on the amount of methane produced, followed by protein content and then carbohydrates (Sialve, Bernet, and Bernard 2009).

Although there are marked differences in the composition of the micro-algal classes and species, protein is always the major organic constituent, followed usually by lipid and then by carbohydrate (Lavens and Sorgeloos 1996). Expressed as percentage of dry weight, the range for the levels of proteins, lipids, and carbohydrates are 12-35%, 7.2-23%, and 4.6-23%, respectively (Lavens and Sorgeloos 1996). C. vulgaris is comprised of 14-22% lipids, 51-58% proteins, and 12-17% carbohydrates by dry weight (Becker 2004) according to Becker, while Chisti (Chisti 2007) reports the lipid content of Chlorella sp. as 28-32% by dry weight and Devgoswami et al. (2011) found Chlorella sp. to contain 31% lipids by dry weight when grown under optimum conditions. Bhola (2010) found that lipid content reached up to 40% (dry weight) under stressed conditions. Several studies show that the calorific value of C. vulgaris biomass can be increased by growing them under low nitrogen conditions (Illman, Scragg, and Shales 2000; Scragg et al. 2002; Hu et al. 2008; Sheng and Azevedo 2005; B. Wang et al. 2008).
C. vulgaris, the subject microalgae of this research, is a very small (5 micron) microalgae that has been widely studied (see Figure 1.1). It is popular commercially for its medicinal value as well as for being a very effective supplement for animal feed. It grows quickly and requires little downstream processing to produce human food, animal feed, fertilizer, or fuel.

![Figure 1.1. C. vulgaris in beaker (left). Cells magnified 1000x (right).](image)

Growing algae requires carbon dioxide and nutrients; and therefore, the process of algal methane gas production can also be used for fixing carbon dioxide released to the atmosphere and capturing nutrients released into the environment with wastewater effluent. Algae is a very promising source of biomass in this context as it can sequester a significant quantity of carbon from atmosphere and industrial gases and is also very efficient in utilizing the nutrients from industrial effluents and municipal wastewater (Singh and Olsen 2011). Equation 1.1 shows the growth equation specific to C. vulgaris. The elemental composition of C. vulgaris is based on values obtained by Oh-Hama and Miyachi (1988).

\[
107CO_2 + 10NH_3 + H_3PO_4 + 85.5H_2O + \text{Light Energy} \rightarrow C_{107}H_{174}O_{26}N_{10}P + 131.25O_2 \quad (1.1)
\]

C. vulgaris was chosen as the subject microalgae due to the fact that it is a well-researched, well-known microalga that grows easily and naturally at some WWTPs and has a theoretically high methane yield. Sialve, Bernet, and Bernard (2009) give a methane yield range of 0.63-0.79 L CH₄ per g VS digested based on the lipid, protein, and carbohydrate percentages. Besides carbon, nitrogen, and phosphorus which are major components in microalgae composition (Singh and Olsen 2011), oligo nutrients such as iron, cobalt and zinc are also found (Grobbelaar 2004) and are known to stimulate methanogenesis (Speece 1996).
1.3 Anaerobic Digestion of Algal Biomass

The idea of producing fuel from algae is not a novel concept, with the DOE’s Aquatic Species Program performing pioneering research on algal biochemistry and mass production methods from 1978 to 1996 (Sheehan et al. 1998). Ultimately the program was discontinued in 1996 because of the low price for oil at the time. As oil prices have increased, sustainable energy alternatives, including algal biomass-based fuels, once again have become popular research and commercial endeavors (Amer, Adhikari, and Pellegrino 2011). Microalgae have the ability to mitigate CO₂ emissions and produce oil with a high productivity, thereby having the potential for applications in producing the third-generation of biofuels (C. Y. Chen et al. 2011). Currently there are scores of private companies actively attempting to commercialize fuel derived from microalgae – none are fully operational at this point (Amer, Adhikari, and Pellegrino 2011).

The biogas production potential of some species of algae has been studied and shown (Rui et al. 2009; Saxena, Tandon, and Singh 1984; Sialve, Bernet, and Bernard 2009; Samson and Leduy 1982), but the current research on algae as a supplement to wastewater digestion is lacking. Other substrates, such as landfill waste (Owens and Chynoweth 1993; Shanmugam and Horan 2009; Gunaseelan 1997; Mata-Alvarez et al. 1990; Mata-Alvarez, Macé and Llabrés 2000; Hansen et al. 2004; Yen and Brune 2007), manure (Cantrell et al. 2008; R. Chen et al. 2012; Gonzalez-Fernandez, Molinuevo-Salces, and Garcia-Gonzalez 2011; Labatut, Angenent, and Scott 2011; Nielsen and Heiske 2011; Rao et al. 2010; Vardon et al. 2011; L. Wang et al. 2010), and other vegetation have also been studied (Saxena, Tandon, and Singh 1984) in various combinations, with and without algae. Several studies have shown efficient digestion of microalgae under hydraulic retention times between 10 (Yen and Brune 2007) and 30 days (Samson and Leduy 1982; Golueke, Oswald, and Gotaas 1957), with methane yields ranging between 0.1 and 0.4 L per g VS, respectively.

The use of AD to produce methane gas from algae began several decades ago, with Golueke, Oswald, and Gotaas (1957) being the first to explore the topic. They reported that anaerobic digestion of Scenedesmus spp. and Chlorella spp. generated 0.17 to 0.23 L of methane gas per g of VS introduced, at 35° and 50°C respectively (Golueke, Oswald, and Gotaas 1957). While methane yield from the algal biomass was 40% less than the yield from wastewater sludge at 35°C, the yields based on VS digested were comparable. The maximum
VS destruction was about 45% for the algae, compared to 60% for the wastewater sludge. They suggested that the relatively low digestability and thus yield of microalgal biomass was the result of cell walls resisting bacterial degradation, but being more readily digested by bacteria at the higher temperature (Abdel-Raouf, Al-Homaidan and Ibraheem 2012).

Algae typically yield less methane than wastewater sludge in the current research (Abdel-Raouf, Al-Homaidan and Ibraheem 2012; Salerno, Nurdogan and Lundquist 2009). Woertz et al. (2010) concluded that methane yield is inversely proportional to percent algae after digesting various proportions of algae with wastewater sludge at an organic loading rate of 2 g per VS-day for 20 days at 37.5°C. Ammonia toxicity and recalcitrant cell walls are commonly cited causes of the lower yields from AD of algae (Abdel-Raouf, Al-Homaidan and Ibraheem 2012). Ammonia toxicity might be counteracted by co-digesting algae with high carbon organic wastes. Carbon-rich feedstocks include primary and secondary municipal sludge, sorted municipal organic solid waste, FOGs, food industry waste, waste paper, and various agricultural residues (Abdel-Raouf, Al-Homaidan and Ibraheem 2012). Acclimation of the digester microbial community to algae digestion may also improve the yield (Abdel-Raouf, Al-Homaidan and Ibraheem 2012).

Park and Li (2012) digested algae biomass residue with lipid-rich FOG to evaluate the effect on methane yield and macronutrient degradation. Co-digestion of algal biomass residue and FOG, each at 50% of the organic loading, allowed for an increased loading rate up to 3 g VS per L-day, resulting in a specific methane yield of 0.54 liters CH₄ per g VS-day and a volumetric reactor productivity of 1.62 L CH₄ per L-day (Park and Li 2012). Lipids were the key contributor to methane yields, accounting for 68–83% of the total methane potential (Park and Li 2012). Co-digestion with algae biomass residue fractions of 33%, 50%, and 67% all maintained lipid degradations of at least 60% when the organic loading rate (OLR) was increased to 3 g VS per L d (Park and Li 2012). During co-digestion, the degradation of carbohydrates and proteins did not change significantly with increasing OLR (Park and Li 2012). Lipid degradation during co-digestion was greater than that in the digestion of 100% FOG or 100% algae biomass residue at increased OLRs, suggesting a synergistic effect (Park and Li 2012). Co-digestion of certain substrates can produce synergistic or antagonistic effects. Synergistic effects may arise from the contribution of additional alkalinity, trace elements, nutrients, enzymes, or any other amendment which a
substrate by itself may lack, and could result in an increase in substrate biodegradability, and therefore, biomethane potential (Labatut, Angenent, and Scott 2011). Antagonistic effects can come from several factors, such as pH inhibition, ammonia toxicity, high volatile acid concentration, among others (Labatut, Angenent, and Scott 2011).

Brennan and Owende (2010) state that microalgae can have a high proportion of proteins that result in low C/N ratios (ca. 10), which can affect the performance of the anaerobic digester. This problem may be resolved by co-digestion with a high C/N ratio product (e.g. waste paper) (Brennan and Owende 2010). Samson and Leduy (1983) found that methane yield and productivity were doubled when equal masses of wastewater sludge and *Spirulina* biomass were co-digested (Samson and Leduy 1983). Similarly, Yen and Brune (2007) co-digested algal biomass with waste paper in a 50/50 (w/w) ratio to adjust the C/N ratio to around 20/1 to 25/1, increasing the methane production rate two-fold compared to the digestion of algal biomass alone. A balanced C/N ratio, along with the stimulated increase in cellulase activity, are suggested as likely reasons for the increased methane production seen in the co-digestion of algal sludge and waste paper (Yen 2004). Shanmugam and Horan (2009) also found that wastes with a higher C/N ratio produced methane more rapidly.

Waste-grown algae have widely varying lipid contents, and the technologies for lipid extraction are still under development (Woertz et al. 2009). Thus, anaerobic digestion is likely to be the near-term, appropriate use of algae biomass at wastewater treatment plants. Sialve, Bernet, and Bernard (2009) found that when the cell lipid content does not exceed 40%, anaerobic digestion of the whole biomass, as opposed to the residual biomass post oil extraction, appears to be the optimal strategy on an energy balance basis. In other words, the energy required for oil extraction would exceed the energy gains in algae with less than 40% lipid content. Furthermore, the AD process is appropriate for high moisture content (80–90% moisture) organic wastes (McKendry 2002) and can be useful for wet algal biomass (Singh and Olsen 2011).

**1.4 BENEFITS OF WASTE STREAM ALGAE PRODUCTION**

Wastewaters derived from municipal, agricultural and industrial activities potentially provide cost-effective and sustainable means of algal growth for biofuels, due to the fact that
most wastewaters contain high concentrations of N and P (Abdel-Raouf, Al-Homaidan and Ibraheem 2012). In addition, there is potential for combining wastewater treatment by algae, such as nutrient removal, with biofuel production. Algae can be used in wastewater treatment for a range of purposes, including: reduction of BOD, removal of N and/or P, inhibition of coliforms, and removal of heavy metals (Abdel-Raouf, Al-Homaidan and Ibraheem 2012).

A great deal of research has been dedicated to the removal of waste nutrients via algae growth (Pittman, Dean, and Osundeko 2011). Woertz et al. (2009) treated municipal wastewater in semi-continuous indoor cultures with 2-4 day hydraulic residence times and achieved over 99% removal of ammonium and orthophosphate. The results suggest that CO₂-supplemented algae cultures can simultaneously remove dissolved nitrogen and phosphorus to low levels while generating a feedstock potentially useful for biofuels production (Woertz et al. 2009).

In particular, many studies have focused on *C. vulgaris* as the subject microalgae. Yang et al. (2011) showed that growing *C. vulgaris* with wastewater could eliminate the need for all of the additional of nutrients except phosphorous. Kim et al. (2010) studied the capability of *C. vulgaris* to remove nitrogen in the form of ammonia and ammonium ions from wastewater effluent at a local wastewater treatment plant containing high concentrations of nitrogen and total inorganic carbon. They found that half of the nitrogen concentration was dramatically removed in 48 hours after a 24-hour lag-phase period and total inorganic carbon concentration also concomitantly decreased during the rapid growth-phase. The total biomass weight gained during the entire cultivation period balanced out well with the total amount of inorganic carbon and nitrogen removed from the culture medium, indicating that algal growth was the cause of the nutrient removal (Kim et al. 2010).

Lau, Tam, and Wong also found that *C. vulgaris* could successfully remove nutrients from primary settled municipal wastewater (Lau, Tam, and Wong 1995). After 10 days, over 90% NH₄⁺-N and 80% PO₄⁻³-P were removed from wastewater. The residual concentrations of NH₄⁺-N, P0₄⁻³-P, TKN and total P in wastewater were negatively correlated with the cell numbers and chlorophyll content of the cultures, indicating that more algal growth led to increased nutrient reduction. The sharp initial reduction of COD (>50%) and total organic-N (TON) (>60%) was not related to the algal number or chlorophyll content. This suggests that the removal of COD and TON was mainly due to the metabolism of the indigenous bacteria.
Under an open system, the interaction between algal and bacterial cells was significant which could enhance the simultaneous removal of N, P and organic matter from primary settled sewage (Lau, Tam, and Wong 1995).

Many other researchers have come to a similar conclusion that microalgae can effectively remove nitrogen and phosphorous from wastewater, which it utilizes for its growth (González, Cañizares, and Baena 1997; Bich, Yaziz, and Bakti 1999; Colak and Kaya 1988; Oswald, “Large Scale,” 1988; Oswald, “Micro-Algae” 1988; Przytocka-Jusiak et al. 1984; Rodrigues and Oliveira 1987), as well as remove heavy metals (Rai, Gour, and Kumar 1981) and some toxic organic compounds. In addition, several studies have found that *C. vulgaris* is a particularly robust microalgal strain that is resistive to bacterial conditions and thus grows well in waste streams (Li et al. 2011). R. Chen et al. (2012) found that *Chlorella* spp. was able to thrive in high nitrogen levels (200 milligrams TN per liter) while five other microalgae species died off. Palmer (1969) listed algae in the order of their tolerance to organic pollutants as reported by 165 authors. The list was compiled for 60 genera and 80 species. *Chlorella* spp. was found to be in the top eight most tolerant genera.

The interest in microalgal cultures stems from the fact that conventional treatment processes suffer from some important disadvantages: (a) variable efficiency depending upon the nutrient to be removed, (b) high cost of operation; (c) chemical processes that often lead to secondary pollution, and (d) loss of valuable potential nutrients (N, P) (De la Noue, Laliberete and Proulx 1992). The last disadvantage is especially serious, because conventional treatment processes lead to incomplete utilization of natural resources (Gutersthan and Todd 1990; Phang 1990).

### 1.5 Research Objectives

The research will investigate methane gas production during anaerobic co-digestion of wastewater sludge and the microalgae, *Chlorella vulgaris*. The primary hypothesis of the research is that anaerobic co-digestion of the microalgae, *C. vulgaris*, with wastewater sludge can enhance methane gas yield. To test this hypothesis, two major objectives are pursued:

1. Investigate the increase in the production of methane gas during anaerobic co-digestion of wastewater sludge and the microalgae species, *C. vulgaris*. 
2. Evaluate the influence of operational parameters, such as wastewater sludge and algal biomass loading, detention time, temperature, and alkalinity on the quantity of methane gas generated.

1.6 RESEARCH OUTLINE

The principle focus of the research is to investigate methane gas production during anaerobic co-digestion of wastewater sludge and the microalgae, *C. vulgaris*. A series lab-scale AD experiments were planned, designed and conducted to achieve the objectives of the research. Chapter 2 describes the experimental setup, materials, and analytical methods, followed by the experimental results in Chapter 3 and a discussion of the data with caveats in Chapter 4. Lastly, Chapter 5 summarizes the results, discusses their significance, and provides recommendations for future studies.
CHAPTER 2

RESEARCH METHODOLOGY

In this chapter, the experimental approach, analytical methods, and materials used in the research are presented. The majority of the twenty-four Standard Operating Procedures developed and utilized for the research can be found in Appendix A.

2.1 EXPERIMENTAL APPROACH

Methane production was measured by adapting the Biochemical Methane Potential assay developed by Owen et al. (1979) in 1978. The procedure was also guided by standard methods ASTM E2170-01 and ISO 11734:1995 as well as other published research (Moody et al. 2009; Chynoweth et al. 1993; Hansen et al. 2004; Raposo et al. 2012; Shanmugam and Horan 2009).

To test methane production, lab-scale anaerobic digesters were set-up using 250 mL glass bottles with 3-port caps equipped with two sets of tubing and one-way polycarbonate luer stopcocks for biogas sampling, inoculum addition, and purging (see Figure 2.1). The tubing apparatus consisted of 1/8” outer diameter (OD) PTFE tubing inserted into 1/8” inner diameter (ID) Tygon® which was capped with a two way valve. The connection between the PTFE and Tygon® tubing was sealed with two plastic zip ties double wrapped around the tubes. The PTFE tubing was inserted into the cap port and sealed with a polypropylene fitting and Tefzel ferrule. Each tube apparatus was approximately 6-8” in length and terminated in the headspace of the digester.

After the digester bottles were washed with phosphate-free detergent and allowed to dry, a mixture of *C. vulgaris*, thickened waste activated sludge (TWAS), and DI water was added to each digester and the headspace was purged with compressed nitrogen gas (N₂) at an approximate rate of 2 L per min for 2 minutes in order to create an anaerobic environment. The seed bacteria (inoculum) were extracted from the collection container through a customized cap and tubing system to minimize oxygen interference (see Figure 2.2). The 3-port cap was customized so that 0.25” OD Tygon® tubing weighted with a magnetic stirrer
Figure 2.1. Lab-scale anaerobic digester.

Figure 2.2. Inoculum bottle with customized cap.

was inserted into the inoculum through the cap while the other two ports were plugged. The inoculum bottle cap did not provide an air tight seal but it was meant to inhibit oxygen interference. A plastic syringe was used to extract the inoculum from the Tygon® tubing and
introduce it to the digesters by fastening the syringe luer onto the valve connected to the shorter tube. After the addition of inoculum, the digesters were purged again with N₂ gas for 20 seconds.

The substrate (*i.e.* *C. vulgaris* and TWAS) was added to the 250 mL glass digester bottles in a one-to-one ratio with the inoculum based on volatile solids (VS) for all experiments, with the exception of a slight unintentional variation in Experimental Set 1. For quality control purposes, no less than 10 mL of TWAS was ever sampled. If a smaller sample size was needed, a diluted solution was prepared. The volume of the mixture of algae, TWAS and inoculum was 200 mL for all the experiments, resulting in a 50 mL headspace.

The lab-scale digesters were placed into an incubator/shaker at 150 rpm and biogas was sampled on a schedule determined by the amount of biogas that was being produced. The biogas was analyzed with an Agilent 6890 gas chromatography (GC) to determine the chemical makeup.

The alga was grown in our lab using primary effluent as the nutrient and water source, 24-hour fluorescent lighting and 2.5% CO₂. Primary effluent was analyzed for total phosphorous, total nitrogen, ammonia, nitrate, nitrite, and orthophosphate by filtering the effluent or otherwise correcting for absorbance interference caused by color, turbidity and suspended solids. *C. vulgaris* effluent was analyzed for the same constituents by centrifuging for 45 minutes and then filtering the effluent.

### 2.2 Preliminary Studies

Preliminary studies were conducted to determine the ideal substrate, *i.e.* *C. vulgaris* and TWAS, loading to inoculum ratio such that the biogas production would neither be limited by low substrate nor by overloaded inoculum. Three substrate loading to inoculum ratios were tested: 0.5, 1.0, and 1.5. Digesters were set-up in triplicate following the approach outlined in Section 2.1 and were incubated at 35°C for 185 days. Results showed the best substrate to inoculum ratio to be 1.

Additionally, a second preliminary test was set-up to determine if a low VS loading was able to generate a measurable amount of biogas. Substrate to inoculum ratio was kept at 1 for both the low VS loading (400 mg/digester or 2 mg/L) and the high VS loading (1460 mg/digester or 7.3 mg/L) sets. Again, digesters were prepared in triplicate and incubated at
35°C for 7 days. It was observed that a low VS loading produced an easily measurable amount of biogas, and therefore, it was used for the remainder of the tests. For graphs of preliminary test results, see Appendix B, Figures B.1.-B.3.

2.3 EXPERIMENTAL CONDITION

Several experiments were carried out to evaluate the influence of operational parameters, such as algal biomass loading, temperature, and alkalinity on the quantity of methane gas generated, see Table 2.1.

The first set of experiments, run 1-5, was designed to evaluate the potential of the microalgae, *C. vulgaris*, as a supplementary feedstock. In these experiments, the contribution of the VS from *C. vulgaris* was 0, 30, 56, 80, and 100% of the total substrate VS. Experimental Set 1 was conducted in triplicate and incubated, along with a control run, at the mesophilic temperature of 35°C for 60 days.

The second set of experiments, run 7 and 8, was conducted to determine the effect of colder temperatures on waste mineralization and biogas production. For these experiments, 50% of the substrate VS was from *C. vulgaris*. Experimental Set 2 was incubated in triplicate at 20°C and at 10°C for 30 days, along with controls for each temperature.

The effect of alkalinity was investigated in the third and final set of experiments, run 11-13. Alkalinity was adjusted using sodium bicarbonate (NaHCO₃) and three alkalinity levels were tested: 70 (alkalinity level without addition of NaHCO₃), 1640, and 3210 mg/L as CaCO₃. Quadruplicate digesters, in addition to a triplicate control of DI and inoculum, were set up for each alkalinity level, and the digesters were incubated at 35°C for 30 days. After 15 days, one digester of each alkalinity level was sacrificed and pH and alkalinity were measured. On the final day, pH and alkalinity of the remaining digesters were measured.

At the beginning and end of each experiment, various tests were completed to determine the chemical oxygen demand (COD), total solids (TS), volatile solids (VS), total and fecal coliform populations, and nutrient levels. Calibration curves were created for COD, total phosphorous, total nitrogen, ammonia, nitrate, nitrite, orthophosphate, methane, and carbon dioxide.
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<th>Temp. (°C)</th>
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<th>Alkalinity (mg/L as CaCO₃)</th>
<th>COD loading (mg)</th>
<th>COD from <em>C. vulgaris</em> (mg)</th>
<th>COD from TWAS (mg)</th>
<th>COD from Inoculum (mg)</th>
<th>VS loading (mg)*</th>
<th>VS from <em>C. vulgaris</em> (mg)</th>
<th>VS from TWAS (mg)</th>
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*Does not account for VS due to addition of NaHCO₃ in runs 12, 13, 15, and 16*
2.4 Analytical Methods

Biogas volume was measured using a clean, dry 20 mL Perfektum Micro-Mate glass syringe purchased from Fisher Scientific. The syringe was equipped with a luer lock that could fasten onto the stopcock valves at the ends of the tubing of the lab-scale digesters. The valve was opened and the glass plunger was allowed to be expelled.

The amount of methane (CH\textsubscript{4}) and carbon dioxide (CO\textsubscript{2}) in the biogas was determined using an Agilent 6890 gas chromatography (GC) equipped with thermal conductivity detector (TCD) and sample injection valve. The column utilized was an All Tech Chromosorb 106 80/100 (6’x1/8” x 0.085”). A computer with Agilent ChemStation software was connected to the GC and used to view and export results.

A biogas sample of approximately 18 mL was taken from a digester with a glass syringe. The sample was manually injected through a sampling valve at a constant speed, and the cycle was initiated. Sample valve temperature was kept at 70°C ± 2°C. The gas sample was routed through an All Tech Chromosorb Column and detected with a thermal conductivity detector (TCD). The GC oven was held at 40°C for four minutes, then ramped up by 50°C each minute for three minutes, and held at 190°C for two minutes before being allowed to cool back down to 40°C where it was held for one minute before another sample could be injected. The TCD oven was set at 250°C, the nitrogen gas reference flow at 25 mL per min, and the nitrogen gas make-up flow at 5 mL per min.

The GC was calibrated with 99.8% bone dry carbon dioxide (CO\textsubscript{2}) gas and 99.0% C.P. grade methane (CH\textsubscript{4}) gas. A 30:70 (CO\textsubscript{2}:CH\textsubscript{4}) pre-made standard was analyzed and results were compared to the prepared 30:70 standard to ensure accuracy. A quality control chart was created from seven runs of the 30:70 pre-made standard and calibration checks were run each new day of testing. For each GC run, peak area was converted to moles of gas using a calibration curve, which was converted to percent CH\textsubscript{4} or CO\textsubscript{2} assuming the contribution from other gases was negligible.

COD was determined using Hach Method 8000 in the range of 20-1500 mg/L, based on the Standard Methods for Water and Wastewater, Method 5220-D (Clescerl, Greenberg and Eaton 1998) which is USEPA approved. 2 mL of sample, standard, or diluted sample were pipetted into a Hach high range COD reagent vial, inverted, and digested for 2 hours at
150°C. Once the temperature had cooled to 120°C, the vials were removed from the digester, inverted one time, and allowed to cool to room temperature. Optical density readings were measured with a Thermo Spectronic Genesys 20 spectrophotometer at 620 nm.

Total and volatile solids were determined based on *Standard Methods for Water and Wastewater*, Methods 2540-B, 2540-E and 2540-G (Clescerl, Greenberg and Eaton 1998). Evaporating dishes were first cleaned by heating in a high temperature box furnace (Lindberg/Blue M BF 51766A-1, Asheville, NC) at 550°C for one hour, after which they were allowed to cool to room temperature and weighed. 10 mL of sample was transferred to the dishes, and the dishes were weighed and placed into a drying oven (Yamato DX-400, Santa Clara, CA) at 105°C for at least two hours. After drying, the dishes were removed and cooled in a dessicator until they reached room temperature. They were weighed to determine the TS weight and placed into the 550°C furnace for 45 minutes. The dishes were taken out to partially cool in the open air for five minutes and then placed into a dessicator to cool to room temperature. Once at room temperature, they were weighed to determine the VS weight.

Alkalinity was determined using the titration method outlined in *Standard Methods for Water and Wastewater*, Method 2320-B (Clescerl, Greenberg and Eaton 1998). Hydrochloric acid in the strengths of 0.02 N and 0.1 N were used to titrate a known volume of sample to a pH of 4.5 as measured by an Oakton pH 500 series pH meter with Accumet probe.

Total nitrogen was measured using the Hach persulfate digestion Method 10072, which is similar to Method 4500-N C of *Standard Methods for Water and Wastewater* (Clescerl, Greenberg and Eaton 1998). A total nitrogen persulfate powder pillow was added to each HR total nitrogen hydroxide digestion reagent vial, followed by 0.5 mL of sample. The vials were then capped, shaken vigorously for 30 seconds, inserted into a DRB 200 digester for exactly 30 minutes at 105°C, and removed to cool to room temperature. Next, one total nitrogen reagent A powder pillow was added to each vial, which were capped, shaken for 15 seconds, and allowed to sit for a three-minute reaction period. The same sequence was repeated for total nitrogen reagent powder pillow B with a two-minute reaction period. Lastly, 2 mL of digested sample was transferred into a Total Nitrogen Reagent C vial, which was then capped, inverted slowly 10 times, and allowed to sit for a five-minute
reaction period. After the digestion period, the vials were read in a spectrophotometer at 410 nm. This reading was compared to a NO$_3$--N calibration curve to find the total nitrogen concentration.

Total phosphorous was measured using the Hach Molybdovanadate Method with Acid Persulfate Digestion, Method 10127, which is adapted from Method 4500-B C of Standard Methods for Water and Wastewater (Clescerl, Greenberg and Eaton 1998). First, 5 mL of sample was added to a total phosphorous TNT vial, followed by the addition of a potassium persulfate powder pillow. The vial was then capped and shaken to dissolve the powder, inserted into a DRB 200 digester for 30 minutes at 150°C, and removed to cool to room temperature. 2 mL of 1.54 N sodium hydroxide was added to each vial, which was then capped and inverted to mix. Immediately afterwards, the same sequence was performed for 0.5 mL of molybdovanadate reagent. The vial was then allowed to sit for a seven-minute reaction period. Lastly, the vials were read in a spectrophotometer at 420 nm. This reading was compared to a PO$_4^{3-}$ calibration curve to find the total phosphorous concentration.

Nitrate was measured using the Hach Cadmium Reduction Method 8039. First, a vial was filled with 10 mL of sample, followed by the addition of a NitraVer 5 powder pillow. Then the vial was capped, shaken vigorously for one minute, and allowed to sit for a five-minute reaction period. Lastly, the vials were read in a spectrophotometer at 500 nm. This reading was compared to a NO$_3$--N calibration curve to find the nitrate concentration.

Nitrite was measured using the Hach Diazotization Method 8507, which is adapted from the U.S. EPA Federal Register (May 1, 1979). First, a vial was filled with 10 mL of sample, followed by the addition of a NitriVer 3 powder pillow. Then the vial was capped, shaken to dissolution, and allowed to sit for a 15-minute reaction period. Lastly, the vials were read in a spectrophotometer at 507 nm. This reading was compared to a NO$_2$--N calibration curve to find the nitrite concentration.

Orthophosphate was measured using the Hach ascorbic acid Method 8048, adapted from Standard Methods for the Examination of Water and Wastewater (Clescerl, Greenberg and Eaton 1998). First, a vial was filled with 10 mL of sample, followed by the addition of a PhosVer 3 powder pillow. Then the vial was capped, shaken for 15 seconds, and allowed to sit for a two-minute reaction period. Lastly, the vials were read in a spectrophotometer at
880nm. This reading was compared to a $\text{PO}_4^{3-}$ calibration curve to find the orthophosphate concentration.

Ammonia was determined by Hach salicylate Method 8155, adapted from Clinica Chimica Acta (Reardon, Foreman, and Searcy 1966). First, a vial was filled with 10 mL of sample, followed by the addition of an ammonia salicylate powder pillow. Then the vial was capped, shaken to dissolution, and allowed to sit for a three-minute reaction period. After this reaction, the same sequence was repeated with an ammonia cyanurate powder pillow for 15 minutes. Lastly, the vials were read in a spectrophotometer at 665 nm. This reading was compared to a $\text{NH}_3$-N calibration curve to find the ammonia concentration.

All glassware used for nutrient testing was acid washed using a 1% hydrochloric acid solution. Glassware acid washed for the first time was soaked overnight for at least 12 hours and subsequent washes were done for at least 10 minutes. Glassware was then rinsed in DI water at least five times.

Total and fecal coliform counts were determined using the IDEXX Colisure® method, which is approved by the EPA for coliform quantification in drinking water (Olstadt et al. 2007). All required supplies coming in contact with the digestate and not already sterile were autoclaved or sprayed down with 70% isopropyl or ethyl alcohol, and the entire procedure was performed in a Jouan MSC 12 Class II Type A Biosafety Cabinet.

Depending on the anticipated concentration of coliforms in the digestate, it was diluted as appropriate in a final volume of 100 mL. Triplicate bottles were prepared and one pack of Colisure® was added to each bottle. The bottles were swirled until no large pieces of solid remained. The contents of each bottle were then poured into a Quanti-Tray/2000, and each tray was sealed in a Quanti-Tray sealer. The trays were incubated for 24 hours in a 35°C incubator.

After 24 hours, the trays were read for total coliforms; red or magenta wells were counted as positive and yellow wells as negative. If there were orange or pink wells, the trays were incubated for another 24 hours, up to 48 hours. At this point, any wells still orange or pink were counted as negative, and red or magenta were counted as positive. To count fecal coliforms, the trays were subject to a portable UV lamp in the dark, and fluoresced cells were counted as positive. The cell counts were converted to MPN (aka CFU) by using the IDEXX Quanti-Tray/2000 MPN table.
The initial coliform concentrations in each digester were determined by a weighted average of the CFU measurements of the individual digester components.

2.5 MATERIALS

The TWAS and inoculum were collected from the San Elijo Water Reclamation Facility on the morning of use. TWAS was scooped up and poured into a wide-mouthed plastic container. Inoculum was collected from the inside of an operational anaerobic digester using a spigot and a glass bottle. The bottle was filled to the top with inoculum to minimize oxygen interference. Inoculum was stored at room temperature or in an incubator at the test temperature while preparing the experiment. TWAS was stored in the cold room at 4°C.

The *C. vulgaris* culture was purchased from Carolina Biological (152069 Burlington, NC). The alga was cultured in 10 liter glass bottles using primary effluent from San Elijo Water Reclamation facility as the source of nutrients and water. The culture was mixed by a magnetic stirrer on a stir plate (Fisher Scientific 11-600-100SH, Waltham, MA). A Lithonia fluorescent lighting fixture (Lithonia Lighting 1233, Conyers, GA) and two F40T 12/CW 40 watt bulbs (Philips 410894, Amsterdam, Netherlands) were purchased from Home Depot. CO₂ diluted with air from San Diego State University’s air compression system was supplied to the algae by routing the CO₂ through an Omega mass flow meter (Omega FMA 5506, Stamford, Connecticut) that had been calibrated for nitrogen gas and the air through an Aalborg mass flow meter (Aalborg GFC17, Orangeburg, New York) in order to introduce an air stream to the algae containing 2.5% CO₂.

Lab-scale anaerobic digesters were set-up using 250 mL glass media bottles (Fisher Scientific FB-800-250, Waltham, MA), GL45 3-ported caps with 1/4-28 PTFE insert and polyethylene collar (Vaplock BC-322N, Lake Elsinore, CA) purchased from Western Analytical, EPDM (ethylene propylene diene monomer) gaskets (Western Analytical GS-017, Lake Elsinore, CA), polycarbonate luer one-way stopcocks (Cole-Parmer EW-30600-06, Vernon Hills, IL), 1/16” ID x 1/8” OD PTFE tubing (Cole-Parmer EW-06605-27, Vernon Hills, IL), and 1/8” ID Tygon® R-3603 tubing (Saint Gobain Performance Plastics 14-169-1E, Garden Grove, CA). Eppendorf and Thermo Scientific pipettes (Eppendorf Research 100-1000 µL, Eppendorf Research Plus 1-10 mL, and Thermo Scientific Finnpipette 1-10 mL) along with epTIPS pipette tips, 50 to 1000 uL and 10 mL (Eppendorf, Hauppauge, NY)
were used to measure and transfer media. In some cases, plastic syringes with a luer-lok (Becton Dickinson 309653, Franklin Lakes, NJ) were used to transfer TWAS and inoculum. Perfektum Micro-Mate glass syringes (Popper & Sons 5037, New Hyde Park, NY) purchased from Fisher Scientific with polycarbonate luer large-bore three-way stopcocks (Cole-Parmer EW-30600-23, Vernon Hills, IL) were used for biogas sampling. Digesters were incubated in one of two incubators: New Brunswick Innova 42R Incubator Shaker (New Brunswick Scientific I42R, Edison, NJ) or VWR 1575R Incubator Shaker (Sheldon Manufacturing, Inc. 1575R, Cornelius, OR).

The amount of methane (CH₄) and carbon dioxide (CO₂) in the biogas was determined using a gas chromatography (GC) machine (Agilent 6890, Santa Clara, CA) equipped with thermal conductivity detector (TCD) and sample injection valve. The column utilized was an All tech Chromosorb 106 80/100 (6’x1/8” x .085” SS, Part # C-5000, Bath Serial # 156-705100057, MAOT 275°C, Deerfield, Illinois). The nitrogen gas used as make-up flow was 5.0 grade compressed nitrogen gas (Praxair, Danbury, CT). A computer with Agilent ChemStation software Rev. B.01.03(204) was connected to the GC and used to view and export results.

Six liter summa canisters (Restek TO-Can® 24174, Bellefonte, PA) were used to create calibration standards. GC calibration gases used were 99.8 % CO₂, bone dry (Air Liquid America 23402, Los Angeles, CA) purchased from Supelco, 99.0 % C.P. grade CH₄ (Air Liquid America T109-14, Los Angeles, CA) purchased from Supelco, and a 30.04% CO₂ 69.96% (balance) CH₄ mix (Praxair ME-CD30P-A3, Danbury, CT). Refrigerated liquid nitrogen gas (Praxair, Danbury, CT) was used for cleaning and purging the summa canisters. An Edwards high vacuum pump (Edwards E2M8, Crawley, Sussex, England) and a Fisher Scientific Traceable Manometer (Fisher Scientific 06-664-21, Waltham, MA) were used to fill and vacuum the summa canisters.

COD, total nitrogen, and total phosphorous samples were digested in a Hach DRB 200 reactor (Hach DRB200-03, Loveland, CO). High range (20-1500 mg/L) COD vials were used to determine initial and final COD (Hach 2125915, Loveland, CO). Total phosphorus was determined using the Hach TNT Reagent Set, High Range Molybdovanadate (Hach 2767245, Loveland, CO). Total nitrogen was determined using the Hach TNT Reagent Set, High Range (Hach 2714100, Loveland, CO). Nitrate, nitrite, orthophosphate, and ammonia
were analyzed using the Hach reagents NitraVer 5, NitriVer 3, PhosVer 3, and Ammonia Salicylate with Ammonia Cyanurate, respectively. Nutrient calibration standards included nitrogen standard, 1000 ppm, N-NO3 (Ricca Chemical Company 5459-4, Arlington, TX); nitrogen-ammonia standard solution, 10 mg/L, NH3-N (Hach 15349, Loveland, CO); phosphate standard solution, 50 mg/L, PO4 (Hach 17149, Loveland, CO); phosphate standard solution, 100 mg/L, PO4 (Hach 1436832, Loveland, CO); nitrogen-nitrate standard solution, 100 mg/L, NO3-N (Hach 194749, Loveland, CO); nitrite stock solution, 250 mg/L NO2-N (Hach 2340249, Loveland, CO). COD Standard Solution, 1000 mg/L (Hach 2253929, Loveland, CO) and potassium acid phthalate (Hach 31534, Loveland, CO) were used as calibration standards for COD.

Optical density readings were measured with a Thermo Spectronic Genesys 20 spectrophotometer (Thermo Electron Corp. 4001/4, Waltham, MA). Primary effluent and chlorella effluent samples were filtered through a vacuum filtration system using 0.45 µm filter paper (Hach 1353001, Loveland, CO) prior to nutrient testing or otherwise corrected for color interference.

Distilled water was purified with Milli-Q Advantage 10 (Millipore, Molshein, France) water purification system and was used for dilutions, coliform testing, preparing lab-scale digesters, and final rinsing of glassware.

Alkalinity was measured with hydrochloric acid, 0.02 N (Hach 2330353, Loveland, CO) and 0.1 N (Hach 1481253, Loveland, CO) and an Oakton WD-35617 series pH meter.

Coliform testing was done inside a Jouan MSC 12 Class II Type A Biosafety Cabinet (52020000, Jouan Laboratory Equipment, Winchester, VA). The safety cabinet was sterilized with 70% isopropyl alcohol or 70% ethanol in addition to the UV light built into the cabinet. Glassware, pipette tips, and DI water were sterilized in one of two autoclaves (Consolidated SSR-3A-PB, Boston, MA) (Consolidated ADV-PB, Boston, MA). Total and fecal coliforms were measured using Colisure® reagent with Quanti-tray 2000s (IDEXX 99-27076, Westbrook, ME). Quantri-tray 2000s were sealed with the IDEXX Quanti-tray sealer (WQTS2X-115). A portable UV lamp (IDEXX WL200, Westbrook, ME) was used to determine fecal coliforms.

Total and volatile solids were measured using CoorsTek porcelain evaporating dishes (70 mL) (CoorsTek 60197, Golden, CO), a drying oven (Yamato DX-400, Santa Clara, CA),
a high-temperature box furnace (Lindberg/Blue M BF 51766A-1, Asheville, NC), a
dessicator, and a precision scale (Mettler-Toledo AE200, Columbus, OH) calibrated with a
100 gram calibration weight (Mettler-Toledo ME-216504, Columbus, OH).
CHAPTER 3

RESULTS

In this chapter, the experimental results of the AD of wastewater sludge with *C. vulgaris* and the influence of operational parameters are presented.

3.1 POTENTIAL OF ALGAE AS A SUPPLEMENTARY FEEDSTOCK

The potential of algal biomass as a supplementary feedstock to anaerobic digesters to generate a reliable supply of biogas, was evaluated by varying the contribution of *C. vulgaris* to the substrate VS. Experiments were conducted at 0, 30, 56, 80 and 100% of the total substrate VS contribution from *C. vulgaris*. The results are presented in Figure 3.1. The error bars presented in the figure span one standard deviation above and below the mean and were obtained from triplicate experiments. Analysis of variance (ANOVA) performed on the data using an $\alpha$ value of 0.05 resulted in an $F$ value of 0.43 which is less than the $F_{crit}$ of 2.53. This shows that there is no significant difference between the cumulative gas productions from the various ratios. Figure 3.2 shows the biogas production of Experimental Set 1 per initial VS and initial COD. Again, no significant difference is detected.

![Figure 3.1. Cumulative volume of biogas production for varied proportions of C. vulgaris to TWAS over time.](image)
Figure 3.2. (a) Cumulative volume of biogas per mg of initial VS for varied proportions of *C. vulgaris* to TWAS over time. (b) Cumulative volume of biogas per mg of initial COD for varied proportions of *C. vulgaris* to TWAS over time.
3.2 Effect of Temperature on Anaerobic Digestion Performance

To evaluate the effect of temperature, experiments were conducted at 10 and 20°C for 30 days, see Figure 3.3. The contribution of VS from *C. vulgaris* was 50% of the substrate VS. The volumes of biogas measured at 10 and 20°C have been adjusted for temperature and the values in the figure represent equivalent volumes at 35°C. The results show that the lowered temperature has a significant effect on the biogas production.

![Figure 3.3. Cumulative volume of biogas production at 10 and 20°C for a 1-to-1 ratio of *C. vulgaris* to TWAS over time.](image)

The results of the experimental runs at 10 and 20°C are compared to the results at 35°C after 30 days from experimental runs 3 and 11, where the contribution of substrate VS from *C. vulgaris* is 56 and 50%, respectively. Figure 3.4 shows the results from all four runs, normalized by initial VS. This shows a clear trend of decreased biogas with decreased temperature. The volumes of biogas measured at 10 and 20°C have been adjusted for temperature and the values in the figure represent equivalent volumes at 35°C.
3.3 EFFECT OF ALKALINITY ON ANAEROBIC DIGESTION PERFORMANCE

Alkalinity plays an important role by providing a buffering capacity for change in pH. Three levels of initial alkalinity were tested: 70, 1600, and 3200 mg/L as CaCO₃. Figure 3.5 shows the effect of alkalinity on biogas production over 30 days at 35°C for digesters consisting of 50% substrate VS from *C. vulgaris*. The ANOVA for the data, using an \( \alpha \) value of 0.05, resulted in an \( F \) value of 0.23 and \( F_{crit} \) of 3.28, indicating that no significant difference in biogas production is seen between the varied initial alkalinity levels.

After 30 days, the alkalinity levels in the digesters had increased to 670, 2100, and 3450 mg/L as CaCO₃, respectively, while pH remained relatively constant. The digesters with the lowest initial alkalinity experienced the greatest natural alkalinity increase, while the digesters with the highest initial alkalinity experienced only a slight natural increase over the 30 days. Table 3.1 shows the values for pH and alkalinity of the three sets at day 0, day 15, and day 30.
Figure 3.5. (a) Cumulative volume of biogas at differing initial alkalinity levels for a 50:50 mix of *C. vulgaris* and TWAS over time (b) Normalized cumulative volume of biogas at differing initial alkalinity levels for a 50:50 mix of *C. vulgaris* and TWAS over time.

Table 3.1. Alkalinity and pH of Digester in Test 5 at Day 0, 15, and 30

<table>
<thead>
<tr>
<th>Exp Run</th>
<th>Starting*</th>
<th>After 15 days°</th>
<th>Ending (after 30 days)β</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Alkalinity</td>
<td>pH</td>
</tr>
<tr>
<td>11</td>
<td>6.69</td>
<td>70</td>
<td>6.87</td>
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<tr>
<td>12</td>
<td>7.54</td>
<td>1640</td>
<td>7.40</td>
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<tr>
<td>13</td>
<td>7.71</td>
<td>3210</td>
<td>7.59</td>
</tr>
</tbody>
</table>

*Calculated based on the alkalinity of the components with spot checking
° Based on triplicate measurements of one digester from each set
β Based on measurements from three digesters per set

3.4 BIOGAS COMPOSITION

Percent methane and carbon dioxide are determined for Experimental Sets 1 and 3 to be 72% ± 0.63 and 28% ± 0.63, respectively, by gas chromatographic analysis. No significant difference in biogas composition is detected between the various experimental runs.

3.5 COMPOSITION OF RESIDUALS

To evaluate the usefulness of the residuals as a fertilizer, total nitrogen and phosphorous were determined. Figure 3.6 shows the percent of nitrogen and phosphorus on the basis of weight, *i.e.* g of total nitrogen as N or g of total phosphorus as P per g of TS. The
Figure 3.6. Percent total nitrogen and phosphorous post-digestion for Exp. Set 1.

Results show that total nitrogen content of the residuals varied in the range of 9-17% as N while for total phosphorus the range was 3-7% as P (7-16% as P₂O₅). Commercial fertilizers contain a wide range of nutrient levels, from 0-82% for N and 0-48% for P₂O₅; therefore, the residuals could be utilized as a fertilizer.

An important aspect of AD is that it reduces harmful pathogens, viruses and bacteria. To demonstrate this reduction, TC and FC were measured pre- and post-digestion. Table 3.2 presents the initial TC and FC and the reduction in both. FC were reduced at least 77% in all experimental runs. The residuals meet the EPA requirements for pathogen reduction (FC < 2x10⁶ CFU per g) for land application (U.S.EPA 1999).

**3.6 ALGAE GROWTH AS WASTEWATER TREATMENT**

*C. vulgaris* and other microalgae have been shown in the literature to be effective at removing nutrients in wastewater. To verify the usefulness of *C. vulgaris* production as a method of wastewater treatment, nutrients in the growth media were measured pre- and post-growth. Table 3.3 compares the nutrient characteristics of the primary effluent that was used to grow the *C. vulgaris* with the effluent of the algae after it had been growing for two weeks. Results show reductions in ammonia and orthophosphate of 95 and 61%, respectively.
Table 3.2. Reductions in Total and Fecal Coliforms

<table>
<thead>
<tr>
<th>Exp. Run</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>7</th>
<th>8</th>
<th>11</th>
<th>12</th>
<th>13</th>
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</thead>
<tbody>
<tr>
<td>Initial TC (CFU/g TS)</td>
<td>7.3E6</td>
<td>5.6E6</td>
<td>4.1E6</td>
<td>2.8E6</td>
<td>1.8E6</td>
<td>4.6E6</td>
<td>4.6E6</td>
<td>4.1E6</td>
<td>4.1E6</td>
<td>4.1E6</td>
</tr>
<tr>
<td>Initial FC (CFU/g TS)</td>
<td>2.4E6</td>
<td>1.7E6</td>
<td>1.1E6</td>
<td>5.8E5</td>
<td>1.7E5</td>
<td>1.15E6</td>
<td>1.15E6</td>
<td>1.1E6</td>
<td>1.1E6</td>
<td>1.1E6</td>
</tr>
<tr>
<td>Final TC (CFU/g TS)</td>
<td>2.8E6</td>
<td>5.0E5</td>
<td>7.9E4</td>
<td>1.2E5</td>
<td>2.5E5</td>
<td>2.7E6</td>
<td>3.75E5</td>
<td>1.6E5</td>
<td>1.8E4</td>
<td>4.743</td>
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<tr>
<td>Final FC (CFU/g TS)</td>
<td>3.2E5</td>
<td>3.2E4</td>
<td>1.8E4</td>
<td>5.1E3</td>
<td>1.3E4</td>
<td>1.99E5</td>
<td>3.1E4</td>
<td>5.5E4</td>
<td>1,105</td>
<td>400</td>
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<tr>
<td>% TC reduced</td>
<td>67.61</td>
<td>69.84</td>
<td>73.12</td>
<td>79.26</td>
<td>90.17</td>
<td>40.99</td>
<td>91.90</td>
<td>96.03</td>
<td>99.54</td>
<td>99.88</td>
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<tr>
<td>% FC reduced</td>
<td>99.89</td>
<td>93.58</td>
<td>76.90</td>
<td>95.82</td>
<td>94.97</td>
<td>82.77</td>
<td>97.33</td>
<td>95.12</td>
<td>99.90</td>
<td>99.96</td>
</tr>
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</table>

Table 3.3. Characteristics of Primary Effluent pre- and post- *C. vulgaris* Growth

<table>
<thead>
<tr>
<th>Unit</th>
<th>Primary Effluent Characteristics</th>
<th>Chlorella Effluent Characteristics*</th>
<th>Percent Reduction</th>
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</thead>
<tbody>
<tr>
<td>Nitrite mg/L N-NO₂⁻</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate mg/L N-NO₃⁻</td>
<td>4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonia mg/L N-NH₃</td>
<td>38.8</td>
<td>1.8</td>
<td>95</td>
</tr>
<tr>
<td>Total N mg/L N-NO₃⁻</td>
<td>44.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orthophosphate mg/L P-PO₄³⁻</td>
<td>7.1</td>
<td>2.8</td>
<td>61</td>
</tr>
<tr>
<td>Alkalinity mg CaCO₃/L</td>
<td>285</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*After growing in primary effluent for 2 weeks, effluent after centrifuge
CHAPTER 4

ANALYSIS AND DISCUSSION

The effects of substrate loading, temperature, and alkalinity on biogas production, bacterial density, and nutrient levels will be discussed in this chapter.

4.1 BIOMASS PRODUCTION AND SYSTEM EFFICIENCY

The results show that the microalgae, *C. vulgaris*, can produce at least the same amount of methane as wastewater sludge and is therefore a good candidate for anaerobic digestion (Figure 3.1). No synergistic effect was observed during co-digestion of *C. vulgaris* and wastewater sludge, thus any increase in methane production is due to additional biomass. However, if additional digester space is available, *C. vulgaris* offers a viable co-digestion substrate due to the fact that no antagonistic effects were observed and methane production matched that of wastewater sludge. The inverse exponential curve pattern of gas production seen in this research is also shown in the literature. Data shows that the methane productions obtained from mixed cultures of the microalgae *C. vulgaris* and *Scenedesmus* sp. (Yen and Brune 2007; Golueke, Oswald, and Gotass 1957; P. H. Chen 1987) and from *C. vulgaris* alone (Ras et al. 2011), fit an inverse exponential curve, characterized by a rapid increase between 10 and 30 days hydraulic loading rate before reaching a plateau above 30 days hydraulic loading rate (Ras et al. 2011). These studies use organic loading between 1 and 2.6 g VS per L-day.

The volume of biogas produced per mass of VS digested and COD oxidized is presented in Figure 4.1 with the error bars representing one standard deviation above and below the mean obtained from triplicate experiments. The results show that the volume of biogas produced varied in the range of 0.47 to 0.57 mL per mg of VS digested (0.23 to 0.27 mL per mg of VS introduced. This agrees with the results reported in the literature. Golueke, Oswald, and Gotaas (1957) found biogas production from a mix of *Chlorella* spp. and *Scenedesmus* spp. to be 0.45 mL per mg of VS digested and from TWAS to be 0.47 mL per
mg of VS digested. Samson and Leduy (1983; 1986) determined a yield of 0.26-0.34 mL per mg VS introduced from anaerobic digestion of the blue-green algae, Spirulina maxima. Yuan et al. (2011) also studied a blue alga but only obtained a yield of 0.19 mL of methane gas per mg VS introduced. Cecchi, Paven, and Mara-Alverez (1996) found that AD of the macroalgae Ulva rigida and Gracilaria confervoides from a lagoon with wastewater sludge produced a maximum of 0.31 mL of methane gas per gram of VS introduced. Ras et al. (2011) performed an experiment that coupled the algae production and digestion process using C. vulgaris and found a methane production of 0.24 mL per mg of VS introduced for a 28 day retention time. Table 4.1 provides a summary of the methane yields achieved by select studies on the anaerobic digestion of microalgae with and without co-digestants.

Similarly, the volume of biogas produced varied in the range of 0.35 to 0.53 mL per mg of COD oxidized. Biogas composition was found to be 72% methane (CH₄) by GC analysis, and was relatively constant in all experimental runs. Therefore, CH₄ produced was in the range of 0.25-0.38 mL per mg of COD oxidized. Through stoichiometric conversion, CH₄ production is directly related to organic degradation; 1 mg COD oxidation ideally yields 0.395 mL CH₄ at 1 atm and 35°C. Therefore, the values obtained are comparable to the theoretical CH₄ yield of 0.395 mL per mg of COD oxidized.

The percent of TS, VS and COD reduced are presented in Table 4.2. The results show reductions of 25 to 42% for TS, 42 to 51% for VS, and 29 to 45% for COD for experiments.
<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>Days</th>
<th>Substrate</th>
<th>mL CH4 / g VS introduced</th>
<th>mL CH4 / g VS digested</th>
<th>VS reduction (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>60</td>
<td>Wastewater sludge</td>
<td>193</td>
<td>393</td>
<td>49</td>
<td>This study</td>
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<tr>
<td>35</td>
<td>60</td>
<td>Algae (C. vulgaris)</td>
<td>172</td>
<td>336</td>
<td>51</td>
<td>This study</td>
</tr>
<tr>
<td>35</td>
<td>30</td>
<td>Wastewater sludge</td>
<td>278</td>
<td>473</td>
<td>58.5</td>
<td>(Golueke 1957)</td>
</tr>
<tr>
<td>35</td>
<td>30</td>
<td>Algae (Scenedesmus and Chlorella)</td>
<td>168</td>
<td>450</td>
<td>41.3</td>
<td>(Golueke 1957)</td>
</tr>
<tr>
<td>50</td>
<td>30</td>
<td>Algae (Scenedesmus and Chlorella)</td>
<td>233</td>
<td>437</td>
<td>54</td>
<td>(Golueke 1957)</td>
</tr>
<tr>
<td>35</td>
<td>16</td>
<td>Algae (C. vulgaris)</td>
<td>147</td>
<td>--</td>
<td>--</td>
<td>(Ras et al. 2011)</td>
</tr>
<tr>
<td>35</td>
<td>28</td>
<td>Algae (C. vulgaris)</td>
<td>240</td>
<td>--</td>
<td>51 (COD reduction)</td>
<td>(Ras et al. 2011)</td>
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<tr>
<td>30</td>
<td>28</td>
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<td>--</td>
<td>--</td>
<td>(Salerno 2009)</td>
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<tr>
<td>30</td>
<td>28</td>
<td>Microalgae</td>
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<td>--</td>
<td>--</td>
<td>(Salerno 2009)</td>
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<tr>
<td>30</td>
<td>28</td>
<td>Microalgae</td>
<td>163</td>
<td>--</td>
<td>--</td>
<td>(Salerno 2009)</td>
</tr>
<tr>
<td>30</td>
<td>28</td>
<td>Microalgae</td>
<td>115</td>
<td>--</td>
<td>--</td>
<td>(Salerno 2009)</td>
</tr>
<tr>
<td>55</td>
<td>7.5</td>
<td>50% Algae: 50% MSW</td>
<td>212</td>
<td>--</td>
<td>--</td>
<td>(Gunaseelan 1997)</td>
</tr>
<tr>
<td>35</td>
<td>10</td>
<td>Algae (Chlorella and Scenedesmus)</td>
<td>143</td>
<td>--</td>
<td>--</td>
<td>(Yen and Brune 2007)</td>
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<tr>
<td>35</td>
<td>10</td>
<td>50% Algae (Chlorella and Scenedesmus): 50% Waste Paper</td>
<td>293</td>
<td>--</td>
<td>--</td>
<td>(Yen and Brune 2007)</td>
</tr>
<tr>
<td>35</td>
<td>10</td>
<td>Waste Paper</td>
<td>113</td>
<td>--</td>
<td>--</td>
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<td>35</td>
<td>10</td>
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<td>(Yen 2004)</td>
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<tr>
<td>30</td>
<td>33</td>
<td>Spirulina maxima</td>
<td>260</td>
<td>--</td>
<td>--</td>
<td>(Samson and Leduy 1982)</td>
</tr>
<tr>
<td>37.5</td>
<td>20</td>
<td>Algae 100%: 0% Wastewater Sludge - Algae 0%: 100% Wastewater Sludge (in 20% increments)</td>
<td>260-430</td>
<td>--</td>
<td>--</td>
<td>(Woertz et al. 2010)</td>
</tr>
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</table>
Table 4.2. Reductions in Total and Volatile Solids and COD

<table>
<thead>
<tr>
<th>Exp. Run</th>
<th>0% of substrate VS from <em>C. vulgaris</em></th>
<th>30% of substrate VS from <em>C. vulgaris</em></th>
<th>56% of substrate VS from <em>C. vulgaris</em></th>
<th>80% of substrate VS from <em>C. vulgaris</em></th>
<th>100% of substrate VS from <em>C. vulgaris</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial TS (mg/L)</td>
<td>1700</td>
<td>1900</td>
<td>2100</td>
<td>2300</td>
<td>2400</td>
</tr>
<tr>
<td>Initial VS (mg/L)</td>
<td>1200</td>
<td>1300</td>
<td>1300</td>
<td>1400</td>
<td>1400</td>
</tr>
<tr>
<td>Initial COD (mg/L)</td>
<td>2100</td>
<td>2100</td>
<td>2200</td>
<td>2200</td>
<td>2200</td>
</tr>
<tr>
<td>% TS reduction</td>
<td>42</td>
<td>33</td>
<td>30</td>
<td>25</td>
<td>31</td>
</tr>
<tr>
<td>% VS reduction</td>
<td>49</td>
<td>51</td>
<td>48</td>
<td>42</td>
<td>51</td>
</tr>
<tr>
<td>% COD reduction</td>
<td>40</td>
<td>45</td>
<td>37</td>
<td>39</td>
<td>29</td>
</tr>
</tbody>
</table>

Conducted at 35°C. A VS reduction of 38% or higher was achieved, and therefore, the residuals meet vector attraction reduction requirements for land application (U.S.EPA 1999). These results are relatively similar to those obtained by Converti et al. (2009), with methane conversion efficiencies between 21 and 52% for activated sludge, when performing digestion under detention times between 12 and 25 days.

Reduction of VS and COD lead to methane production. 1 mg of COD oxidized ideally yields 0.395 mL of CH₄ at 35°C. Table 4.3 presents the maximum theoretical and actual CH₄ yields for experimental runs at 35°C based on COD oxidized. Results show methane conversion efficiencies between 64 and 97% based on COD oxidized. These results align with those found in literature. Ras et al. (2011) found a 51% COD reduction when anaerobically digesting *C. vulgaris*, and a methane conversion efficiency of 46% after 28 days.

Table 4.3. Theoretical versus Actual Methane Production and COD Mass Balance

<table>
<thead>
<tr>
<th>Exp. Run</th>
<th>Biogas produced (mL)</th>
<th>CH₄ produced (mL)a</th>
<th>COD Digested (mg)</th>
<th>Theoretical CH₄ produced from COD digested (mL)b</th>
<th>% of Theoretical production from COD digested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68</td>
<td>49</td>
<td>165</td>
<td>65</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>67</td>
<td>48</td>
<td>190</td>
<td>75</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>61</td>
<td>44</td>
<td>161</td>
<td>64</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td>66</td>
<td>48</td>
<td>171</td>
<td>67</td>
<td>71</td>
</tr>
<tr>
<td>5</td>
<td>69</td>
<td>49</td>
<td>130</td>
<td>51</td>
<td>97</td>
</tr>
</tbody>
</table>

a Assuming 72% of biogas is CH₄
b Theoretical methane production at 35°C = 0.395 L CH₄ per g COD
4.2 Influence of Operational Parameters

In this study, we evaluate the effect of two critical operational parameters, temperature and alkalinity. As expected, the cumulative biogas production decreased with a decrease in temperature. Though the gas production was lower at 20°C than at 35°C, substantial production did still occur with a steady increase in biogas, almost matching the production at 35°C by day 30 (Figure 3.4). The biogas production at 10°C was nearly 80% less than that at 20°C, but it did still show increasing biogas production through the duration of the digestion (Figure 3.3).

Three levels of initial alkalinity were tested: 70, 1600, and 3200 mg/L as CaCO₃. Varying initial alkalinity did not show a difference in biogas production (Figure 3.4). However, the digesters with the lowest initial alkalinity experienced the greatest natural alkalinity increase, while the digesters with the highest initial alkalinity experienced only a slight natural increase during the 30 day digestion period. The increase in alkalinity could be due to the ammonification, where organic nitrogen is transformed to ammonium nitrogen and bicarbonate. Since *C. vulgaris* is composed of 51-58% proteins (Becker 2004), it therefore, may be the source of the organic nitrogen in the anaerobic digesters (Abril and Frankignoulle 2001). Thus, adding algae as a supplementary feedstock to the AD system may also serve as a source of alkalinity. It should also be noted that the presence of too much protein in anaerobic digesters may pose an adverse effect since ammonia produced during the ammonification of proteins is known to inhibit microorganisms responsible for degrading organics in anaerobic digesters (Parkin and Owen 1986). In a study by Park and Li (Park and Li 2012), the additional alkalinity provided by 50% algae biomass residue during co-digestion with lipid waste helped retain the lipid degradation efficiency when the organic loading rate was increased up to 4 g VS per L-day. Lipid-rich substrates are easily degradable (mostly short-chain fatty acids), but are prone to produce biochemical inhibition due to long chain fatty acid accumulation coming from the hydrolysis of neutral lipids (Labatut, Angenent, and Scott 2011).

4.3 Quality of Residuals

Bacteria testing reveals that for all *C. vulgaris* to TWAS ratios, total and fecal coliforms are significantly reduced during AD. This is important in meeting EPA
requirements for using the dewatered digestate (biosolids) as fertilizer. In all runs except 10°C, total coliforms were reduced an average of 87% and fecal coliforms were reduced an average of 97%. As expected, the low temperature led to a lower digestion rate and thus less destruction of coliforms.

High levels of nitrogen and phosphorous were measured in the digestate, revealing its value as a fertilizer. Nutrient testing shows *C. vulgaris* to be useful in removing nitrogen, particularly ammonia, and phosphorous from primary wastewater effluent during its growth, potentially supplementing wastewater treatment processes and reducing treatment costs. Results show reductions in ammonia and orthophosphate of 95 and 61%, respectively.
CHAPTER 5

SUMMARY, SIGNIFICANCE AND RECOMMENDATIONS

This chapter will summarize the major findings of the studies, discuss their significance, and provide recommendations for future research.

5.1 SUMMARY

The results of the study show that the microalgae, C. vulgaris, can produce at least the same amount of methane as wastewater sludge and is therefore a good candidate for AD. Biogas yields varied in the range of 0.47-0.57 mL per mg of VS digested. Reductions of 42 to 51% in VS and 29 to 45% in COD were achieved at 35°C. Decreased temperatures resulted in lower biogas yields, while initial alkalinity did not appear to affect biogas production. On average, TC and FC concentrations of 6.3x10^5 and 1.0x10^4 CFU per gram of TS, respectively, were measured in the digested waste. Thus, the residual meets the EPA requirements for pathogen reduction (FC < 2x10^6 CFU per g TS) and vector attraction reduction (> 38% reduction in VS) for land application. The total nitrogen and phosphorus content of the residuals were determined to be in the range of 9-17% as N and 3-7% as P (7-16% as P_2O_5), respectively, revealing the residuals’ usefulness as a fertilizer.

5.2 SIGNIFICANCE

The research presented here shows that microalgae can be co-digested with wastewater sludge, or by itself, to produce methane gas in amounts similar to current AD processes at WWTPs. This is significant because the algae can be grown with the nutrient and CO₂ waste streams at a WWTP, thus creating a carbon-neutral energy source and reducing infrastructure costs (see Figure 5.1). This system is beneficial because it can (a) increase energy generation at WWTPs, thereby providing a decentralized energy source and increasing energy stability while decreasing energy costs, (b) increase renewable energy generation and decrease greenhouse gas emissions, and (c) improve treatment of wastewater and potentially reduce treatment costs.
Figure 5.1. Wastewater treatment schematic with integrated AD energy recovery from sludge and algal biomass.

Anaerobic digestion is a mature technology, however, there is plenty of room for optimization and improvements of the process to enhance methane gas yield that can be used for the generation of carbon-neutral energy. Increasing the amount of biogas at WWTPs could (a) make energy generation economical for small WWTPs where currently the amount of methane gas produced is not high enough to make the added energy infrastructure cost-effective and (b) make the process even more cost-effective for larger WWTPS. In addition to the current benefits of AD, such as reduction of pathogens, reduction of waste volume, and organic fertilizer output, the use of algal biomass in AD has several benefits: (1) it develops a carbon-neutral energy source since the algae consume the CO₂ released during energy generation, (2) it eliminates or minimizes the discharge to the environment of nutrients contained in wastewater effluent since algae utilize the nutrients and the water, and (3) it requires less land area to grow compared with other bioenergy crops (Cooke and Fang 2008; Hossain et al. 2008), particularly if integrating with the existing infrastructure of a WWTP.

Throughout the U.S., WWTPs are significant energy users with growing future energy demands due to additional treatment requirements. For example, the State of California’s water-related (water and wastewater) energy use consumes about 19% of the
state’s electricity (CEC 2005). The percentage is even higher for Southern California, including the San Diego region, where water is transported over distances of hundreds of miles. The Energy Commission Demand Office estimates that a total of about 9,000 GWh of electricity is used annually by both water and wastewater facilities in California (CEC 2005) with wastewater treatment averaging 2,500 kWh per million gallon throughout the state (CEC 2005). Therefore, onsite renewable energy generation at WWTPs can play a significant role in reducing fossil-based fuels and associated climate change emissions. As a novel idea with significant social impacts, this research has the potential to develop a carbon-neutral energy source in an economically and environmentally responsible way – contributing to energy security, especially in overloaded areas such as California and the San Diego region.

California has 137 WWTPs with anaerobic digesters and an estimated excess digestion capacity of 15 to 30% (EBMUD 2008). Therefore, this additional capacity can be utilized to generate more carbon-neutral energy by adding algae as a supplementary feedstock to the digesters. In addition, co-locating an algae growth facility for digestion or co-digestion at a WWTP provides multiple infrastructure and economic synergies; the waste streams of the WWTP, including CO₂, water, and nutrients, can be recycled to grow the algae and no new digestion or energy generation facilities would need to be built.

In summary, AD of *C. vulgaris* has the potential to increase renewable, carbon-neutral energy supply, enhance energy security, integrate with existing energy generation infrastructure, and improve the wastewater treatment process and cost-efficiency. A detailed economic analysis and energy balance of an integrated algae growth reactor and digestion system at a WWTP is beyond the scope of this research, and is recommended for a future study.

5.3 RECOMMENDATIONS

For both the sludge and the algae, approximately 50% of the digestable material remained after biogas production ceased, meaning that if the remaining portion could be digested, the biogas yield could increase further. In other studies of ordinary mesophilic methane fermentation, only about 40% of algal energy has been released (P. H. Chen and Oswald 1998). The 60% remaining in algal biomass is resistant to release through decomposition at least in part because many of the cells and walls remain intact through the
fermentation process (P. H. Chen and Oswald 1998; Sánchez Hernández and Travieso Córdoba 1993; Sialve, Bernet, and Bernard 2009; Gonzalez-Fernandez, Molinuevo-Salces, and Garcia-Gonzalez 2011; Golueke, Oswald, and Gotaas 1957). If the cell wall can be disrupted, algae may be able to provide more energy than wastewater sludge. In addition, algal pre-treatment techniques may also release further energy from the sludge. Further study on cost and energy efficient pre-treatment methods are needed as the next step in enhancing methane gas yield from anaerobic co-digestion of microalgae and wastewater sludge.
REFERENCES


Li, Y., W. Zhou, B. Hu, M. Min, P. Chen, and R. R. Ruan. 2011. "Integration of Algae Cultivation as Biodiesel Production Feedstock with Municipal Wastewater


———. 2006. *Biosolids Technology Fact Sheet: Multi-Stage Anaerobic Digestion*. Washington, DC.


APPENDIX A

STANDARD OPERATING PROCEDURES
1. PURPOSE
This protocol describes the process of testing the bio-chemical methane potential (BMP) of microalgae co-digested with wastewater sludge.

2. MATERIALS

2.1. 250mL reactor bottles

2.2. Caps, (BC-322N Western Analytical) BOTTLE CAP GL45 3-ported 1/4-28 PTFE insert (replace with EPDM gasket), blue Polyethylene collar

2.3. Hard tubing, (EW-06605-27 Cole Parmer) PTFE tubing, 1/16"ID x 1/8"OD

2.4. Soft tubing, ¼" OD

2.5. Connectors/Adaptors:

1. (AQ-115X) TUBING CONNECTION 1/8" OD tubing x 1/4-28 Blue Polypropylene fitting natural Tefzel ferrule

2. (PL-104X 10/P) PLUGS 1/4-28 natural PFA Tef

3. Zip ties

2.6. Nitrogen gas

2.7. Plastic 30 ml syringes

2.8. Glass syringes, (Micro-mate) 20mL

2.9. Graduated cylinders, from 10-50mL

2.10. Can koozies

3. MACHINERY

3.1. Incubator(s) with shakers
4. PROCEDURES FOR OVERALL EXPERIMENT SET-UP

4.1. VS Inoculum to VS Sludge Ratio Testing

4.1.1. Determine the VS, TS, and COD of Inoculum and TWAS (See SOP #0005 and SOP #0006)

4.1.2. Determine volume of inoculum and TWAS to be added to each digester based on various ratios of VS of inoculum to VS of TWAS - 1:1, 1:1.5, and 1:0.5

Note: We used 43mL, 65mL, and 22mL of TWAS with 135mL of inoculum and the remainder DI water to 200mL

4.1.3. Follow Digester Setup Procedure (Section 5) and Monitor and Sample as described below (Section 6)

Note: The purpose of this ratio test is to ensure CH₄ production is not limited by substrate or inoculum availability. Therefore, multiple substrate to inoculum ratios should be tested. After correction for endogenous methane production, results are normalized to volume of gas per mass of assayed COD. Normalized results between ratios are compared to insure the assay was not substrate limited. The substrate to inoculum ratio should not affect the final volume of methane produced, but should control the rate at which methane is produced.

4.2. Co-Digestion of Algae and Sludge

4.2.1. Pour appropriate amount of algae into glass bottle and cap

4.2.2. Place on bench overnight to allow settling

4.2.3. Syphon off supernatant

4.2.4. Use the remaining dense algae for testing

4.2.5. Determine VS, TS, and COD (See SOP #0005 and SOP #0006) of each algae (chorella and scenedesmus) and correlate to Optical Density (optional, See SOP #0008)

4.2.6. Determine mL of Algae, TWAS, and inoculum to add to each digester

4.2.6.1. Use the VS inoculum to VS substrate ratio determined in Section 4.1
4.2.6.2. Based on this VS/VS ratio, calculate the volume needed for each algae/TWAS ratio

<table>
<thead>
<tr>
<th>Exp. Run</th>
<th>Target Algal Species</th>
<th>Fraction of by VS (%) of Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wastewater Sludge</td>
</tr>
<tr>
<td>1</td>
<td>Chlorella</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

4.2.6.3. Follow Digester Setup Procedure (Section 5) and Monitor and Sample as described below (Section 6)

4.3. Alkalinity Test

4.3.1. Using optimum algae:sludge ratio found in section 4.2, follow Digester Setup Procedure (Section 5) for 3 sets of digesters (4 digesters each set): control (no alkalinity adjustment), and two sets with above average operating alkalinity (3000 mg/L and 5000 mg/L). Also set up a triplicate control for each set without substrate (just inoculum and DI).

4.3.2. Determine the alkalinity (See SOP #0004) of all the digester components (inoculum, TWAS, chlorella, scenedesmus)

4.3.3. Calculate the alkalinity of each digester based on the alkalinity and amounts of the components.

4.3.4. Make additional digesters (duplicate) from at least 2 of the tests to check the alkalinity against the calculated values.

4.3.5. If values are similar, calculate the amount of NaHCO₃ needed for each set.

Note: amount of NaHCO₃ to add (in grams) =

\[
\text{[Alkalinity Increased Desired (mg/L) / 50,000 (mg CaCO₃/eq)] * 61 (g HCO₃/eq) * [1 + 23/84] * volume (in liters) you are adding to}
\]
4.3.6. Add enough NaHCO₃ to the first set of digesters to increase the alkalinity by 2,000 mg/L.

4.3.7. Add enough NaHCO₃ to the second set of digesters to increase the alkalinity by 4,000 mg/L.

4.3.8. Test the pH of the additional digesters; this is the starting pH.

4.3.9. Retest the additional digesters to ensure they are at the desired alkalinity.

4.3.10. Put into incubator as above and do not touch 3 digesters from each set until final day

4.3.11. Monitor and Sample as described below (Section 6)

4.3.12. Halfway through the experiment (15th day in this case) sacrifice 1 digester from each set and test the alkalinity and pH

4.3.13. At the end of the test (day 30), analyze pH and alkalinity of remaining 3 digesters from each set.

4.4. Temperature Test

4.4.1. Using optimum algae:sludge ratio found in section 4.2, follow Digester Setup Procedure (Section 5) for 3 sets of digesters (3 digesters each set) (plus a set of controls for each) (total 18 digesters)

4.4.2. Place one set in an incubator at 10°C and one at 20°C

4.4.3. Monitor temperature with an external thermometer

4.4.4. Monitor and Sample as described below (Section 6)

5. PROCEDURES FOR SETTING UP DIGESTERS

5.1. Clean and dry all bottles, caps, syringes, tubing, and graduated cylinders

5.2. Prepare caps

5.2.1. One port capped, two ports with 1/8” OD hard tubing attached with ferrule and tubing connection – one with longer soft tubing (for syringe) and one with shorter soft tubing.
5.2.2. Zip tie soft tubing around hard tubing by double wrapping and plug soft tubes with 2-way valves.

5.2.3. Keep hard tubing ends in headspace.

5.2.4. *Put a small piece of red tape around the longer tube to mark it for the syringe.*

*Note: Optional, may cause stickiness*

5.2.5. Tighten all fittings.

5.3. Prepare each test in triplicate

5.4. Label caps 1 – X and record in lab book and excel what will go in which digesters.

5.5. Cover bottles with a koozie and electrical tape or heavy duty aluminum foil to inhibit light transfer.

5.6. Add determined amount of substrate to the 250mL bottles

5.7. Add DI water (calculated amount to fill to 200mL once inoculum is also added)

5.8. Cap digesters

5.9. Purge with N₂ gas at a flow rate of at least 1 L/min for at least 2 minutes for all digesters

5.9.1. Flush bottles in the hood with hood exhaust turned on.

5.9.2. Connect tubing from N₂ canister to the valve on the longer tubing and open the valve on the short tubing to allow gas to flow out

5.9.3. Close short tube valve, close long tube valve, release Nitrogen gas tubing

5.9.4. Open short tube valve to release extra pressure and then close.

5.10. Add inoculum

5.10.1. Cap inoculum bottle with a customized 3-port cap to allow inoculum extraction, nitrogen entering, and nitrogen exiting (if tubes are in the head space)

5.10.2. Use soft tubing connected to a 30 ml plastic syringe to extract inoculums

5.10.3. *Keep nitrogen gas blowing into tubing of inoculum bottle while extracting*
Note: Optional, will only work if tubes are in head space and using a low gas flow rate

5.10.4. Start with digester 1 and continue in sets of 9

5.10.5. Shake inoculum bottle before each extraction

5.10.6. Extract required amount of inoculum and insert into short tubing valve of digester

5.10.7. Attach syringe, open long tube valve to release pressure, open short tube valve and insert inoculum

5.10.8. Close valves and detach syringe

5.10.9. Repeat if more inoculum is needed

5.10.10. Go back to hood with nitrogen gas

5.10.10.1. Open long tube valve

5.10.10.2. Open short tube valve

5.10.10.3. Insert nitrogen gas into short tube and flush for 10-20 seconds.

5.10.10.4. Close short tube valve, close long tube valve, release Nitrogen gas tubing

5.10.10.5. After all valves are closed, quickly open and close the longer tube valve to release any excess pressure. Do this multiple time in a half circle motion until all gas pressure is released.

5.11. After completing 9 digesters, cover bottles, put into incubator and record start time

5.12. Place on a shaker at 150 rpm at desired temperature

Note: For the purpose of accuracy, never add less than 10mL of substrate or inoculum to a digester. If less is called for, use a dilution and always add at least 10mL to the digester and dilution.
6. **MONITORING AND SAMPLING**

6.1. **Volume Measurement**

6.1.1. Attach a clean, dry glass syringe to the digester to be sampled

6.1.2. While bracing the plunger, turn valve to release gas

6.1.3. If more than 20mL of gas appears to have been produced, push plunger to about the 15mL mark and close valve. Release plunger. Read gas level and repeat until all gas has been released.

6.1.4. Record reading on Volume Data Sheet

6.1.5. Save at least 20mL if testing the gas constituents (6.2 below), or perform GC measurement first before wasting gases.

6.2. **Gas Chromotography Measurement**

See SOP #0012
1. PURPOSE
This protocol describes the process of calibrating the Gas Chromatography with TCD for CO₂ and CH₄.

2. MATERIALS
2.1. Standard CO₂, 99.8 % grade (Scotty)
2.2. Standard CH₄, 99.0 % grade (Scotty)
2.3. 30/balance CO₂/CH₄ calibration grade (Praxair)
2.4. 20mL Glass Syringe, (Micro-mate)
2.5. 5x Summa Canisters
2.6. Fischer Scientific Digital Flow Sensor

3. MACHINERY
3.1. GC (HP 6860) with TCD and sample injection valve
3.2. GC Control Computer
3.3. Vacuum Pump

4. PROCEDURE FOR GC CALIBRATION
4.1. FLUSH CANISTERS
4.1.1. Place canisters in oven at 60°C and keep them inside during the whole procedure
4.1.2. Attach flow sensor connected to vacuum pump to first canister and vacuum until -750 mm Hg pressure is reached
4.1.3. Switch valve to nitrogen side and fill canister with Nitrogen gas until about 20 mm Hg pressure
4.1.4. Repeat filling and vacuuming seven times
4.1.5. During last nitrogen fill cycle, fill to 100 mm Hg pressure
4.1.6. Take sample, making sure to purge once, and inject into GC to test for any residual gases

4.2. CREATE CALIBRATION STANDARD DILUTIONS

4.2.1. At room temperature, vacuum nitrogen out of clean canisters until -750 mm Hg and fill with CO₂ and CH₄ to a total pressure of 150 mm Hg in the following ratios: 90 mm Hg:810 mm Hg (CO₂:CH₄/10:90), 180 mm Hg:720 mm Hg (CO₂:CH₄/20:80), 270 mm Hg:630 mm Hg (CO₂:CH₄/30:70), 360 mm Hg:540 mm Hg (CO₂:CH₄/40:60), 450 mm Hg:450 mm Hg (CO₂:CH₄/50:50)

4.2.2. Extract three 18-20mL samples for each canister and run in GC according to SOP 12. Run each set in triplicate and flush with gas to be filled before filling each time. Use this data to make a GC calibration curve plotting moles of CO₂ and CH₄ against peak area.

Note: Moles of sample_gas = (1*0.002)/(0.0821*293)*Percent-of-Gas(in decimal)

4.2.3. Run seven 18-20mL samples of the 30:70 pre-made standard and compare results to self-made 30:70 standard to ensure they are very similar. Make a control chart from these seven samples to use as a calibration check each sample day.

5. QA/QC

5.1. Always make sure reference gas is flowing through before turning on the detector, otherwise you will burn the detector.

5.2. Always inject the gas sample at a slow, consistent rate.

5.3. An injection sample of 20mL is preferred with a minimum sample size of 10mL
1. PURPOSE
This protocol describes the process of testing the alkalinity of digester sludge solution.

2. MATERIALS
   2.1. HCl (0.02 N)
   2.2. 100-200 ml Beaker
   2.3. 50-100 mL Beaker (x2)
   2.4. 50 ml Pipette with turn valve
   2.5. Pipette clamp and stand

3. MACHINERY
   3.1. pH meter

4. PROCEDURE FOR ALKALINITY TESTING
   4.1. Attach pipette to clamp on stand.
   4.2. Pour approximately 10-20 mL of HCL into a small beaker and pour this into the pipette to clean it.
   4.3. Open the valve and let the HCL go into a waste beaker.
   4.4. Fill the large beaker with 20 ml of digester sludge solution, insert pH probe, and place under pipette.
   4.5. Add 10 ml of HCl to pipette.
   4.6. Slowly add HCl to digester solution until pH reaches 4.5.
   4.7. Once pH 4.5 is reached, determine amount of HCl added.
   4.8. Multiple volume of HCl added by its normality and 50,000 and divide by the mL of solution tested (20 mL in this case) to get the alkalinity in mg CaCO3/L.

NOTE: The pH will naturally vary during digestion, which we will measure with a pH meter at the beginning and end of the alkalinity digestion test. No manual adjustment of the pH will be made.
5. COMMENTS

5.1. Be sure to wear safety goggles, gloves, and lab coat when handling HCl.

1. PURPOSE

This protocol describes the process of testing the COD of a sample.

2. MATERIALS

2.1. Hach 0-1500 mg/L COD Reagent Vials
2.2. 40 ml Vials
2.3. 1000 mg/L COD Standard Solution
2.4. Potassium acid phthalate (KHP)
2.5. DI Water
2.6. Kim Wipes

3. MACHINERY

3.1. COD Reactor
3.2. Spectrophotometer

4. PROCEDURE FOR COD CALIBRATION

4.1. Make a 3000 mg/L COD standard from the KHP by weighing out the appropriate amount and adding it to DI water.

4.2. Dilute the 3000 mg/L stock to make concentrations of 250, 500, 750, 1000, 1250, and 1500 mg/L.

*Note:* Do not dilute one standard to make another. Use the 3000 mg/L base for all of the standards. Check the dilution by also running the 1000 mg/L standard to ensure accuracy.

4.3. Follow the Procedure in Section 5 below to determine the COD of these standards.
4.4. Create a COD calibration curve (COD (mg/L) vs Absorbance at 620nm

4.5. Create a COD-OD calibration curve by plotting the OD readings of the 0, 250, 500, 750, 1000, 1250, and 1500 mg/L standards versus their COD concentration. This curve can be used to determine the COD of unknown samples by reading their OD.

5. PROCEDURE FOR COD DETERMINATION

5.1. Standard Procedure

5.1.1. Hold the vial at a 45 degree angle and add 2 ml of the diluted (2x or 3x) sample or standard to be tested to the Hach COD reagent vial.

5.1.2. If you are blanking with reacted reagent, add 2 ml of DI water to another COD reagent vial to act as a blank.

Note: we created our calibration curve by blanking with DI water (unreacted); therefore, we zero our spectrometer with unreacted DI water before each reading.

5.1.3. Cap the vials and invert to mix.

5.1.4. Place sample, standards, and blank vials into the COD reactor at 150°C for 2 hours.

5.1.5. After reactor has finished its 2 hour run, wait for it to cool to 120°C, invert all vials to mix, and let cool to room temperature.

5.1.6. Once cool, clean the vials with a kim wipe and use the spectrophotometer to get absorbance readings of the vials at 620nm.

5.1.7. Use the COD calibration curve to determine the COD

5.2. Using Optical Density

5.2.1. Use the COD-OD calibration curve created in step 4.3 above to determine the COD of unknown samples based solely on the optical density.

6. QA/QC

6.1. Run a 1,000 mg/L COD standard each test day to ensure consistency
1. PURPOSE
This protocol describes the process of determining the volatile solids (VS) and total solids (TS) content of a sample.

2. MATERIALS
2.1. Evaporating Dishes
2.2. Graduated Cylinder or 20 ml Syringe
2.3. Pipette and tips for 10 mL

3. MACHINERY
3.1. 105°C Oven
3.2. 550 °C Oven
3.3. Dessicator
3.4. Scale

4. PROCEDURE FOR VS/TS TESTING (Based on Standard Methods 2540-B, 2540-E and 2540-G)
4.1. Clear the evaporating dish of any contaminants by heating it at 550°C for 1 hour, then let dish cool to room temperature and store in desiccator until needed. Weigh dish immediately before use.
4.2. Transfer 10 ml of sample onto dish, weigh and then place dish in drying oven at 105 °C for at least 2 hours. This weight minus dish weight is total sample weight.

Note: According to standard methods, the sample should be evaporated on a water bath or in a drying oven first, and then dried at 103-105°C for at least 60 minutes.
Note: The sample size should yield a total solids residue between 2.5 and 200 mg.

4.3. Remove from oven, allow dish to cool in dessicator to room temperature and weigh.

4.4. Repeat drying and cooling until weight change is less than 4% of previous weight or 0.5 mg, whichever is less. This weight minus the dish weight is the TS weight.

4.5. Place dish into furnace at 550°C for 45 minutes.

Note: If semi-solid samples are to be measured, such as wastewater sludge, follow standard method 2540-G: use 25-50 grams of wet sample and, after TS determination, heat at 550°C for 1 hr. If the residue contains large amounts of organic matter, first ignite it over a gas burner and under an exhaust hood in the presence of adequate air to decrease losses due to reducing conditions and to avoid odors in the laboratory.

4.6. Remove from furnace and partially cool the dish in air. Then transfer to dessicator for final cooling to room temperature.

4.7. Weigh dish as soon as it is cooled, and repeat heating and cooling cycle until weight change is less than 4% of previous weight or 0.5 mg, whichever is less. The TS plus dish weight minus this weight is the VS weight.

4.8. % TS = TS weight / Total sample weight found in step 4.2

4.9. % VS = VS weight / Total substrate weight found in step 4.2

5. COMMENTS

TS cycle: 120 minutes, 30 minutes, if <4% change finished, else continue 30 min cycles VS cycle: 45 minutes, 15 minutes, if <4% change finished, else continue 15 min cycles
1. PURPOSE

This protocol describes the process of growing algae for use in laboratory experiments.

2. MATERIALS

   2.1. 4 L Flask or larger container

   2.2. Primary Wastewater Effluent

   2.3. Magnetic Stirrer Bar

   2.4. 99.9% CO₂ tank with regulator and associated connectors, valves, and tubing

   2.5. Air source

   2.6. Diffuser

   2.7. Aluminum Foil

   2.8. Culture of Algae to be grown

3. MACHINERY

   3.1. Stir Plates

   3.2. T-12 Fluorescent Light Fixture and 2x 40W Bulbs

   3.3. Air and CO₂ Flow Regulators

4. GROW ALGAE

   4.1. Setup up the light fixture so that it will be no more than two inches above the 4 L flask.

   4.2. Setup a 5% CO₂ stream by connecting the CO₂ tank and air source to their flow regulators set at 1 ml/min and 20 ml/min, respectively. Then combine the two flows into one tube that is capped with a diffuser.
Note: We are using a N₂ flow controller instead of a CO₂ controller. Therefore, we adjust the flow based on gas K factor (see flow meter manual) so that 1 mL/min on the N₂ flow controller equals 0.739 mL/min CO₂ in reality.

4.3. Place a stirrer bar into the 4 L flask and place the flask on top of the stir plate. This unit should be centered under the light fixture.

4.4. Fill the flask with 0.5 L of algae solution and 2 L of primary effluent and place the tube with diffuser as close to the bottom as possible. Cover the mouth of the flask with foil.

Note: We started with about a 20 mL culture in 1 L of effluent

Note: We use a glass rod to secure the tubing to in order to get the diffuser to the bottom

4.5. Turn on the stirrer so that a gentle swirl is created in the flask. This should prevent any algae from settling.

4.6. Add fresh effluent to the flask at least once every two weeks. If the flask is full, pour out half of the solution and replace with effluent.

4.7. Continue growing this poured half in another bottle if needed.

Note: pH of the algae can be buffered with Na₂CO₃ if needed to prevent acidification. pH of chlorella should be between 7 and 8. For maximum growth, light is on 24/7.
1. PURPOSE
   This protocol describes the process of collecting and storing thickened waste activated sludge (TWAS) and inoculum.

2. MATERIALS
   2.1. 2 L Glass Bottle
   2.2. Ported cap with attached 30mL plastic syringe
   2.3. Wide-Mouth Container
   2.4. Cooler

3. MACHINERY
   3.1. None

4. PROCEDURE FOR TWAS AND INOCULUM COLLECTION AND STORAGE
   4.1. For collecting TWAS, use a wide mouth bottle to make it easier to collect since it must be manually scooped up.
   4.2. Use a standard 2 L glass bottle for inoculum, equipped with cap with pressure releasing syringe attached. When collecting it, leave as little headspace as possible in the bottle and make sure the cap is airtight to minimize oxygen in the bottle.
   4.3. Keep the bottles in a cooler during transport to minimize temperature fluctuations. This is more important for the inoculum since temperature affects its performance.
   4.4. TWAS can be stored in a 4°C room or refrigerator for up to 3 days.
4.5. Inoculum should be used as soon as possible, but can be stored for up to 24 hours in an incubator at 35°C or whatever temperature you are testing.

**NOTE:** Be sure to vent the inoculums bottle frequently or gas pressure will build up inside the bottle. A cap vented with a syringe may also be used to prevent gas buildup.
1. **PURPOSE**

This protocol describes the process of measuring the proportions of CO₂ and CH₄ with a Gas Chromatography with TCD.

2. **MATERIALS**

   2.1. Sample in glass syringe with valve

   2.2. 30/70 CO₂/CH₄ calibration check sample

3. **MACHINERY**

   3.1. GC (Agilent 6890) with TCD and sample injection valve

       3.1.1. Column Specifications:
               ALL TECH Chromosorb 106 80/100
               6’x1/8” x .085” SS
               Part # C-5000
               Bath Serial # 156-705100057
               MAOT 275°C

   3.2. Computer with Agilent ChemStation software

4. **PROCEDURE FOR GC MEASUREMENT**

   4.1. Make sure GC and computer are on and open method “Digester” in the ChemStation software

   4.2. Ensure all gases are flowing and all temperatures are as needed

       4.2.1. Sample valve temperature should be 70°C ± 2°C

       4.2.2. Keep FID oven on 250°C even though not using

       4.2.3. Sample valves should look like this: oxidizer / / methanizer

       4.2.4. Aux 3 is the Nitrogen carrier gas
4.2.5. Use the method “DIGESTER.M” -> for details on this method see the Comments below.

4.3. Create new Sequence

4.3.1. Edit Sequence Table and Parameters

4.3.2. Click “Run” on the Sequence Table

4.4. Load 20mL sample in glass syringe and inject into GC sample valve at steady speed (about 10 seconds for 20mL)

4.5. Press “Start” on the GC

4.6. Look at the peak graphs to make sure everything is looking right

4.7. Flush syringe with N₂ or ambient air before next sample

4.8. When run is finished, wait until temperature is cooled and run next sample

4.9. After finished with all runs, turn off the filament and close the method on the software

5. QA/QC

5.1. Always make sure reference gas is flowing through before turning on the detector, otherwise you will burn the detector.

5.2. Always inject the gas sample at the same steady speed.

5.3. An injection sample of 20 mL is preferred with a minimum sample size of 10mL

5.4. Run a calibration check sample as the first run each sampling day and check against control chart. Use the 30:70 standard as the calibration check.
6. COMMENTS

Method Details
We were not using the FID detector so only the Heater and Makeup flow were checked (on)
1. PURPOSE
This protocol describes the process of determining the total (TC) and fecal coliform (FC) counts (CFU) in digester liquid using the IDEXX Colisure method.

2. MATERIALS
2.1. Quanti-Tray/2000 and Colisure packs (IDEXX)
2.2. 100 mL bottles (number depends on number of dilutions) – autoclaved
2.3. 100 mL volumetric flask (x2) – autoclaved
2.4. ~500 mL beaker (x2) – one for waste, the other autoclaved
2.5. Autoclaved DI water (~550 mL per test)
2.6. 1 ml and 10 ml pipettes and autoclaved tips
2.7. Marker and labeling tape
2.8. 70% ethanol for sterilizing

3. MACHINERY
3.1. Incubator at 35°C
3.2. Autoclave
3.3. Laminar flow hood
3.4. Quanti-Tray sealer
3.5. 6 W, 365 nm UV light (IDEXX)
4. PROCEDURE FOR SETTING UP COLISURE TEST

4.1. After autoclaving the required supplies, sterilize the entire laminar flow hood with ethanol solution and then the UV light for 15 minutes.

Note: The entire procedure must be performed in the hood.

4.2. Spray pipette bodies down with ethanol as well as gloved hands. Alternatively, use antibacterial gel on skin if no gloves are worn.

4.3. For this example, we will be doing 100x, 1000x, or 2000x dilutions.

For 100x, 1 ml of sample plus 99 ml of DI water. To do this, measure out 100 ml DI water with the volumetric flask, then remove 1 ml with the 1 ml pipette. Afterwards, pour the 99 ml DI water into a 100 ml bottle. Add 1 ml of sample with the pipette. Prepare two more bottles to complete the triplicate.

For 1000x, To do this, measure out 100 ml DI water with the volumetric flask, then remove 1 ml with the 1 ml pipette. Afterwards, pour the 99 ml DI water into a 100 ml bottle. Add 1 ml of sample with the pipette(this makes 100x dilution). Prepare two more bottles to complete the triplicate. Next, in a small beaker make a 10x dilution, (90ml H₂O+10ml sample). Remove 1ml from the bottles with 100x dilution. Then add 1ml of the 10x dilution in the small beaker to the 100x dilution bottle which should have 99ml of solution in it. (99ml of 100x + 1ml of 10x)

For 2000x, To do this, measure out 100 ml DI water with the volumetric flask, then remove 1 ml with the 1 ml pipette. Afterwards, pour the 99 ml DI water into a 100 ml bottle. Add 1 ml of sample with the pipette(this makes 100x dilution). Prepare two more bottles to complete the triplicate. Next, in a small beaker make a 20x dilution, (190ml H₂O+10ml sample). Remove 1ml from the bottles with 100x dilution. Then add 1ml of the 20x dilution in the small beaker to the 100x dilution bottle which should have 99ml of solution in it. (99ml of 100x + 1ml of 10x)
Note: Take care not to let the pipette body touch the volumetric flask when removing the 1 mL of DI water. If this is not possible, the 100 mL of DI water may be poured into the bottle and then the 1 mL removed. IMPORTANT: If this is done with a used bottle, make sure to dispose of the pipette tip afterwards, do not continue to use it for DI water.

4.4. Add one pack of Colisure to each bottle and swirl until no large media particles remain. Try to minimize foam production.

4.5. Open a Quanti-Tray/2000 by curling the tray back and pour in the contents of one 100 ml bottle. Take caution to not touch the inside of the Quanti-Tray. At this point, you may reuse the bottle for the same digester if you do not have enough bottles by rinsing it at least twice with DI to remove as much foam as possible.

4.6. Place the tray in the Quanti-Tray sealer insert and seal the tray in the sealer. Remember that the sealer needs about 10 minutes to warm up after turning it on and is ready when the green light is on.

4.7. Place the tray in a 35°C incubator in a dark location for 24 hours.

5. PROCEDURE FOR READING QUANTITRAYS

Total Coliforms

5.1. After 24 hours, read trays by counting the number of red or magenta squares (positive). If a square is still yellow after 24 hours, it is negative.

5.2. If there are orange or pink squares, continue incubating for another 24 hours (up to 48 hours total).

5.3. At 48 hours, count the number of magenta or red squares as positive. At this time, squares that are still orange or pink are negative.
**Fecal Coliforms**

5.4. After counting TC, count the number of large and small squares that fluoresce by using a portable UV lamp in a dark room.

**Total and Fecal Coliforms**

5.5. Record the number of positive large squares and positive small squares for both Total and Fecal.

5.6. Use the given chart to find the MPN for Total and Fecal Coliforms based on the number of positive large and small squares.

5.7. Quantitray-2000 can read up to 2,419 MPN, so dilutions may be necessary. If this is the case, multiply the MPN found on the chart by the dilution factor to obtain the MPN.

**6. QA/QC**

6.1. Tests should be done in at least triplicate

6.2. Take care to keep hands, surfaces and tools sterile
1. **PURPOSE**

   This protocol describes the process of tests to be run at the end of a digestion cycle.

2. **MATERIALS**

   2.1. See SOPs #0005, 0006, 0017, 0021, and 0022

3. **MACHINERY**

   3.1. See SOPs #0005, 0006, 0017, 0021, and 0022

4. **PROCEDURE FOR POST ANALYSIS**

   4.1. Autoclave necessary glassware, D.I. Water, and pipet tips day before starting coliforms.

   4.2. Test each digester (excluding controls) for Total Coliforms and Fecal Coliforms using the Quantitray 2000 method in triplicate (see SOP #0017)

   *Note:* This should be done first so that the digesters are not exposed to open air before being sampled for coliforms.

   4.3. Test each digester for COD in triplicate (see SOP #0005)

   4.3.1. Run triplicate of 1000 standard calibration check each testing day

   4.4. Test each digester for TS/VS in duplicate(triplicate if possible) (see SOP #0006)

   4.4.1. Keep samples in the cold room for up to one day while waiting for oven space

   4.5. Test each digester for Total Nitrogen (see SOP #0021) and Total Phosphorous (see SOP #0022) in duplicate

   4.5.1. Run single calibration standard for both N and P (~50 mg/L) each testing day
5. QA/QC

5.1. Do not open digesters until in the laminar flow hood. Make the dilutions needed for the coliform testing before exposing the digestate to the air to do the other tests.

6. COMMENTS

Place all used digesters and dilutions in cold room for one week post testing in case tests need to be re-run.
Standard Operating Procedure #0021:  
Total Nitrogen Determination

1. PURPOSE

This protocol describes the process of determining the total nitrogen in a sample (specifically anaerobically digested substrate) by the Hach persulfate digestion method (Method 10072). This is similar to method 4500-N C of the Standard Methods for Water and Wastewater.

2. MATERIALS

2.1. Assorted glass beakers for dilutions and holding chemicals

2.2. HCL for acid washing

2.3. Nitrate-nitrogen standard

2.4. HACH Total Nitrogen Test N Tube High Range, 10-150 mg/L (cat # 27141-00)

2.5. Kim wipes

3. MACHINERY

3.1. Digester – DRB 200

3.2. Spectrometer

4. PROCEDURE FOR PHOSPHATE TNT METHOD (Method 10127)

4.1. Turn on the DRB 200 and heat to 105°C

4.2. Use a funnel to add one Nitrogen Persulfate Powder Pillow to each vial

4.3. Add 0.5 mL of sample to a TNT vial

Note: Prepare diluted sample as appropriate, normally (2x)

4.4. Cap tightly and shake vigorously for at least 30 seconds to mix

Note: The persulfate reagent may not dissolve completely after shaking. This will not affect accuracy.
4.5. Insert the vials in the DRB 200 reactor and close the cover

4.6. Heat for EXACTLY 30 minutes

4.7. After the timer expires, immediately remove the hot vials from the reactor and allow them to cool in a rack to room temp (18-25°C)

**Note:** At this point you can stop the test and resume 4.8 the following day.

4.8. Add the contents of one Total Nitrogen Reagent A Powder Pillow to each vial

4.9. Cap and shake for 15 seconds

4.10. Start the timer (a three-minute reaction period will begin)

4.11. Add the contents of one Total Nitrogen Reagent B Powder Pillow to each vial

**Note:** The reagent will not completely dissolve. This will not affect accuracy. The solution will begin to turn yellow.

4.12. Cap and shake for 15 seconds

4.13. Start the timer (a two-minute reaction period will begin)

4.14. After the timer expires, pipet 2 mL of digested, treated sample into one TN Reagent C vial (separate vial)

4.15. Cap the vials and invert ten times to mix. Use slow, deliberate inversions for complete recovery.

**Note:** The tubes will become hot to the touch

4.16. Start the timer (a five-minute reaction period will begin)

4.17. Zero the spectrometer with DI, Wipe the vial with a Kim wipe, and Read in a spectrometer at 410 nm

**Note:** Make calibration curve (10-150mg/LNO₃⁻-N)

5. **QA/QC**

5.1. Run a standard each new day of testing
1. PURPOSE

This protocol describes the process of determining the total phosphorous in a sample (specifically anaerobically digested substrate) by the HACH Molybdovanadate Method with Acid Persulfate Digestion (Method 10127 Adapted from *Standard Methods for the Examination of Water and Wastewater* (4500 B-C)).

2. MATERIALS

2.1. Assorted glass beakers for dilutions and holding chemicals

2.2. HCL for acid washing

2.3. Phosphate Standard, 50 mg/L (might need to get 100 mg/L)

2.4. HACH Total Phosphate Test N Tube High Range, 1-100 mg/L (cat # 27672-45)

2.5. Kim wipes

3. MACHINERY

3.1. Digester – DRB 200

3.2. Spectrometer

4. PROCEDURE FOR PHOSPHATE TNT METHOD (Method 10127)

4.1. Turn on the DRB200 and heat to 150°C

4.2. Add 5 mL of sample to a TNT vial

**Note:** Prepare diluted sample accordingly, normally (5x). ex. 5ml sample+20ml H₂O

4.3. Use a funnel to add one Potassium Persulfate Powder Pillow to each vial

4.4. Cap tightly and shake to dissolve

4.5. Insert the vials in the DRB 200 reactor and close the cover
4.6. Set the instrument timer to 30 minutes and start (a 30 min heating period will begin)

4.7. After the timer expires, carefully remove the hot vials from the reactor and allow them to cool in a rack to room temp (18-25°C)

**Note:** At this point you can stop the test and begin with 4.8 the next day

4.8. Add 2 mL of 1.54N NaOH to each vial, Cap and invert to mix

4.9. Add 0.5 mL of Molybdovanadate reagent to each vial, Cap and invert to mix

4.10. Start the timer for 7 minutes (A seven min reaction period will begin)

**Note:** Read the sample between 7 and 9 minutes after adding the Molybdovanadate reagent.

**Note:** Make calibration curve (0-100mg/L)

4.11. Zero the spectrometer with DI, Wipe the vial with a Kim wipe, and Read in a spectrometer at 420nm

5. **QA/QC**

5.1. Run a standard each new day of testing

6. **COMMENTS**

6.1. The final samples will contain molybdenum. In addition, the final samples will have a pH of less than 2 and are considered corrosive.
APPENDIX B

RESULTS FROM PRELIMINARY TESTS
Figure B.1. Cumulative biogas production at various sludge to inoculum ratios.

Figure B.2. Cumulative biogas production for high vs low VS loading.
Figure B.3. (a) Biogas produced per mg COD introduced in high and low VS loading. (b) Biogas produced per mg VS introduced in high and low VS loading.