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Anaerobic Co-digestion of Chlorella vulgaris and Dairy Whey for Enhanced Methane Production

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Anaerobic Co-digestion of *Chlorella vulgaris* and Dairy Whey for Enhanced Methane Production

by

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Abstract

The anaerobic digestion process is an additional step that can be implemented at wastewater treatment facilities for the production of biogas (i.e. methane) that can be used to generate energy and significantly reduce the facility's energy cost. An emerging area of interest with anaerobic digestion is the inclusion of high-strength degradable organic waste (in addition to wastewater solids) that can lead to increased methane production by methanogens. *Chlorella vulgaris* (*C. vulgaris*), a species of green microalgae, is a ubiquitous green alga often present at water-water treatment plants. I investigated its usefulness in an existing wastewater treatment process. Two investigations were conducted, the first to investigate the biomass growth potential of *C. vulgaris* in wastewater (primary clarifier and secondary clarifier effluents) and associated nutrient (ammonia and phosphorus) uptake, and the second to investigate the potential for methanogens to produce methane-rich biogas from anaerobic co-digestion of *C. vulgaris* with dairy whey. I hypothesized that (1) *C. vulgaris* would grow well in both wastewater effluents, but achieve the greatest total biomass production when cultured in primary clarifier effluent; and (2), including *C. vulgaris* in the anaerobic digestion of wastewater solids and dairy whey (i.e. co-digestion) would result in the production of biogas volumes greater than that produced from the digestion of only wastewater solids and only dairy whey. A growth experiment was conducted to measure algal biomass growth in primary and secondary clarifier effluents, and an anaerobic digestion trial was conducted to measure biogas volume and composition (% methane). Both hypotheses were supported by the results. The most biomass production was observed in primary clarifier wastewater

effluent (605 mg/L). The highest biogas volumes (827 ml) and methane concentrations (56.8%) were obtained from anaerobic co-digestion of 32 ml (48% feed ratio) *C. vulgaris* (15 mg/L volatile solids) with 32 ml (48% feed ratio) of dairy whey (~1000 mg/L volatile solids). The data from the anaerobic digestion experiment was used to calculate potential savings at an existing wastewater treatment facility. The results indicated that including *C. vulgaris* in the anaerobic co-digestion of wastewater solids and a high-strength organic feedstock (i.e. dairy whey) could result in significant financial savings to wastewater treatment systems with anaerobic digesters. Further site-specific studies are needed to determine more accurately what the maximum digester loading rates of *Chlorella* and dairy whey (or other high-organic strength feedstocks) are, and subsequent methane production and energy savings.

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Background

The typical wastewater treatment process incorporates a primary physical treatment followed by a secondary biological treatment (Kerri, 2004). Primary treatment involves the physical removal of large floating or settled solids, mostly fats, oils and greases, are collected as primary sludge (Kerri, 2004). After primary treatment, the wastewater continues on (as primary clarifier effluent) to secondary treatment, which utilizes bacteria and other microorganisms to oxidize dissolved and suspended organic matter (Kerri, 2004). This creates a secondary sludge, which is then separated and removed from the water (now called secondary clarifier effluent) (Kerri, 2004). These two sludge types are combined, and then incinerated, land-filled or composted (LAWPCA, 2013). The secondary clarifier effluent is treated further (tertiary treatment and/or disinfection) and discharged to a nearby receiving water (Kerri, 2004).

A common secondary biological treatment design is the activated sludge process, which was first presented in 1914 in Manchester, England by scientists Edward Ardern and William T. Lockett (Bengston, 2013). Their paper "Experiments on the Oxidation of Sewage without the Aid of Filters," was later published in the Journal of the Society of Chemical Industry (Bengston, 2013). In the activated sludge process, the wastewater is aerated and the organic matter is converted into microbial cells and carbon dioxide (Sustarsic, 2009). This process allows for the formation of biological flocs (particles of solids) that readily settle out of the water when placed in a quiescent environment, typically a secondary clarifier. Unfortunately, the activated sludge process is expensive in terms of energy consumption (Oswald, 2003).

The passage of the U.S. Clean Water Act in 1972 provided funding for the construction of wastewater treatment plants across the country. While this was a monumental advance from an environmental and human health standpoint, there is still much research to be done in terms of upgrading and optimizing these facilities. Water and wastewater systems are estimated to consume over 4% of the United States' electrical energy (Electric Power Research Institute, Inc., 2002). In municipal wastewater treatment, the largest proportion of energy is used in biological treatment, generally in the range of 30% to 60% of plant energy usage (Williams, 2011). The United States relies heavily on fossil fuels for energy production, including oil, natural gas, and coal. Because these fuels are non-renewable and contribute to air pollution, including greenhouse gas emissions, water pollution, and land degradation, the reliance on them is unsustainable, and development of more sustainable energy sources must be explored (Williams, 2011). An alternative technology with great potential for generating energy necessary for secondary treatment is the process of anaerobic digestion, which produces biogas in the form of methane that can be harnessed as fuel, and thus offsets the need to purchase fossil fuel-based electricity.

Anaerobic digestion is a naturally occurring process in which anaerobic bacteria metabolize organic materials in the absence of oxygen (Figure 1). The biomass entering the process is typically in the form of large biological molecules such as proteins, carbohydrates and lipids, which are then hydrolyzed into smaller molecules such as amino acids, sugars and fatty acids (Agrawal, 2013). During the second step of anaerobic digestion, acidogenesis, fermentative bacteria degrade simple sugars, amino acids and fatty acids into acetate, carbon dioxide and hydrogen (70%), as well as into volatile fatty

acids (VFA) and alcohols (30%) (Agrawal, 2013). During the next step, acetogenesis, the biomass is converted into carbon dioxide (CO_2), hydrogen (H_2) and acetic acid, which can be directly utilized by methanogens (methane-forming archaeans). Methanogens are able to use CO_2 and H_2 as their sole food source, and ultimately convert the acetic acid into methane during the final step of anaerobic digestion, called methanogenesis. Biogas produced from anaerobic digestion has two main components: methane (CH_4, about 55–70% by volume) and carbon dioxide (CO_2, 30–40%) (Bohutskyi, 2013). Depending on the source of the biogas, other minor components include nitrogen (N, <2%), hydrogen (H), oxygen (O_2, <1%), hydrogen sulfide (H_2S) (0–50 ppm) (Bohutskyi, 2013).

Figure 1. Degradation steps of the anaerobic digestion process.
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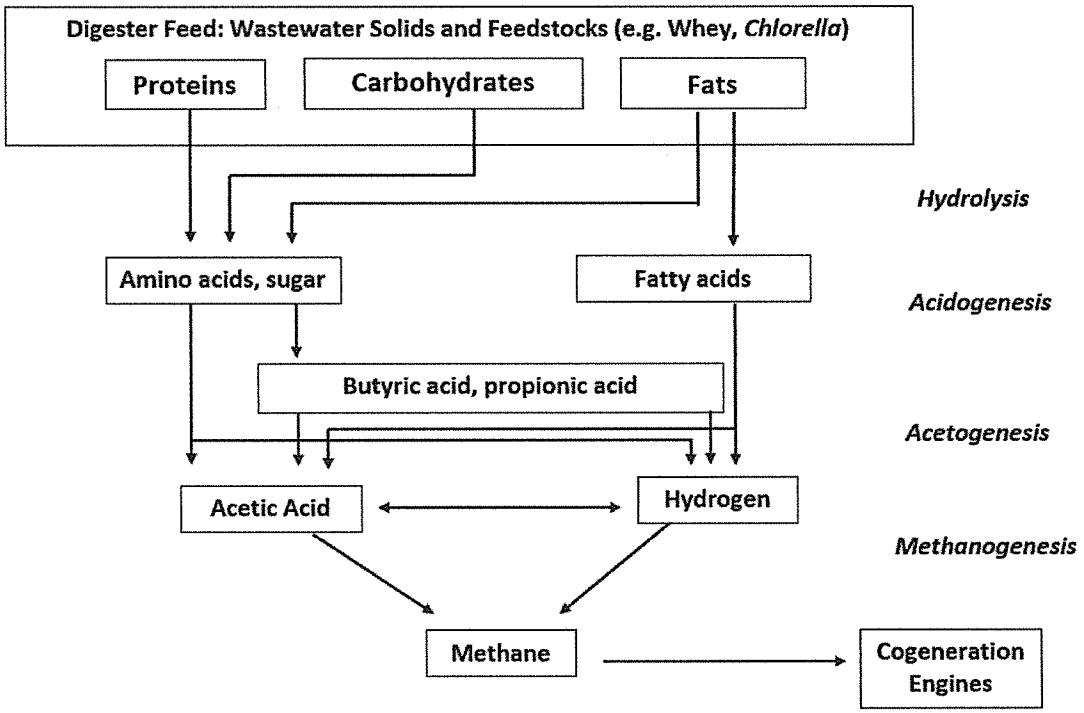


The first documented anaerobic digestion plant was built in 1859 at a leper colony in Mumbai (formerly Bombay), India (Meynell, 1976). Just over 30 years later in England (1895), anaerobic digesters were being used at sewage treatment plants, while the biogas captured was used to fuel street lamps (McCabe, et. al, 1957). However, it was not until the 1970's that anaerobic digesters began to be constructed as a part of large-scale industrial pretreatment processes, wastewater treatment processes, and biogas production. The continued use and further development of the dual role of anaerobic digestion has been stimulated by the sharp rise in fossil fuel prices and by increasingly stringent pollution control regulations (Abassi, *et al.* 2012). At the most basic level, the process of anaerobic digestion has a twofold benefit for wastewater treatment plants: it reduces the amount of solid material to be processed (and subsequently incinerated, landfilled or composted), and it produces methane-rich biogas which can potentially be used as an energy source for thermal energy and electricity production.

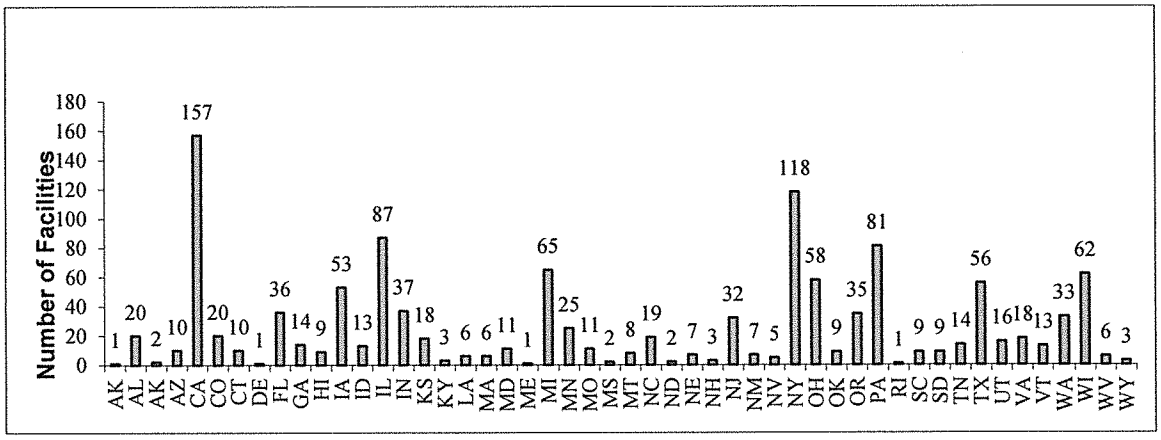
Reports of anaerobic digestion using an algal carbon source go back to the 1950's, when Clarence Golueke was one of the first scientists studying the feasibility of algal photosynthesis followed by biomass anaerobic digestion for methane production (Golueke, *et al.* 1957). Golueke's collaborator, William Oswald, was a well-known pioneer and expert in the field of engineering, specializing in wastewater treatment and algal biotechnology. Oswald and Golueke *et al.* (1957) found that the volume of gas produced per pound of volatile matter destroyed by digesters containing either raw sludge or containing algae was comparable. Later, Benneman *et al.* (1977) found anaerobic digestion to be the most practical method (over direct burning) for conversion of algal biomass grown in ponds, with more than half of the heat (10,000 BTUs per pound of

algae) converted into methane gas with digester loadings, temperatures and detention times similar to those used for wastewater sludge. In 2012, Abdel-Raouf *et al.* reported that anaerobic digestion of wastewater-grown algae for biogas production is likely the most appropriate short-term use of algal biomass at wastewater treatment plants because efficient extraction methods (for use in biofuels) are still being developed. In the last years of his life, William Oswald attested that microalgae could make an important contribution to the global quest for greenhouse reductions, and that it will become an important component in global renewable energy production and greenhouse gas abatement (Oswald 2003). He predicted the rapid improvement of processing algal biomass derived from wastewater treatment, including increases in methane production (Oswald, 2003).

In March 2014, the White House released The Climate Action Plan Strategy to Reduce Methane Emissions (United States, The White House, 2014). The U.S. Department of Agriculture (USDA), the U.S. Environmental Protection Agency (EPA) and the U.S. Department of Energy (DOE) worked with industry leaders to create a 'Biogas Opportunities Roadmap' for the country. In its discussion of biogas production, the report lists anaerobic digester systems enabling algae biomass and biofuel production as one of the potential comprehensive solution research topics. There are over 16,000 wastewater treatment facilities in the United States, and as of 2013 there were approximately 1200 anaerobic digesters associated with wastewater treatment plants (7.5%) (Figure 2). The biogas produced from anaerobic digestion of sewage sludge is an environmentally friendly fuel and the expansion of biogas production systems will be an

important contribution to the global conversion from fossil to renewable energy systems (Olsson, 2013).

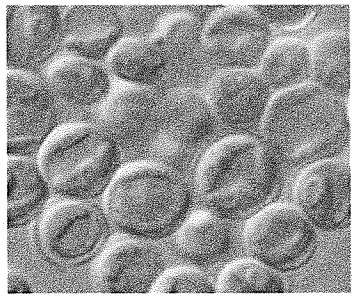
Figure 2. Anaerobic Digesters at Wastewater Facilities in the United States. Retrieved from: http://www.wrrfdata.org/biogas/biogasdata.php



The unicellular green algae *Chlorella vulgaris* (Figure 3) is a promising candidate for testing at wastewater treatment plants that have anaerobic digesters. *Chlorella*, and other chlorophytes, occur naturally in wastewater and are the primary algal taxa present in waste stabilization ponds, both in order of abundance and frequency of occurrence (Abdel-Raouf *et al.*, 2012). *Chlorella* has been cultured in existing wastewater side-streams, including primary clarifier effluent (Wang *et al.* 2012), a mixture of final effluents (after sand filtration), centrate from solids dewatering (Ficara *et al.*, 2014), and secondary treated wastewater (Gomez *et al.*, 2012), thus eliminating the need to use arable cropland or clean water. Recently, *Chlorella* has been cultured in a wider range of industrial wastewater streams as well, including those from rubber latex processing, olive

oil mills, and dairy waste (Prajapati, 2013). Research on understanding the digestibility of *Chlorella* during the anaerobic digestion process has been an emerging topic of interest in recent years, with some studies focusing on pretreatment methods (Passos, 2013) and others looking at co-digestion with high-organic strength waste (Yen, 2007).

Figure 3. *Chlorella vulgaris* cells. Retrieved from Algae Research and Supply



Anaerobic digestion of algae is potentially a more viable option for biogas (i.e. methane) production than digestion of biomass from higher plants, due to less recalcitrant cell wall composition and greater efficiency of biomass production (Korres *et al.* 2013). Higher methane yields appear to be related to easily degradable microalgae that either lack cell walls, or have protein-based cell wall lacking cellulose/hemicellulose (Torres, 2013). Cellulose hydrolysis is considered the rate-limiting step in digesters fed high-cellulosic content, such as waste paper (Yen *et al.*, 2005); whereas *Chlorella* cell walls contain glucosamine polymers, such as chitin and chitosan, that are more easily degradable (Eckhardt, 2010) than the cellulose and hemicellulose found in the cell walls of land plants (Torres, 2013). Studies show that biomass productivities are significantly greater for microalgae than for land plants, with productivity projected at 70 metric

tons/hectare/year of ash-free dry weight (i.e. organic matter) in specialized growth reactors such as high rate ponds (Figure 4) and tubular photobioreactors (Figure 4) as compared with terrestrial crops such as soybeans (3 metric tons/hectare/year), corn (9 metric tons/hectare/year) and switchgrass (10-13 metric tons/hectare/year) (Abdel-Raouf, 2012). Therefore microalgae have the ability to fix CO_2 while capturing solar energy with an efficiency 10 to 50 times greater than that of terrestrial plants (Wang, *et al.* 2008), offering a strong potential to reduce anthropogenic carbon emissions.

Figure 4. Examples of photobioreactors used for growing algae.
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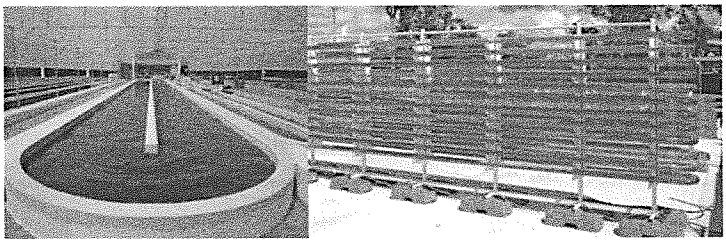


The composition of microalgae typically consists of 5-23% carbohydrate, 6-52 % protein, and 7-23% lipid (Sialve *et al.* 2009). Variations in composition among taxa could conceivably impact anaerobic digestion (Jegede, 2012), though documented correlation between algal lipids, carbohydrates and proteins and methane yield have not been reported (Torres, 2013). Lakaniemi *et al.* (2011) used two chlorophyte species, *C. vulgaris* (freshwater) and *Dunaliella tertiolecta* (marine), as a feedstock (i.e. anaerobic digester feed) and reported the methane yield was approximately 12 times higher for *Chlorella* than for *D. tertiolecta* per added gram volatile solid (VS), possibly due in part to cellular leakage from storage (i.e. cell age). In a comparison of *Chlorella* with

cyanobacteria, Jegede *et al.* (2012) found that digestion of *Chlorella* and cyanobacteria at similar operating conditions and hydraulic retention times yielded similar results and trends, although methane production rates from *Chlorella* were slightly higher than for the cyanobacterium.

Pretreatment methods to disrupt the algal cell wall, such as thermal, chemical and ultrasonic methods, have also been used to maximize methane yields (Jegede, 2012). Nielsen at al. (2011) determined that maceration significantly facilitated methane yield for some algal species (*Ulva latuca*), but had no positive impact on others (e.g., *Saccharina latissima)*. The authors attributed this result to the indigestible dietary fibers found in *U. latuca* that were unavailable to microbes for metabolism unless made accessible through maceration (Nielsen *et al.* 2011). They obtained the highest methane yield (340 ml/g VS) from unmacerated *S. latissima*, suggesting that a higher methane yield is to be expected from species that have more readily degradable cell structures.

There are many other advantages to using waste-grown algae for anaerobic digestion at wastewater treatment plants. They require fewer resources for growth than other photosynthetic organisms since they have no additional requirements such as fertile cropland or clean water and can be cultured in nutrient-rich wastewater side streams (Ficara, 2014; Sahu, 2013). In facilities with existing digesters, CO_2 is readily available in flue gas (gas exiting the anaerobic digestion process) that can be directly used as a carbon source for photosynthesis by the algae. Algal productivity using flue gas is similar to that of using pure CO_2 and is barely impacted by the low levels of SO_x and NO_x contained in flue gases (Negoro *et al.*, 1993 as cited by Torres *et al.*, 2013). Douskova *et al.* (2009) observed that *Chlorella* growth was higher in cultures grown on flue gas (10-

13% CO_2) than on control cultures supplied with a mixture of pure CO_2 (11%) and air (Douskova *et al.*, 2009).

An added benefit to facilitating *Chlorella* productivity at wastewater treatment plants is that all algae including *Chlorella*, have significant phycoremediation capacities, meaning that they are capable of removing or biotransforming pollutants, including nutrients, from water (Sivasubramanian, 2015). Therefore, growing *Chlorella* in wastewater, can potentially reduce the nutrient loads (particularly of P and N) to receiving waters, thus helping to prevent anthropogenic eutrophication (human-caused nutrient overload) downstream from the plant.

One confounding factor of using algae as a feedstock in anaerobic digestion is the potential negative impact of low carbon to nitrogen (C:N) ratio on methanogen metabolism. Previous authors have found that methane yield from the anaerobic digestion of algae can be reduced by ammonia toxicity. Algal biomass typically has a high protein content (40-50% with a carbon to nitrogen ratio of 6:1), which contributes to high total ammonia concentration in the sludge (Salerno, 2009). A high C/N ratio is an indication of rapid consumption of nitrogen by methanogens and results in lower gas production (Verma, 2002). On the other hand, a lower C/N ratio causes ammonia accumulation and pH values exceeding 8.5, which is toxic to methanogenic bacteria (Verma, 2002). At alkaline pH and high ammonia concentrations, acetate (a main substrate for methanogens) is converted to ammonium acetate or ammonium bicarbonate and results in depletion of acetate available to methanogens (Shanmugam and Horan as cited by Prajapati, 2013). Therefore careful attention to the optimal ratio of algae to other feedstock biomass and digestion period must be observed. Krustok *et al.* (2013) found

that during the first 25 days of digestion, a mixture containing 12% microalgae gave the highest biogas production relative to (25% and 37% microalgal biomass). They surmised that the higher proportion of algal biomass increased pH levels and ammonia production, inhibiting the digestion process. Others have noted that methanogen ammonia acclimation can occur, leading to significant increases in biogas production rate occurring many weeks into the digestion process (Salerno, 2009), but the most feasible method for balancing the C/N ratio is the co-digestion of algal biomass with suitable carbon-rich substrate (Prajapati, 2013). Salerno *et al.* (2009) co-digested algae with soybean oil, and found that the highest methane yield came from a mixture of algae and soybean oil, when compared to algae or soybean oil alone. Thus, it is possible that in practice, addition of high-strength, high-carbon waste may balance the high-nitrogen nature of the waste-grown algae.

Several studies have demonstrated that the addition of microalgae to primary and secondary wastewater solids increases methane production over wastewater solids alone. Rusten *et al.* (2011) anaerobically co-digested *Chlorella* and wastewater sludge and found that after 10 days, the specific methane gas produced (ml CH_4/g VS fed) was 74% greater than the methane gas production for the wastewater sludge alone. In combination with secondary sludge, Wang *et al.* (2013) found that co-digestion with *Chlorella* increased the biogas yield over *Chlorella* alone by 73-79%. Likewise, Salerno *et al.* (2009) reported methane yield and productivity doubled when equal masses of wastewater sludge and *Spirulina maxima* biomass were co-digested over algae biomass alone. Olsson *et al.* (2013) concluded that co-digestion of microalgae and wastewater sludge is more efficient in terms of biogas production compared to using sludge alone

under mesophilic (human body temperature, ~37°C) conditions. In the same study, they reported the highest methane yield after a 35-day incubation period (12% microalgae and 88% sewage sludge digestion). It appears that in mixture, microalgae contribute to the overall biodegradability of the biomass source, as Mahdy *et al.* (2014) found that microalgae anaerobic biodegradability (as measured by ml of methane produced per gram chemical oxygen demand [COD] in the treatment) was higher than that of secondary sludge. They also reported that when compared to pretreated microalgae biomass and primary sludge substrates alone, that co-digestions enhanced methane yields 13-17%. (Mahdy *et al.*, 2014).

Project Objectives and Hypotheses

I sought to further test *Chlorella vulgaris* as an appropriate algal candidate for growth in wastewater side streams and subsequent anaerobic co-digestion with dairy whey for methane gas production. The first objective was to determine in which wastewater side stream (primary clarifier effluent or secondary clarifier effluent) *C. vulgaris* would achieve the highest total biomass and to assess associated nutrient uptake (ammonia and phosphorus). In the two streams for phycoremediation potential, both primary and secondary clarifier effluents can be suitable for algal growth, but primary effluent contains more ammonia and phosphorus, and thus would likely support greater productivity while simultaneously reducing ammonia and phosphorus levels in streams.

The second objective was to compare the biomethane potential that exists when *C. vulgaris* is included in the anaerobic digestion of wastewater solids and high-strength organic waste (dairy whey) under mesophilic conditions. Although earlier studies have

indicated co-digestion of algae with wastewater sludge is promising, the existing literature does not explore co-digestion of *C. vulgaris* with wastewater solids and high-strength organic wastes. The addition of different feedstocks is currently being explored by workers investigating efficient production of biogas, and my investigation could potentially be useful for wastewater treatment facilities looking to increase methane production.

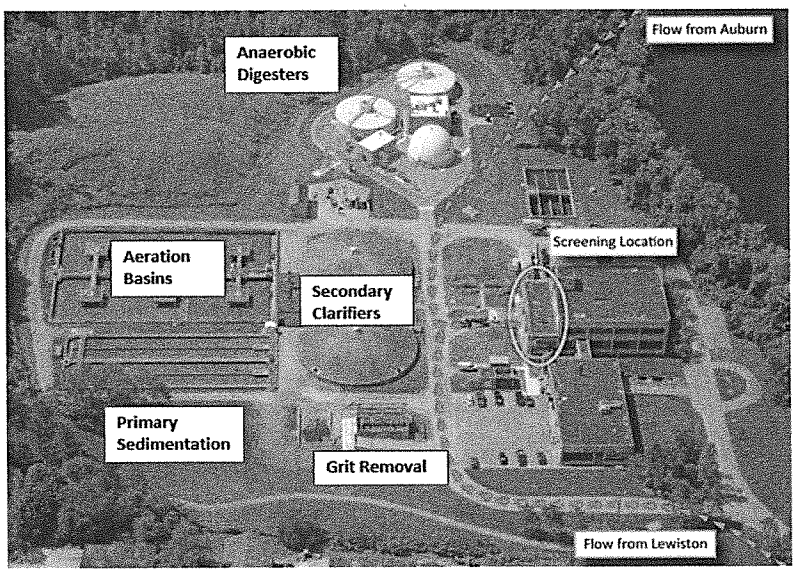
I therefore hypothesize that (1) *C. vulgaris* would grow well in both wastewater effluents, but achieve the greatest total biomass production when cultured in primary clarifier effluent; and that (2) including *C. vulgaris* in the anaerobic co-digestion of wastewater solids and a high-strength organic feedstock (i.e. dairy whey) will result in methane production values greater than digestion of only wastewater solids and high-organic strength feedstock. Lastly, my third objective was to use the data generated in the anaerobic digestion experiment to calculate potential energy savings at a Maine wastewater treatment facility that currently utilizes anaerobic digesters. This information could potentially be used in an economic analysis of the method proposed and aid managers in decision of whether to adopt similar technologies.

Materials and Methods

Field site

The Lewiston-Auburn Water Pollution Control Authority (LAWPCA, Figure 5) has been the wastewater treatment plant servicing Lewiston and Auburn, Maine since 1974 (LAWPCA, 2013). In 2013, the LAWPCA became the first publicly owned wastewater treatment plant in Maine to complete construction of anaerobic digesters (City of Lewiston, 2013). The facility digests wastewater solids, but also takes in high-organic strength waste for introduction to the anaerobic digesters for additional methane production (C. Cwik, personal communication, February 10, 2016). The high-organic strength waste comes from various sources, including whey from yogurt manufacturing, chicken processing waste, and glycol from airport deicing operations. These wastes have a higher chemical oxygen demand (i.e. are a richer food source for anaerobic microorganisms) than wastewater solids (LAWPCA, 2013).

Figure 5. The Lewiston Auburn Water Pollution Control Authority (LAWPCA)



Growth Experiment

An experiment was setup to test hypothesis 1: *Chlorella vulgaris* would grow well in both wastewater effluents, but achieve the greatest total biomass production when cultured in primary clarifier effluent. The independent variable was treatment (which wastewater effluent *Chlorella* was grown in) and the dependent variable was amount of cells grown (i.e. total biomass).

Primary clarifier effluent and secondary clarifier effluent were obtained from the Lewiston Auburn Water Pollution Control Authority (LAWPCA). Because primary clarifier effluent occurs earlier in the treatment process (i.e. before biological treatment), it naturally contains higher levels of ammonia and phosphorus than secondary clarifier effluent, which is why I hypothesize it will allow for the greater total biomass production over secondary clarifier effluent. The ammonia levels at the beginning of the growth trial were 29.8 mg/L for primary effluent and 7.03 mg/L for secondary effluent. The reactive phosphorus levels at the beginning of the growth trial were 13.0 mg/L for primary effluent and 2.18 mg/L for secondary effluent.

A slant culture of *Chlorella vulgaris* was obtained from Carolina Biological Supply Company (PO Box 6010, Burlington, NC 27216-6010. Item # 152075). Bolds Basal Medium was prepared in the Aquatics Research Laboratory at Lewiston Auburn College in Lewiston, Maine (Appendix A). *C. vulgaris* was aseptically transferred to a 10ml sterile tube of Bold's Basal Medium (BBM). The tube was placed into a Thermo Scientific Precision low temperature incubator illuminated with Sylvania 34 Watt T12 cool white fluorescent bulbs, where it remained for approximately three weeks. The tube was removed from the incubator, was vortexed, and 0.5 ml (approximately 1680 cells)

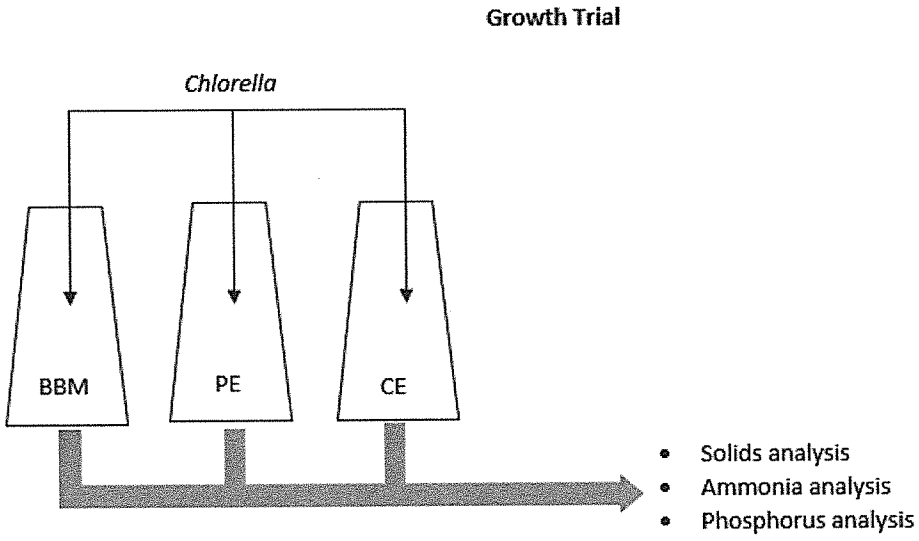
was transferred into the test flasks (Figure 6). All conditions were run in triplicate, with three flasks filled with BBM, three with filtered/UV treated primary clarifier effluent, and three with filtered/UV treated secondary clarifier effluent, for a total of nine flasks. Filtration of the effluents was done using 0.2 um Nalgene Rapid-Flow Sterile Disposable Filter Units with Nylon Membrane. Both primary clarifier and secondary clarifier effluents were filtered into sterile 1-liter Pyrex media bottles and then treated with ultraviolet (UV) light for two hours. Before the growth experiment was set up, aliquots of each filtered and UV treated wastewater effluent were placed in the growth chamber for approximately one week and then microscopically analyzed to verify there were no bacteria present. Temperature/incubation length was 25° C for four weeks in a Fitotron growth chamber. Light conditions were 12hrs:12hrs (light: dark) using Philips PL-L 55 830 4P fluorescent bulbs. A light meter was used to determine total lux inside of the growth chamber, which was set to 8160 lux. The flasks were aerated using a fish pump bubbler.

Flasks were removed from the incubator weekly and swirled to mix the contents. While in suspension, 10 ml was removed from each flask for growth and nutrient (ammonia and reactive phosphorus) analyses. Manual cell counts using a hemocytometer were done on the original culture tube that was used to inoculate the flasks, and also done on each test flask for the first two weeks to create a standard growth curve to relate absorbance to cell concentration (Appendix B). Using a spectrophotometer (Hach Company DR2800), the absorbance (optical density at 600 nm wavelength [OD_{600}]) was measured and used to calculate growth rate over time. At the end of the 29 day growth period, the total biomass yield was also determined using a total suspended solids

method (Standard Methods for the Examination of Water and Wastewater., method 2450D-1997).

$$TSS = \frac{\text{dried weight (mg)} - \text{initial weight (mg)}}{\text{Volume (ml)}} \times 1000$$

Figure 6. Experimental Design for Growth Trial



Ammonia and phosphorus uptake by *C. vulgaris* was determined by analyzing ammonia and reactive phosphorus levels weekly using Hach (Hach Company, Loveland, Co.) reagent kits and a spectrophotometer (Hach Company DR2800). The kits, which are commonly used in wastewater treatment plants, were provided by the LAWPCA and are considered appropriate methods according to the Environmental Protection Agency (http://www.hach.com/epa). The reactive phosphorus (EPA 365.1), ammonia (EPA

350.1) methods these kits employ are equivalent to the respective methods referenced in parentheses under the U.S. Code of Federal Regulations (CFR), Title 40, Chapter 1, Subchapter D, Part 136: Guidelines Establishing Test Procedures for the Analysis of Pollutants.

Anaerobic Digestion

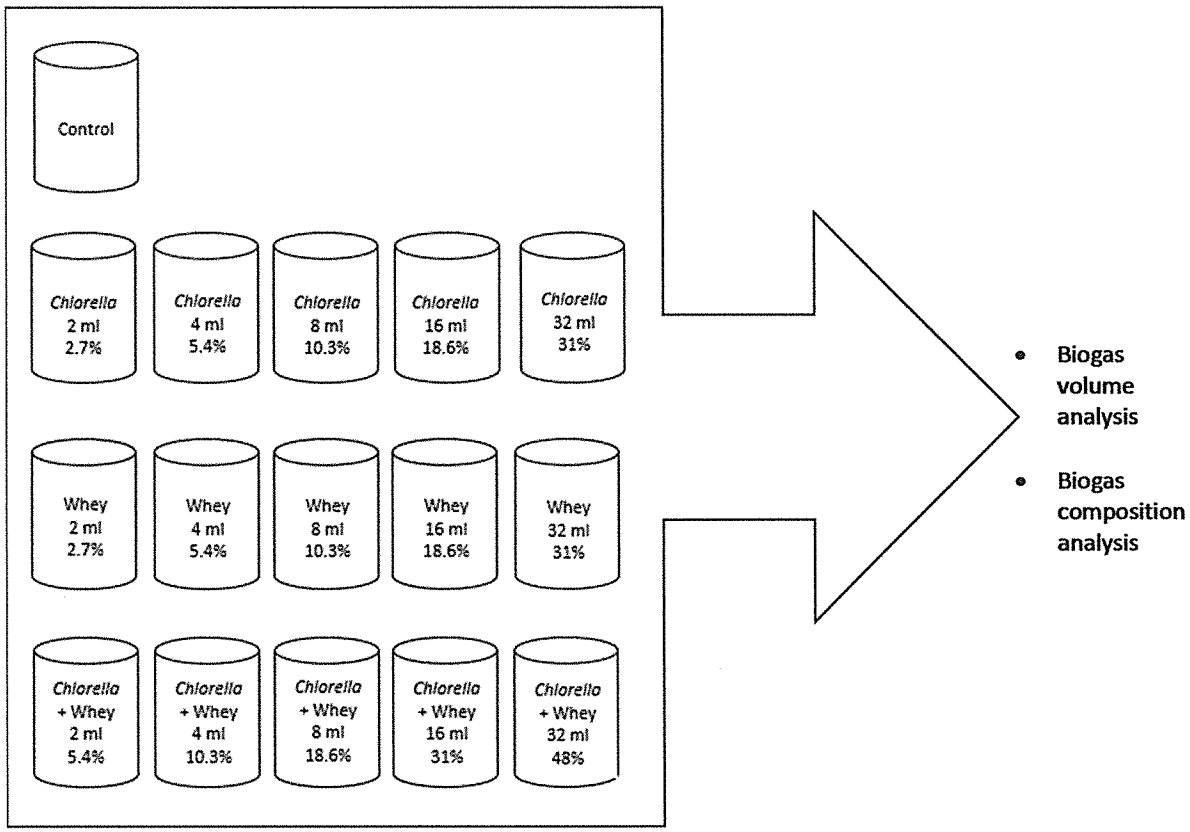
An experiment was conducted to investigate hypothesis 2: including *C. vulgaris* in the anaerobic digestion of wastewater solids and dairy whey (i.e. co-digestion) would result in the production of biogas volumes greater than that produced from the digestion of only wastewater solids and dairy whey. The independent variables were treatment (what was fed into the digester) and dosage (how much was fed in to the digester). The dependent variables were volume of biogas produced and percentage of methane in the biogas.

Chlorella vulgaris cultures were grown in flasks within a Thermo Scientific Precision low temperature incubator illuminated with Sylvania 34 Watt T12 cool white fluorescent bulbs. Every 1-2 weeks, the algal growth was poured off from the flasks into a sterile 1-liter Pyrex media bottles and stored in the refrigerator. BBM media was added to bring the flasks back to the desired volume, and the flasks were returned to the incubator to allow for continued algal growth. To obtain a denser culture of *C. vulgaris,* the cells were allowed time to settle in the refrigerated Pyrex bottle and media was carefully decanted. After approximately two months of collection, the culture was tested for total suspended solids concentration (Standard Methods for the Examination of Water and Wastewater, method 2450D-1997). The total suspended solids concentration of the

C. vulgaris culture was 467 mg/L and 93% volatile solids (VS). The algal biomass obtained was then used for the anaerobic digestion experiment.

Solids from a mesophilic anaerobic digester were obtained from the LAWPCA. Dairy whey was obtained from Stonyfield Farm Inc. in Londonderry, New Hampshire. Laboratory reports on whey composition were obtained from LAWPCA. Whey contained approximately 965 mg/L total solids (TS) and 88% volatile solids (VS). The ammonia and phosphorus concentrations for whey were approximately 60 mg/L and 2100 mg/L, respectively. Treatment volumes were determined by taking into consideration the current loading rates at LAWPCA and providing a range that spanned above and below typical loading rates. Between 7-17% of what is fed to the anaerobic digesters at LAWPCA is feedstock; therefore experimental treatments covered a range of 2-31% whey, 2-31% *Chlorella vulgaris* and 5-48% co-digestion of *C. vulgaris* and whey (Figure 7).

Figure 7. Experimental Design for Anaerobic Digestion Experiment



All digestion treatments were analyzed in triplicate (three bottles for each treatment), with dairy whey only, *C. vulgaris* only and co-digestion of *C. vulgaris* with dairy whey. 200-ml serum bottles were used as digestion vessels. All bottles were seeded with 70 ml of digester solids and the amounts of each feedstock shown in (Table 1). Due to limited incubation space, the digestion vessels containing whey only were analyzed first, then *C. vulgaris* only and the co-digestion bottles were analyzed as a second set. The oxygen in the headspace of each bottle was purged with helium gas (obtained from Matheson Gas Co.), and then sealed with a rubber septum and aluminum-crimp seal. All

bottles were incubated in a Thermo Fisher air bath at 35°- 37° C until gas production in all bottles ceased (approximately 34 days). Bottles were mixed once daily by manual agitation.

Table 1. Anaerobic Digestion Experimental Setup

	Digester (ml)	Whey (ml)	*C. vulgaris* (ml)	Working volume	% Feedstock
Control	70	0	0	70	0.0
Whey	70	2	0	72	2.8
Whey	70	4	0	74	5.4
Whey	70	8	0	78	10.3
Whey	70	16	0	86	18.6
Whey	70	32	0	102	31.4
C. vulgaris	70	0	2	72	2.8
C. vulgaris	70	0	4	74	5.4
C. vulgaris	70	0	8	78	10.3
C. vulgaris	70	0	16	86	18.6
C. vulgaris	70	0	32	102	31.4
Whey + *C. vulgaris*	70	2	2	74	5.4
Whey + *C. vulgaris*	70	4	4	78	10.3
Whey + *C. vulgaris*	70	8	8	86	18.6
Whey + *C. vulgaris*	70	16	16	102	31.4
Whey + *C. vulgaris*	70	32	32	134	47.8

The biogas volume was measured following the method used by Owen *et al.*, 1978 (as reported by Salerno *et al.*, 2009). Additional biogas measuring specifics were passed along directly by Salerno (per com., 2016) which involves sampling with a glass syringe (20-50 ml) equipped with a 20-gauge needle. Volume determinations were made by allowing the syringe plunger to move and equilibrate between the bottle and atmospheric pressure. Readings were verified by drawing the plunger past the

equilibrium point and releasing, where the plunger should return to the original equilibration volume. In order to continue the assay, the gas was wasted. Alternatively, biogas volume may be measured utilizing a manometer, which measures gas pressures in milibars.

Biogas composition (methane and carbon dioxide concentrations) was measured daily for the first week of the experiment, and then again during the last week using a modified CO2meter.com portable Carbon Dioxide and Methane Sampling Data Logger (model CM0191), which uses NDIR (non-dispersive infrared) technology to detect concentrations of methane as a function of transmitted light. Because the volume of gas being analyzed was small, the meter was modified so that the same aliquot of gas could be continuously cycled through the meter until a stable and final reading could be obtained. The modification consisted of tubing with a needle attached on both the inlet and outlet ports on the meter. For sample analysis, both needles were inserted through the rubber septum of each serum bottle and the headspace gas was pumped in and out until the reading stabilized (approximately 10 seconds). The LAWPCA currently uses a Bacharach Fyrite unit, which is a crude measurement for determining methane concentration. The method involves introducing gas into a sealed plastic column where chemical absorption of a certain gas allows for liquid displacement and a measurement can be read (in this case carbon dioxide being absorbed by red dyed potassium hydroxide). The portion of gas that is not carbon dioxide is assumed to be methane. Biogas from the LAWPCA's anaerobic digestion process was analyzed using the Bacharach Fyrite unit and the modified NDIR meter. The Bacharach measured 35%

carbon dioxide (an assumed 65% methane) and the NDIR meter measured 35.1% CO2, and 51.3% methane.

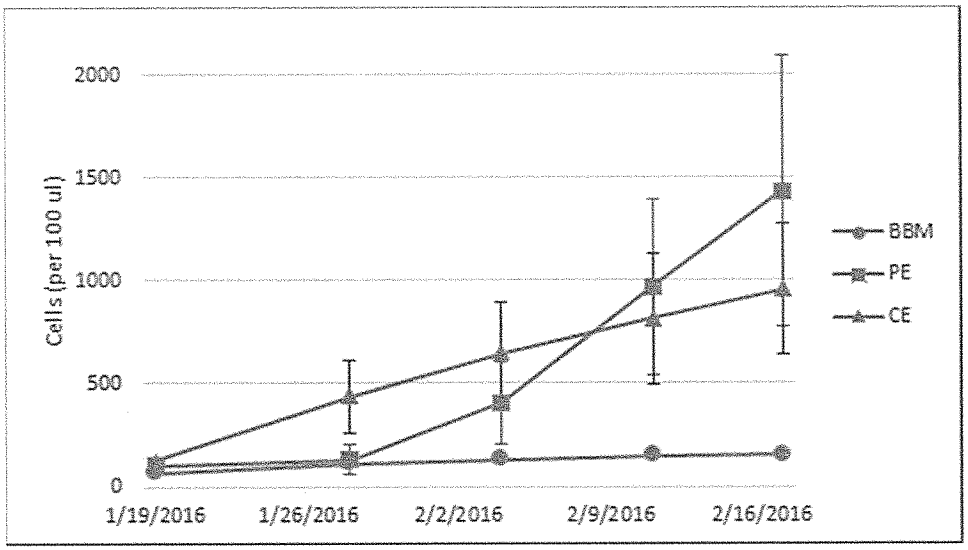
I analyzed all anaerobic digestion data using JASP software version 0.7.5.5 (JASP Team (2016). ANCOVA was utilized to determine if biogas volume and methane concentration differed significantly with feedstock (treatments = *Chlorella* alone, whey alone, *Chlorella* + whey). All of the studies were conducted in triplicate. All results are presented as means of the replicates including +/- standard error bars) with $P < 0.05$ significance level.

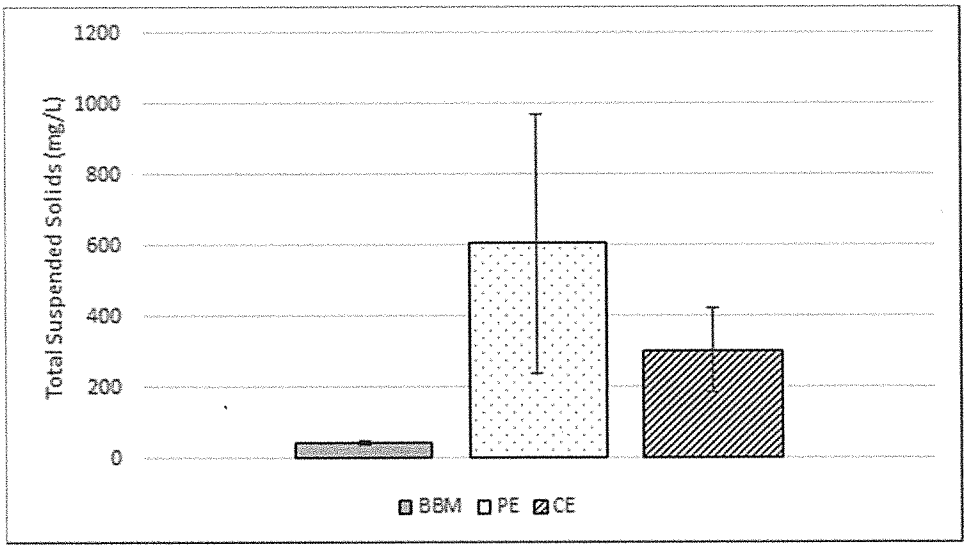
Results

Growth Experiment

The higher *Chlorella* biomass was obtained from growth in primary effluent. The average calculated cell count for the control (BBM), primary clarifier effluent and secondary clarifier effluent was 158.4, 1434, 954.7 (per 100 uL), respectively (Figure 8). Total average biomass yield for the control (BBM), primary clarifier effluent and secondary clarifier effluent was 45.1 mg/L, 605 mg/L, and 302.7 mg/L, respectively (Figure 8).

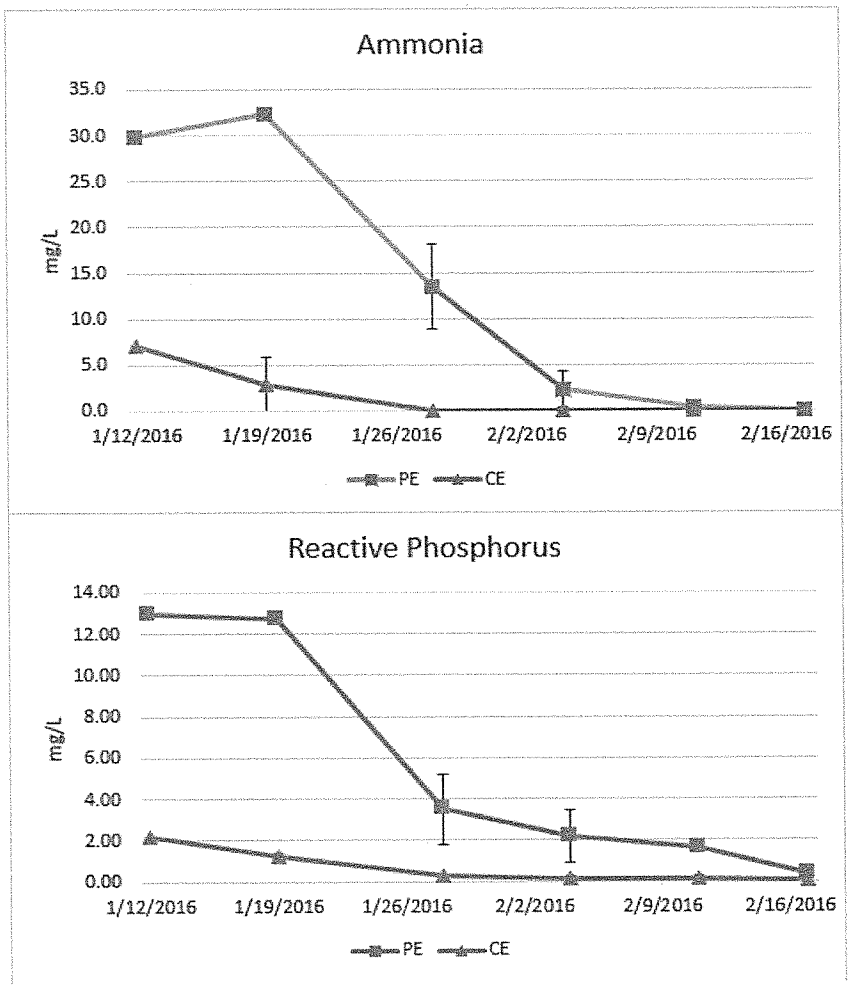
Figure 8. *Chlorella vulgaris* Growth. Cell density over time in each wastewater effluent, as a function of manual cell counts and light absorbance (top). Final cell density displayed as total suspended solids (bottom).





Ammonia and reactive phosphorus (soluble form directly taken up by cells) were reduced to zero in both wastewater effluents (Figure 9). Primary clarifier effluent contained 29.8 mg/L ammonia and 13.0 mg/L reactive phosphorus at the beginning of the experiment, and took 36 days to reach zero for both. Secondary clarifier effluent contained 7.03 mg/L ammonia and 2.18 mg/L reactive phosphorus, and took 16 and 30 days, respectively, to reach zero.

Figure 9. Reduction of ammonia and phosphorus by *Chlorella vulgaris* in two different wastewater effluents.



Anaerobic Digestion

The largest amount of biogas and highest percent methane was obtained from the anaerobic co-digestion of *Chlorella* and dairy whey. For the digesters containing whey only, the greatest volume of biogas and highest peak methane concentration was seen in the 16 ml dosage (Figure 10). For the digesters containing *Chlorella* only or *Chlorella* and dairy whey, the greatest volume of biogas and highest peak methane concentration was seen in the 32 ml dosage (Figure 10). There was a significant effect on biogas produced for both treatment (what was fed into the digesters) and dosage (amount of what was fed) ($p<0.001$, Figure 11). The relationship between treatment (what was fed into the digesters) and peak methane percentage produced was significant ($p = <0.001$, Figure 11), but the dosage (amount of what was fed) did not have a significant effect ($p = 0.244$, Figure 11).

Figure 10. Biogas Production. Total biogas volume produced for each treatment (top). Peak Methane percent achieved for each treatment (bottom).

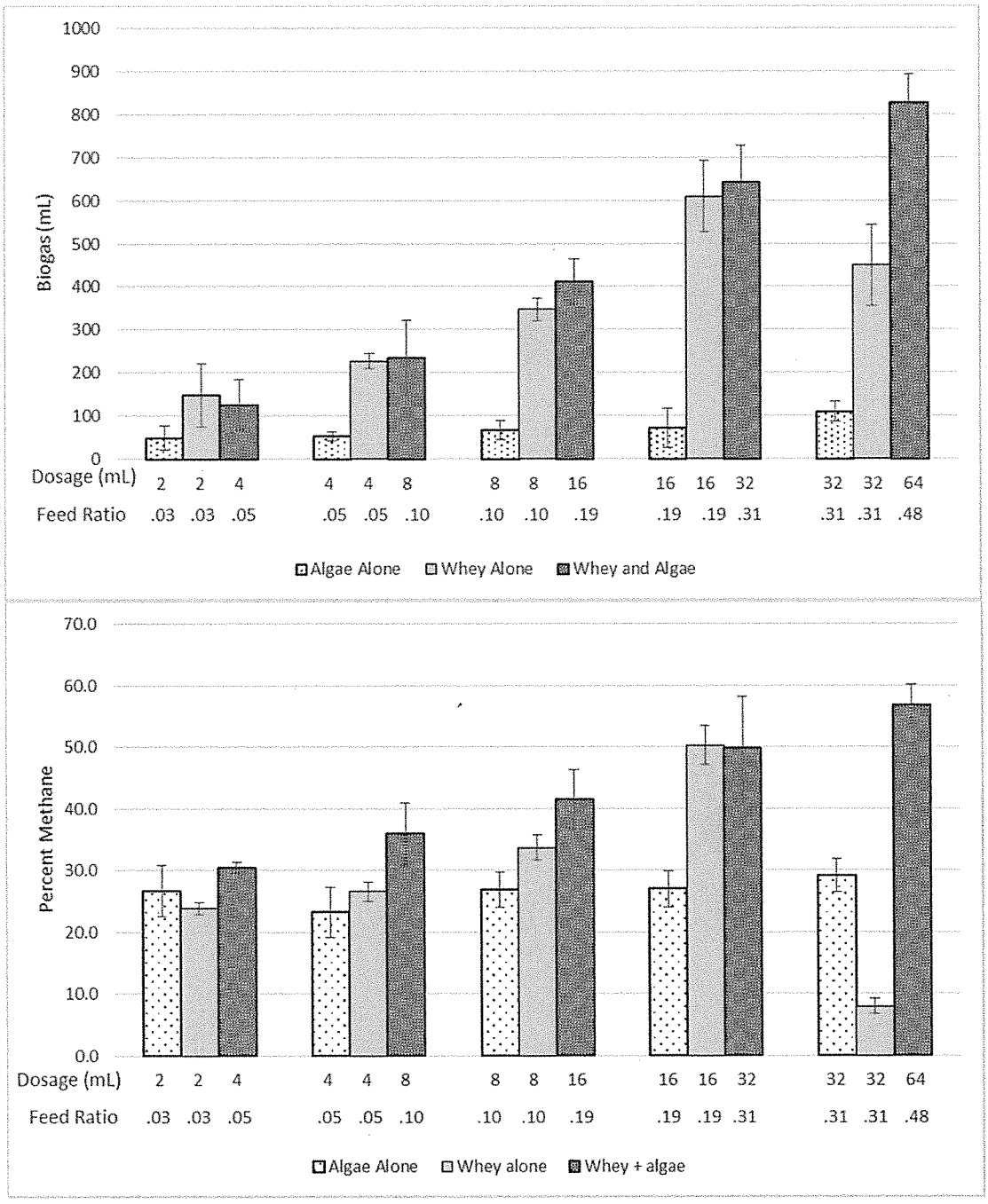


Figure 11. ANCOVA Analysis of Biogas Production and Composition

ANCOVA - Total Biogas Produced

Cases	Sum of Squares	df	Mean Square	F	p
Treatment	1.180^6	3	393439	23.32	< .001
Dosage	715539	1	715539	42.41	< .001
Residual	725550	43	16873		

Note. Type III Sum of Squares

ANCOVA - Peak % Methane

Cases	Sum of Squares	df	Mean Square	F	p
Treatment	2433.4	3	811.13	8.191	< .001
Dosage	138.4	1	138.44	1.398	0.244
Residual	4258.3	43	99.03		

Note. Type III Sum of Squares

Economic Analysis

According to the experimental data presented in this study, anaerobic co-digestion of *Chlorella vulgaris* with dairy whey has the potential to increase biogas production by up to 36%. This makes *C. vulgaris* an ideal candidate for further studies in increasing biogas production and energy co-generation during anaerobic digestion. An economic assessment of how this additional biogas might add to current savings at LAWPCA is as follows:

According to LAWPCA data, when whey is added to the anaerobic digestion process, it boosts microbial activity, resulting in the production of 0.239 pounds of volatile solids per gallon of whey introduced, producing 3.7 ft^3 of biogas (T. Peaslee, per com., 2016). With an additional 36% from the addition of *C. vulgaris*, 5.04 ft^3 of biogas would be generated per gallon.

Combined capacity for solids storage in the anaerobic digesters is 184,492 ft^3. The model maximum organic loading rate is 0.150 lbs VS/ ft^3 (pounds of volatile solids per cubic foot), which totals 27,674 lbs. VS. When taking into consideration the current average loading rate of wastewater solids (14,948 lbs VS), the remaining capacity would allow for an additional 12,725 lbs VS. Factoring in the 58.9% VS reduction (LAWPCA 2015 average), this equals the potential to destroy 7,495 additional VS lbs, creating 116,172 ft^3 of biogas. (LAWPCA 2015 average gas produced for each lb VS destroyed = 15.5 ft^3).

The cogeneration unit consists of two Liebherr Ettlingen G9408 engines and a Leroy Somer generator, supplied by Tech 3 Solutions Inc. Utilizing the extra 116,172 ft^3 of biogas by running each engine at 9000 ft^3 gas per hour for 12.9 hours would create 230

KwH (kilowatt hours), which in turn produces 1,173,777 BTUs (thermal output) per hour. The 2015 average cost per KwH for LAWPCA was $0.11, making the average potential electrical savings $25.30 per hour, per engine. Utilizing the generated thermal output (processed via boilers and heat exchangers) equates to using 1173.7 ft^3 less natural gas per hour, adding an additional average potential savings of $18.20 per hour (Average 2015 cost of natural gas = $1.55 per 100 ft^3).

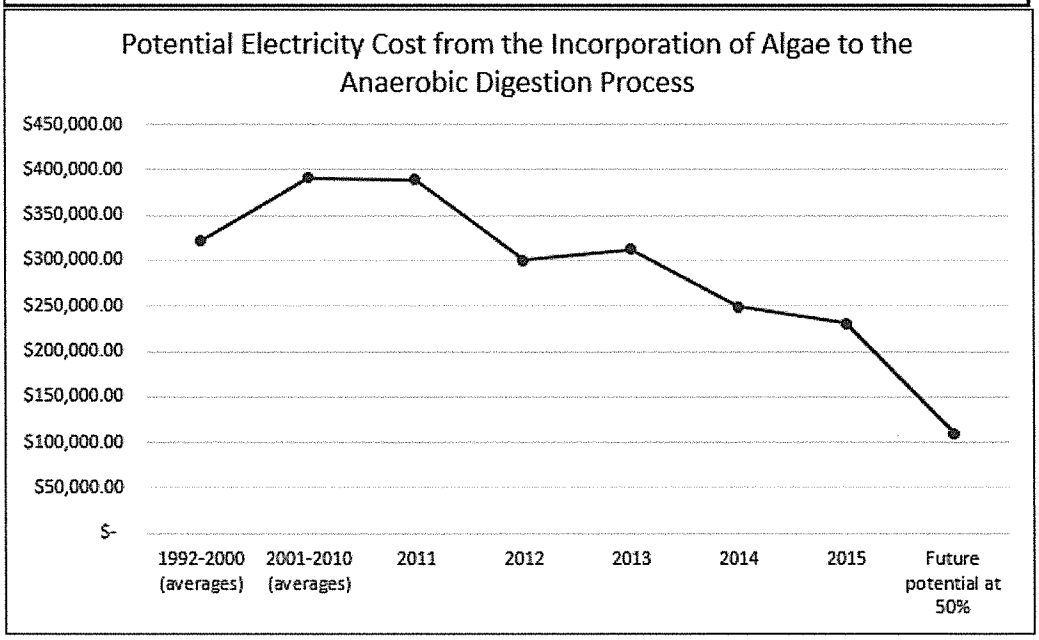
If both engines were used at maximum capacity, 5934 kWh per day extra could be produced, translating to a maximum electrical savings of $653 per day. Engine design is for operation at 100% for one engine and 80% for the second engine, meaning that the existing equipment has the capacity to make more electricity. For this reason, it was decided (T. Peaslee, per com., 2016) that achieving 50% of the projected maximum electricity savings would be reasonable and realistic, and this value ($238,998) is displayed in the "Potential Electricity Cost from the Incorporation of Algae to the Anaerobic Digestion Process" graph (Figure 12).

Figure 12. Electrical Cost and Potential Savings at the LAWPCA

Electrical Power Usage

	Yearly Power Cost	Average KwH-daily	Days per year	Average Cost per KwH
1992-2000 (averages)	$ 321,311.00	9773	365.3	$ 0.091
2001-2010 (averages)	$ 390,696.00	10010	365.1	$ 0.107
2011	$ 389,471.00	8901	365	$ 0.120
2012	$ 300,039.00	8768	365	$ 0.094
2013	$ 312,140.00	8803	365	$ 0.092
2014	$ 248,995.00	6395	365	$ 0.107
2015	$ 231,005.00	5679	365	$ 0.111
Future potential at 50%	$ 109,568.00	2697	366	$ 0.111

*Assumes same electrical use and cost as in 2015, incorporating 50% of the maximum possible projected $238,998 average KwH savings

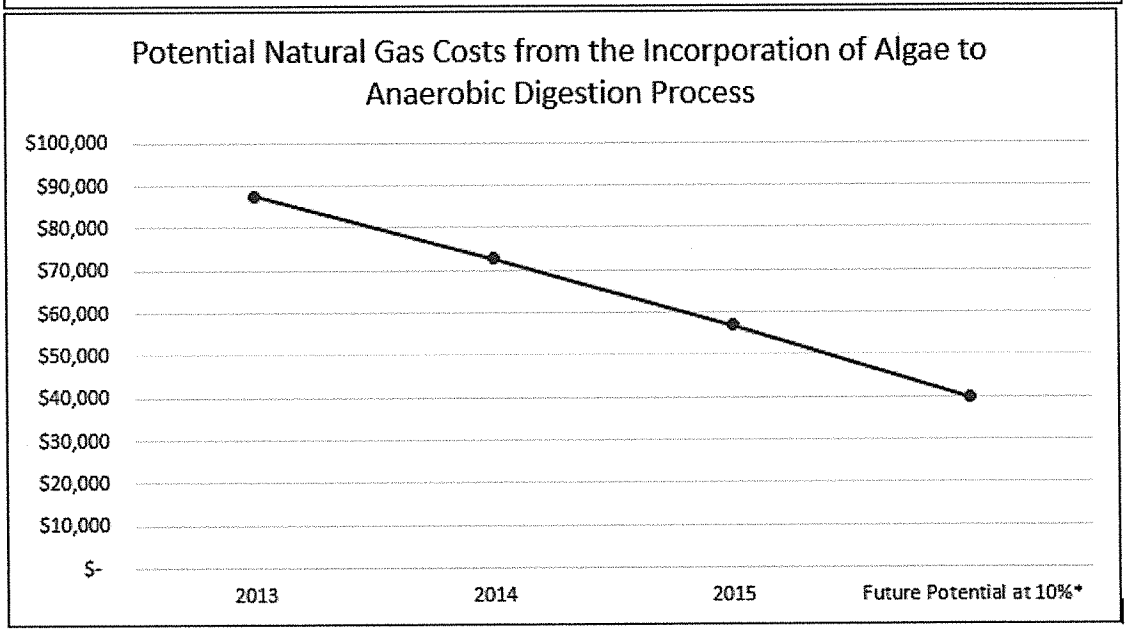


The equivalent of 302 CcF (100 cubic feet) of natural gas can be thermally generated per day, translating to a maximum natural gas savings of $469 per day. This thermal output is used to heat the anaerobic digestion process (must be maintained at approximately 35° C for proper microbial activity and methane production), which in turn reduces the need to purchase natural gas. Because there is a limit to how much thermal output can be used at a given time, it was decided that 10% (T. Peaslee, per com., 2016) of the projected maximum natural gas equivalent could realistically be utilized. This value ($171,185) is displayed in the "Potential Natural Gas Cost from the Incorporation of Algae to the Anaerobic Digestion Process" graph (Figure 13). Efficient utilization of the additional biogas produced in terms of internal use (and probable equipment addition/upgrades) or energy distribution back to the grid was not part of this study.

Figure 13. Natural Gas Cost and Potential Savings at the LAWPCA

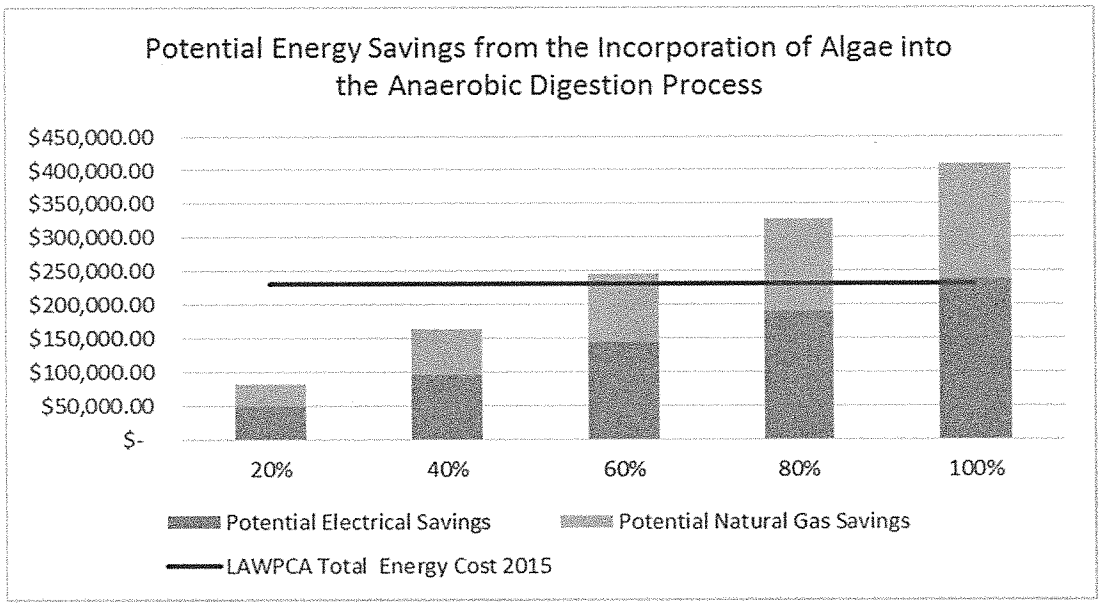
Natural Gas Usage	Treatment Plant Use (CcF)	AD Use (CcF)	Total Usage (CcF)	Total Cost
2013	50505	18090	68595	$ 87,528
2014	49409	3399	52808	$ 72,713
2015	36733	5489	42222	$ 56,891
Future Potential at 10%*				$ 39,772

* Assumes same use as 2015 and $1.55/CcF natural gas cost, incorporating 10% of the maximum possible projected $171,185 average natural gas cost savings



With more research and additional treatment plant equipment, it is probable that a greater portion of the additional energy produced could be utilized. The maximum projected annual electrical savings is estimated to be $238,345 ($653 per day), and the maximum projected annual natural gas savings is estimated to be $171,185 ($469 per day), for a total potential savings of $409,530 (Figure 14). The total electrical cost for 2015 at LAWPCA was $231,005, and the total natural gas cost was $56,891, for a total facility energy cost of $278,896.

Figure 14. Total Potential Energy Savings at the LAWPCA



It should be noted that the projected 36% increase in biogas production suggested in this study is based upon the addition of an equal volume of *C. vulgaris,* without reducing or replacing any amount of whey. Theoretically, similar if not better results could be obtained by replacing a portion of whey with a higher total solids concentration culture of *C. vulgaris* (the loading rate for the current experiment was 467 mg/L).

Discussion

The main goal of this research was to investigate the growth of *Chlorella vulgaris* in wastewater and its potential to generate methane gas during the anaerobic co-digestion of *C. vulgaris* and dairy whey. I was secondarily interested in its ability to positively impact wastewater water quality. I hypothesized that (1) *C. vulgaris* would grow well in both wastewater effluents, but achieve the greatest total biomass production when cultured in primary clarifier effluent; and (2), including *C. vulgaris* in the anaerobic digestion of wastewater solids and dairy whey (i.e. co-digestion) would result in the production of biogas volumes greater than that produced from the digestion of only wastewater solids and only dairy whey. Both hypotheses were supported, and an economic analysis of the data provided promising projections for electrical and natural gas savings at wastewater treatment facilities.

Chlorella vulgaris grew well in both primary and secondary effluent wastewater side-streams, but the most biomass was achieved from growth in primary effluent. These findings are in agreement with other studies, including Wang *et al.* (2012) and Wang *et al.* (2009), which also concluded that primary effluent supported better growth of *Chlorella vulgaris* than secondary clarifier effluent. *C. vulgaris* is a mixotroph, meaning it is able to exploit either autotrophic or heterotrophic metabolisms without a preference (Belotti *et al.*, 2013). In conditions where nutrients are available, *Chlorella* will readily utilize them. Belotti *et al.* (2013) found that phosphorus starvation switches off photosynthetic machinery, thus reducing photosynthetic activity resulting in a lowered growth rate (Belotti *et al.*, 2013). The high levels of ammonia and reactive phosphorus

found in primary clarifier effluent likely allowed for more cellular growth than in secondary clarifier effluent, which contained much lower levels. *Chlorella's* rapid response to large variation in N and P suggests it could survive and efficiently phycoremediate waste streams with a wide range of N and P concentrations.

The complete uptake of ammonia and phosphorus by *C. vulgaris* in each effluent was also an important part to this experiment because it showed that in addition to being able to grow *Chlorella* in wastewater, simultaneous and complete nutrient phycoremediation is also feasible. Other studies have found that microalgae can effectively accumulate nitrogen and phosphorous from wastewater (Wang *et al.* 2009; Wang *et al.*; 2012; Prajapati, 2013), however it was important to demonstrate this at the field site used in this study. In addition, algae have the capacity to remove heavy metals, as well as some toxic organic compounds (Abdel-Raouf *et al.* 2012). Algae have also been used for the removal of coliform bacteria (Abdel-Raouf *et al.* 2012). The role of *Chlorella* in these processes needs further investigation.

Phycoremediation of wastewater can be useful for wastewater treatment facilities that must remove ammonia and/or phosphorus before discharging treated water into a receiving water (e.g. river, stream). High levels of nitrogen are particularly a problem in marine waters, whereas high levels of phosphorus pose problems in freshwater. Both nutrients are present in municipal wastewater treatment plant discharge, creating a potential to cause detrimental environmental effects (Maine Department of Environmental Protection). Anthropogenic eutrophication during summer months can cause algal blooms, which in turn can result in low dissolved oxygen availability, turbid

water, and even death of flora and fauna (e.g. fish kills) (Maine Department of Environmental Protection).

The conventional activated sludge process was developed in the early part of the twentieth century, at a time when knowledge of eutrophication did not exist as it does today. This means that most current activated sludge processes are not specifically designed to remove nitrogen and phosphorus, creating a need to upgrade with nutrient removal technology. Emerging technologies fall in one of two categories: chemical or biological nutrient removal. Using algal-based technology is more environmentally friendly, and would avoid the need to pursue chemical options. This, therefore, represents an untapped biological technique for nutrient removal.

The anaerobic digestion of *Chlorella* occurred at a slow rate, with peak biogas production occurring during weeks three and four, likely due to the time it takes for the cell walls to be degraded (Bohutskyi and Bouwer, 2012). Conversely, the digestion of dairy whey occurred very quickly, with gas production being observed immediately and peaking within the first week, likely due to its liquid form. These results were also observed for the co-digestion treatment containing 32 ml of *Chlorella* and 32 ml dairy whey (48% total feed ratio). Results were high during week one, likely from the digestion of the dairy whey, then peaked again at week four, indicating the algal cells had been degraded.

Anaerobic digestion treatments containing only dairy whey produced more biogas overall (609 ml) and a higher peak methane concentration (50.3%) than treatments containing only *C. vulgaris*. The largest feed rate (32 ml/ 31% feed ratio) of dairy whey appeared to be too high, generating 450 ml biogas and only a peak methane concentration

of 8%. Whey has a low pH (~ 4); therefore, over-feeding can result in a drop in pH and subsequent loss of methanogenic activity (i.e. digester souring). Patil *et al.* (2012) proposed a two phase digestion process with pH and temperature control in which higher biogas production can be achieved from the digestion of whey (maximum study yield: 2,990 ml of biogas containing 50% methane). pH control measures such as this could be implemented in future studies.

Ammonia toxicity is a commonly cited problem in the anaerobic digestion of algae. Co-digesting *C. vulgaris* with dairy whey appeared to have a synergistic effect on biogas production and composition, likely due to a balance in the carbon to nitrogen ratio. Co-digestion with whey (high-carbon) likely balances the high-nitrogen found in the *C. vulgaris*. Yen and Brune (2005) reported the highest amount of methane production from the co-digestion of algae with waste paper, citing the balanced carbon to nitrogen ratio as a likely reason. Spierling (2011) reported the highest amount of methane production from the co-digestion of algae with food waste. At the time this paper was written, no other studies were found in the literature that researched co-digestion of algae with dairy whey.

In the present study, all co-digestion treatments of *C. vulgaris* and whey averaged 126 ml or greater for biogas production and 30.4% or greater for methane concentration over the 34 day (approximate) digestion period. The treatment with the highest loading rate, which contained equal amounts of *C. vulgaris* and dairy whey (32 ml each/ 48% feed ratio), resulted in the greatest amount of biogas (827 ml) and percent methane concentration (56.8 %). Since the highest dosage of *C. vulgaris* and dairy whey resulted in the most biogas production and methane concentration, it is possible that this was not the maximum possible loading rate. Due to limited glassware and incubation space,

digestion with dairy whey only was conducted first, which indicated the maximum loading rate was somewhere between 16 and 32 ml (19-31% feed ratio). It was expected that the same loading rates would be appropriate for the subsequent experiment that included *C. vulgaris* only and co-digestion of *C. vulgaris* with dairy whey. Because the co-digestion treatments were able to tolerate the maximum loading rates used in this study, further studies should be done to increase the dosage amounts until the maximum, and optimal, feed rate is found.

Because of the limited number of existing studies, there is not a clear indication of what the ideal loading rate for co-digestion might be. Olsson *et al.* found that 12% algae (and 88% waste activated sludge) resulted in the highest methane volume. Wang *et al.* (2013) did not find a significant difference in methane concentrations between 4%, 11%, and 41% *Chlorella* loadings. It seems that loading rates of approximately 40% algae tend to yield some of the highest biogas volumes and methane concentrations, whereas digesting 100% algae yields relatively low methane concentrations. Salerno *et al.* (2009) co-digested with biodiesel glycerin to simulate feedstock that might be available to treatment plants. They found that 90 ml inoculum, 18 ml or 36 ml algae in addition to 0.082 ml glycerin resulted in approximately the same biogas production (1013, 1173 ml, respectively). They noted that low amounts of glycerin were used, suggesting that a higher amount may have resulted in more biogas production. At the LAWPCA, high-strength feedstock has been shown to significantly increase biogas production.

Algal cell wall resistivity to degradation is a commonly cited cause for lower biogas yields during anaerobic digestion, therefore, pretreatment methods (e.g. mechanical, thermal, biological) to increase digestibility have been investigated. Passos

et al. (2013) found that final biogas yield was significantly higher (12–78% increase) for microalgae pretreated with microwaves. Ometto (2014) reported that methane production by *Chlorella sorokiniana* could be enhanced by 42% with ultrasound pretreatment. It is expected that the biogas production potential of *C. vulgaris* reported in the present study can be significantly enhanced through appropriate pretreatment methods; however, these methods create an additional energy expenditure that should be considered. Further studies utilizing the two most promising pretreatment technologies (high-temperature thermal hydrolysis and enzymatic addition) are recommended to investigate the costs/feasibility for large scale applications (Ometto, 2014).

There are many variables that can account for the different methane yields reported in the literature, including whether or not the cells were pretreated, and type and operational conditions (e.g. temperature, length of digestion) of the anaerobic digesters used. Despite the range of results in the literature, there seems to be a consensus that algal addition will improve the digestibility of wastewater solids and generate more biogas (Mussgnug, 2010; Yuan *et al.*, 2012; Wang *et al.* 2012; Prajapati 2014). The results of this study are comparable with the literature findings in that they support the utilization of algal biomass as a feasible substrate for anaerobic digestion to produce biogas.

It should be noted that the current preferred method for methane gas analysis is gas chromatography; however, a GCMS unit equipped to do such a specialized analysis could not be found for this project (dozens of calls were made to laboratories and universities in New England). A second option was to analyze via Fournier Transform Infrared Spectroscopy (FTIR) at Bates College. Unfortunately, despite generous support from their staff, the unit was found to not be suitable for high-level methane analysis.

Other studies have supported the economic feasibility of co-digestion with algae. Yuan *et al.* (2012) found that that addition of algae to existing anaerobic digesters can improve overall digestion efficiency. Peng and Colosi (2016) found that the when algae is anaerobically co-digested, the energy return on investment of a typical wastewater treatment plant increases from 0.53 without algae to 0.66 with algae. Kusin and Horan (2015) reported that the energy and revenue potential of the biomass generated from cultivating *C. vulgaris* in sludge liquor makes the use of *C. vulgaris* for sludge liquor treatment more economical than conventional nutrient removal processes (Kusin and Horan, 2015).

In conclusion, *Chlorella vulgaris* can grow in and phycoremediate wastewater, enhance methane production when anaerobically co-digested with a high-organic strength waste, and result in economic savings to wastewater treatment facilities. Further site-specific studies are needed to determine more accurately what the maximum digester loading rates of *Chlorella* and dairy whey (or other high-organic strength feedstocks) are, and subsequent methane production and energy savings. Initially, larger ($\geq$ 1 liter) batch-reactors that more closely resemble actual anaerobic digesters (inlet and outlet for feeding and wasting as opposed to a closed system that is fed once and sealed) should be constructed. With the ability to remove digested solids over time, other parameters such as pH, volatile solids, alkalinity and volatile acids could be monitored. If desired results are obtained, even larger scale algal growth units could be built and a large-scale application could be initiated.

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Appendix A

Bold's Basal Media

Stock Solutions

A: $NaNO_3$ (10.0 g/400 ml)
B: $MgSO_4 \cdot 7H_20$ (3 g/400 ml)
C: NaCl (1.0 g/400 ml)
D: K_2HPO_4 (3g /400 ml)
E: KH_2PO_4 (7 g/400 ml)
F: $CaCl_2 \cdot 2H_2O$ (1 g/400 ml)
Prepare all above stock solutions in 500 ml media bottles.
Place caps on loosely and autoclave for 15 minutes. Store at 4 degrees C

Stock Solutions

G (Trace Elements):

$ZnSO_4 \cdot 7H_2O$ (0.8g)
$MnCl_2 \cdot 4H_2O$ (0.14g)
$MoO_3 \cdot$ (0.07g)
$CuSO_4 \cdot 5H_2O$ (0.15g)
$Co(NO_3)_2 \cdot 6H_2O$ (0.04g)
Distilled water to 100 ml

H (EDTA stock):

$EDTANa_2$ (5.0g)
KOH (3.1 g)
Distilled water to 100 ml

I (Fe solution):

$FeSO4 \cdot 7H_2O$ (0.49g)
conc. H_2SO_4 (0.1 ml)
Distilled water to 100 ml

J (Boron solution):

H_3BO_3 (1.14g)

Prepare above stocks in 100 ml media bottles
Place caps on loosely and autoclave for 15 minutes. Store at 4 degrees C

To prepare 1 liter of media:
Add 10 ml of each stock A-F to 940 ml distilled water
Add 1 ml of each stock solution G-J
Autoclave at 121 degrees C (15 PSI for 15 minutes)

Appendix B

Linear Regression - Cell Counts and Absorbance