NATIONAL RENEWABLE ENERGY LABORATORY

Final Report

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Executive Summary

The economic viability of algal biofuels requires extracting value from the entire algal feedstock, not just the lipid fraction. Lipid-extracted microalgae contain large amounts of fixed carbon and energy, plus most of the inorganic nutrients (nitrogen and phosphorous) that were used to grow the algae. Anaerobic digestion (AD) is a promising avenue for conversion of extracted microalgae into biogas/biopower and a nutrient rich effluent that could potentially be recycled to algal growth systems. This approach has been widely assumed in process modeling, and removal of the AD component in NREL's techno-economic models results in a significant increase in the fuel selling price. However, there has been relatively little research done to support the concept under process-relevant conditions. The purpose of this project was to answer specific questions regarding yields, loading rates, retention times, inhibitors, and nutrient recycle. We have demonstrated good biogas yields from five disparate microalgal feedstocks, both for extracted and non-extracted materials, and successfully scaled up to multi-liter digesters for the industrially-relevant strain *Nannochloropsis salina*. The specific results from these digestions generally support the modeling assumptions, and the anticipated issues (*e.g.*, ammonia toxicity, C/N ratios, and cell wall recalcitrance) were either not encountered or were overcome through careful optimization. We have also demonstrated that algal AD effluent can serve as a superior nitrogen source for re-growth of the original strain. Publication of these results will provide important data to the algal biofuels industry and help to provide confidence around the feasibility of this process component.

Background and Objectives

The cultivation of microalgae is a promising avenue for the generation of renewable, drop-in biofuels on a massive scale. Microalgae grown under certain conditions can accumulate as much as 50% of their dry cell mass in the form of lipids that are amenable to conversion into renewable diesel and jet fuel substitutes. However, the economics of generating fuel from algae are challenging. To be economically viable, value must be extracted not only from the lipid fraction but from the entire algal feedstock. The residual microalgal biomass remaining after extraction of lipids

contains large amounts of fixed carbon and energy, plus most of the inorganic nutrients (nitrogen and phosphorous) that were used to grow the algae. Anaerobic digestion (AD) is a proven means of converting the fixed carbon in a variety of organic waste streams into biogas and biopower while releasing nitrogen and phosphorus for re-use as fertilizer. AD of algal biomass residues has long been proposed as part of an algal biorefinery (Golueke *et al*. 1957), but historically experimental data on algal AD have been sparse, conflicting, or insufficient in scope to produce meaningful results. *Thus, the overall goal of this project has been to advance the economic viability of algal biofuels by filling knowledge gaps on the conversion of algal residues to biogas/biopower via AD*.

Figure 1 shows the overall Algal Lipid Upgrading (ALU) process schematic as modeled by Davis *et al*. (2011). In this pathway, algae are concentrated through a three-stage dewatering process and extracted with solvents to remove the lipid fraction for upgrading to fuel. As shown, AD is used to convert spent algae after lipid removal into three process streams: 1) biogas, composed primarily of methane, which can be combusted in a turbine for biopower generation, 2) a stream of liquid effluent containing nutrients required for algal growth [primarily nitrogen (N) and phosphorus (P)], and 3) solid effluent components, referred to as sludge or digestate. Combustion of the biogas in the turbine generates both electrical and heat energy that can be integrated to serve various process demands, and the CO_2 from combustion can be recycled to the algal growth system. AD thus permits the potential recycling of both expensive nutrients and carbon.

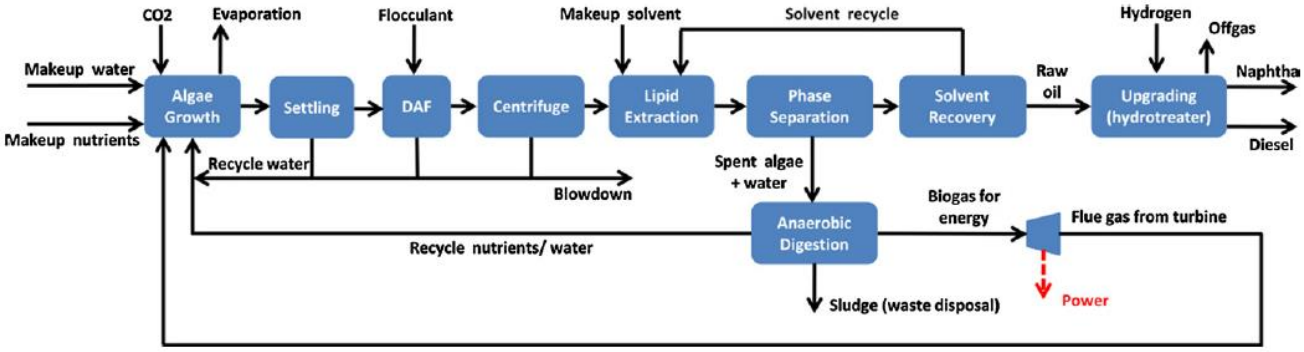

Figure 1. Overall schematic of ALU baseline process (from Davis *et al*., 2011).

Previous work on AD of algal feedstocks has raised challenges relative to more conventional agricultural or organic waste feedstocks. AD utilizes a natural consortium of microorganisms to convert a variety of carbonaceous materials (carbohydrate, protein, and lipid) to biogas, which is composed primarily of methane and CO_2 (with smaller amounts of hydrogen and other gases). The actual process is quite complex and involves three types of anaerobic organisms that catalyze three separate steps: 1) hydrolysis/acidogenesis in which complex macromolecules are broken down into organic acids such as propionate and butyrate, 2) acetogenesis to generate acetate and other simple compounds, and 3) methanogenesis which converts these substrates to methane and CO_2. While the consortia of microbes can be quite adaptable, some feedstocks, such as those high in nitrogen (typically in the form of protein), can pose challenges. In a review of previous studies on AD of algae, Sialve et al. (2009) showed that the methane yield of microalgal AD has been reported to range from 0.09 to 0.45 L/gVS (liters of gas per gram of volatile solids). Such a wide range shows the difficulties of working with such feedstocks and is unacceptable for process modeling. Sialve and others have suggested that the challenges that microalgae pose appear to arise from 1) poor degradability of algal cell walls, 2) high protein contents [*i.e*., low carbon-to-nitrogen (C/N) ratios] that lead to toxic levels of ammonia in the digesters, and 3) high sodium content from the algal growth medium. These concerns have been reiterated as recently as 2013 by Schwede *et al*. in a paper demonstrating very low yields of biogas from un-pretreated *N. salina*.

The specific goals of this project were to: 1) optimize biogas production from spent microalgae of at least three disparate species to increase potential levels of biopower production, 2) generate meaningful data in scaled-up reactors for yields, loading rates, retention times, and inhibitors, 3) seek to understand the fate of nitrogen and phosphorus and test nutrient recycle to support algal growth, and 4) assess the impacts of the findings on the process economics and life cycle analysis.

Technical Approach

The project was organized around the following five research tasks (with responsible organizations shown in parentheses):

Task A. Feedstock development (NREL). The purpose of this task was to provide the algal biomass materials needed for AD optimization and scale-up, to extract lipid, to perform compositional analysis, and to experiment with various pretreatment strategies.

Task B. High-throughput screening for biogas optimization (NREL/WSU subcontract). The purpose of this task was to discover optimal conditions for biogas production in algal AD by systematically exploring the parameter space. Small batch studies using the classical Biochemical Methane Potential (BMP) assay provided relatively high throughput to test variables such as species, presence or absence of lipid, extraction methodology, inoculum source, solids loading, etc. in order to determine the best conditions for further study.

Task C. Larger bench-scale experimentation (NREL/WSU subcontract). The purpose of this task was to mimic a more industrially relevant scale of digestion and collect data to inform the modeling effort of Task E.

Task D. Nutrient recycle testing (NREL). The purpose of this task was to test the hypothesis that AD residuals can be recycled to serve as a nutrient source for microalgal cultivation. This involved both effluent characterization and testing in the context of algal cultivation at the shake flask scale.

Task E. Techno-economic Analysis and Life Cycle Assessment (NREL). The purpose of this task was to quantify the impact of optimized AD and biopower production on the economics, net energy production, and greenhouse gas emissions of the algal biofuels process.

The project delivered Milestone Reports for six E-level (internal) Milestones, as shown in Table 1.

Table 1. Project Milestones.

Milestone	Performers	Description and Performance Measure	Completed
A.ML.1	NREL	Minimum of 200 g dry weight of biomass available for each of two algal strains and minimum of 1 kg dry weight of biomass available for third; >50% of oil solvent-extracted from biomass.	8/31/11
B.ML.2	NREL/WSU	Initial high-throughput Biochemical Methane Potential (BMP) screening completed with outputs of methane production rate and specific productivity; optimal conditions chosen for further analysis for 3 algal strains.	2/07/12
B.ML.3	NREL/WSU	BMP screening studies completed with at least four variables explored (species, pretreatment method, organic loading rate, carbon/nitrogen ratio); optimal conditions chosen for scale-up.	3/31/12
C.ML.4	NREL/WSU	Through larger-scale continuous-flow reactor run(s), demonstrate stable biogas production over at least 30 days reaching at least 50% conversion of incoming volatile organic carbon.	6/30/12
D.ML.5	NREL	Reactor effluent characterized and demonstrated to partially (≥20%) replace chemical nitrogen supplementation to support growth of microalgae with less than a 10% impact on growth rate.	3/31/13
E.ML.6	NREL	Techno-economic and LCA models updated using data from Tasks C and D, to include net energy balance upon inclusion of AD of algal residues and 20% nitrogen and phosphorous recycle.	5/31/13

Technical Accomplishments

Generation/Acquisition of Algal Biomass

Microalgae represent an extremely diverse group of organisms with wide variations in biochemistry, composition and genetic makeup. We chose to test at least three genetically diverse species in an attempt to ensure that our conclusions would be more broadly applicable. We initially selected three organisms to work with: 1) *Nannochloropsis* sp., 2) *Chlorella vulgaris* UTEX 395, and 3) *Phaeodactylum tricornutum* CCMP632. This represents a diversity of algal types: a Eustigmatophyte, a green alga, and a diatom, respectively. *Nannochloropsis* sp. was obtained in kilogram quantities through collaboration with Dr. Ami Ben-Amotz at Seambiotic in Israel. This material was generated in their real-world, outdoor production systems. Because *C. vulgaris* and *P. tricornutum* were not available from industrial sources, we chose to generate biomass from these species in-house at NREL. Production of >100 g of each was achieved using NREL's suspended polyethylene bag growth systems and 250 L greenhouse raceway ponds, respectively (Figures 2A, 2B). Both were grown using supplemental CO_2 and relatively replete nitrogen conditions. Based on input from the DOE and the 2011 Peer Review, two additional sources of industrially-produced biomass were identified. *Nannochloropsis salina* (strain CCMP1776) was obtained from Solix BioSystems from their Coyote Gulch outdoor photobioreactor facility in southern Colorado. A second diatom, *Nannofrustulum* sp., was obtained from Cellana in Hawaii. Both algae were available in kilogram quantities as both whole cells and solvent extracted materials. Because of the quality and quantity of the purchased Solix biomass, we chose to use that material for the scale-up work of Task C. The five sources of biomass utilized in this project are summarized in Table 2.

Figure 2. Some of the sources of biomass used in this study. A, NREL hanging bag system; B, NREL greenhouse ponds; C, Solix BioSystems Coyote Gulch facility in Colorado.

Table 2. Algal biomass used in this project. Composition is shown in weight percent. The *N. salina* from Solix BioSystems (highlighted) was used for scale-up and nutrient recycle studies described below.

Species	Source	Scale	Designation – Extraction	Lipid	Protein	Carbs
Nannochloropsis sp.	Seambiotic	kg	**N1** – Whole cells **N2** – Hexane:IPA	10.6 3.0	34.0 32.7	7.6 9.6
Chlorella vulgaris	NREL	100 g	**C1** – Whole cells **C2** – Hexane:IPA	9.8 2.8	35.1 39.0	16.9 12.2
Phaeodactylum tricornutum	NREL	100 g	**P1** – Whole cells **P2** – Hexane:IPA	7.6 6.1	26.5 32.5	19.0 16.1
Nanofrustulum sp.	Cellana	kg	**NF1** – Whole cells **NF2** – Methyl pentane	13.0 2.6	12.5 8.7	9.0 11.0
Nannochloropsis salina CCMP1776	Solix Biosystems	kg	**NS1** – Whole cells **NS2** -- Hexane	37.2 11.8	17.2 26.7	11.5 17.0

For material that was not available in extracted form, lipid extraction was performed at NREL. This was done for the first three biomass materials of Table 2 using multiple runs in Soxhlet reactors. Initial extractions were done using Bligh-Dyer (chloroform/methanol) extraction chemistry, which was chosen because it was considered the "gold standard" for efficient extraction of algae at the laboratory scale. However, as discussed below and in Appendix A, any material that was extracted with chloroform could not be anaerobically digested. Subsequent extractions were performed with hexane/isopropyl alcohol (hexane:IPA) to generate the materials shown in Table 2. The

Nanofrustulum sp. and *N. salina* were obtained from the growers as methyl pentane and hexane extracted materials, respectively, in addition to un-extracted whole cells. All biomass was dried and stored at -20^{o}C to maintain stability and consistency between sources.

Compositional analysis of the biomass samples was conducted at WSU and NREL. This included elemental analysis (C, H, O, N, S) and metals analysis, using standard methods as described in Appendix A. In addition, measurements of primary cellular constituents such as lipid (fatty acid methyl esters; FAME), protein, carbohydrates, and ash were carried out using NREL's published methods (Laurens *et al*., 2012a; Laurens *et al*., 2012b). The complete compositional analysis is described in Appendix A (Tables 1 and 2). Of particular note, some of the materials were quite high in ash content, most notably *Nanofrustulum* sp., which had an ash content of over 50% for the extracted material. The key components of lipid, protein, and carbohydrate are summarized in Table 2 above. Note that most of the materials are relatively low in lipid and high in protein. C/N ratios, related to protein content, ranged from 6.8-15 for the whole biomass and from 5.5-8.5 for the extracted biomass. Such low C/N ratios were viewed as desirable for the project because they can pose important challenges for AD due to ammonia inhibition (preferred C/N ratios are in the 20-30 range), thereby setting the bar high for successful digestion. Note that the lipid extraction tended to be incomplete, with the extracted *C. vulgaris* and *Nanofrustulum* sp. showing the lowest levels of lipid post-extraction at just under 3%. The Solix *N. salina* was relatively high in lipid content at ~37%, with about 12% lipid remaining after hexane extraction. Note that as lipid extraction technologies improve (a commercial facility would not want to lose a third of the oil product), further reductions in C/N ratios could reduce the efficiency of AD to some extent.

Some experimentation was done on various pretreatment options using the solvent-extracted *Nannochloropsis* sp. biomass. This included utilizing a device called a Microfluidizer (manufactured by Microfluidics, Inc.), which passes a slurry of material through a small pore at high pressure. Such a pretreatment can be used to break apart the cell structure, with the intent of making the material more accessible to AD, and is a proxy for industrial scale homogenization procedures. A second method involved treatment with hot water and pressure using an Accelerated Solvent Extractor (ASE350). These materials were sent to WSU for testing, but since un-pretreated materials digested very well (see below), studying pretreatment options seemed unnecessary and was given low priority. Adding a pretreatment step would add extra cost to the process for little if any benefit in AD. However, in an industrial algal biofuels process, a cell rupture step might be included to aid in lipid extraction. Such a lysis step would likely only make AD easier; thus, we can think of these materials as a worst case scenario. Of course, there is certainly some breakdown of cell integrity inherent in the solvent extraction and drying steps performed on these materials.

High-Throughput AD Optimization

Working through the large number of algal biomass samples and potential variables of interest required a relatively rapid methodology for testing AD on a small scale. Because the Biological Systems Engineering department at Washington State University (WSU) is set up for such experimentation and has such an excellent track record for AD research, we chose to work with WSU under subcontract to carry out the AD optimization task. The work centered on the relatively high-throughput Biochemical Methane Potential (BMP) assays (also known as "respirometry" studies). These batch digestion experiments were useful in helping to elucidate optimum conditions for AD of algal materials and provided the groundwork for scale-up under Task C. Details of this work are provided in WSU's Final Report for their subcontract, which is included in its entirety in Appendix A. We therefore present here only some of the highlights.

All of the algal biomass materials described above were dried and shipped by NREL to WSU for the BMP studies. The materials were milled through a 1 mm screen to reduce compaction. BMP assays were conducted in 250 mL bottles in a 16-cell automated Challenger AER System (Fayetteville AR) as shown in Figure 3A. The bottles were maintained at 35±1 ºC and mixed continuously with a magnetic stirrer at 200 rpm. Seed inocula consisted of either anaerobic sludge from a wastewater treatment plant or from a dairy manure digester. Online gas monitoring was used to assess raw biogas and methane production. No additional nutrients or trace elements were added to the BMP bottles (except as noted). Cultures were adjusted to pH 6.95-7.05 and flushed with N_2 gas to induce anaerobic conditions. Triplicate digestions were run for each set of conditions, and BMP runs proceeded for 20-40 days to achieve nearly complete digestion as indicated by leveling off of methane production (for examples, see Figure 3B). Primary BMP performance indicators included volatile solids reduction (VS %), specific methane productivity (SMP; L CH_4 g VS fed^{-1}), total methane productivity (TMP; L CH_4 g VS^{-1}), and 95% methane production time (days to achieve 95% of total realized methane production.

A B

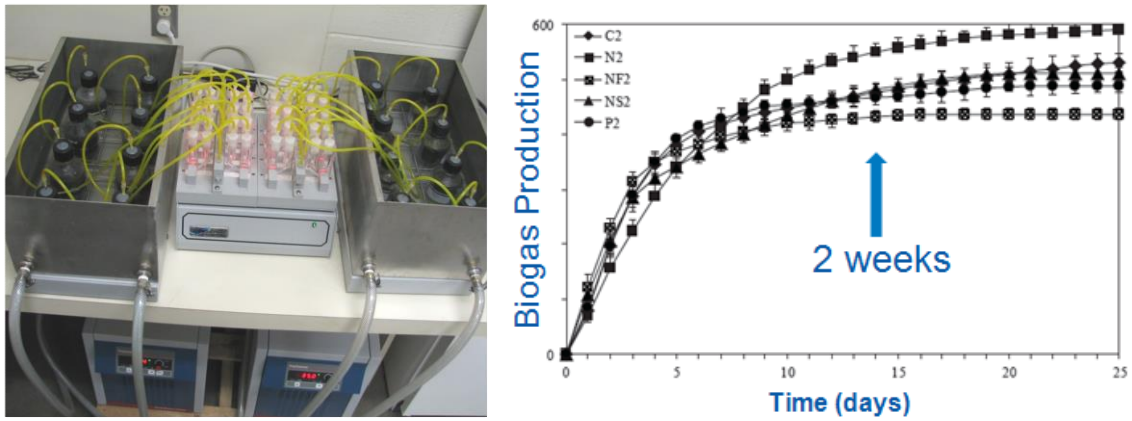

Figure 3. Respirometry studies. A, WSU equipment used for BMP studies; B, sample data for several of the extracted materials showing total biogas evolution over time (scale in mL).

A large number of BMP assays were carried out; further details can be seen in Appendix A and the two Milestone Reports associated with Task B (B.ML.2 and B.ML.3). Some serious initial roadblocks were overcome with careful experimentation and optimization of inoculum source, the ratio of inoculum to substrate, organic loading rates, and issues of extraction solvent inhibition. Table 3 summarizes the results after optimization for the ten biomass sources (see Table 2 for biomass designations).

Table 3. Summary of results from BMP studies. SMP, Specific methane productivity; 95% CH_4 Prod., days in which 95% of total CH_4 production is realized. See Appendix A for more details.

Parameter	C1	C2	N1	N2	NF1	NF2	NS1	NS2	P1	P2
SMP (L CH_4/g VS fed)	0.34	0.31	0.36	0.40	0.51	0.30	0.56	0.38	0.34	0.34
Max CH_4 (L CH_4/L D)	0.046	0.037	0.087	0.037	0.072	0.056	0.074	0.054	0.040	0.050
CH_4 Fraction (%)	63.8	59.1	66.6	67.6	73.2	69.6	66.6	75.0	64.8	69.3
VS Reduction (%)	66.1	64.2	65.9	64.4	76.4	59.3	78.5	73.8	70.6	60.2
95% CH_4 Prod. (D)	9.8	12.0	5.6	13.9	11.4	9.9	12.7	7.8	13.5	9.7

The following important findings resulted from the BMP studies:

1) Following optimization, all ten of the materials digested quite well as shown by the SMP values, which range from a low of ~0.3 L CH_4/g VS fed for extracted *C. vulgaris* and *Nanofrustulum* sp. to a high of 0.56 L CH_4/g VS fed for the un-extracted *N. salina*. Such values compare quite favorably with literature values for AD of other feedstocks and, although variable, represent a much tighter range than seen in the literature for algal feedstocks.

2) The maximum rates of methane evolution (a parameter that correlates with the initial slopes in Figure 3B) were also quite good.

3) Approximately 60-75% of the biogas consisted of methane, which is quite typical for AD of other materials.

4) The volatile solids reduction ranged from ~59-78%, indicating good digestion of the input algal biomass. However, this is quite a broad range, suggesting that it will be difficult to make *a priori* predictions of digestibility for a given source of algal biomass.

5) The digestions reached essential completion quite quickly, with the slowest digestions only taking about two weeks to reach 95% of their ultimate methane production. These data are important in determining the retention times that might be required in a production facility and are well within industry standards. Digestion beyond 95% typically shows very limited economic returns.

6) At least in these batch studies, higher inoculum:substrate (IS) ratios were important for achieving optimal digestion. This was true not only of degradation kinetics but also of the overall biogas production. Using an inoculum consisting of dairy digester sludge produced better results than wastewater treatment plant sludge, at least for the *Chlorella* and *Phaeodactylum* substrates.

7) Higher organic loading rates (OLR) resulted in reduced methane productivity. This coincided with high levels of volatile fatty acids (VFA), up to 10 g/L. In such BMP assays this may be the result of a "shock factor" of having all of the substrate available from the start, and this effect may not translate to continuously fed systems that have adapted to high OLRs.

8) On a qualitative level, the algal species varied considerably in their susceptibility to product inhibition and therefore the optimum OLR and IS ratio.

9) As expected, the extracted materials yielded less methane than the whole biomass (*e.g.*, 0.38 *vs*. 0.56 L CH_4/g VS fed for *N. salina*). This probably reflects the highly digestible lipid fraction in the whole cells and the higher relative fraction of protein in the extracted materials. In fact, overall there was a very good correlation between methane yields and lipid content.

10) Bligh-Dyer (chloroform/methanol) extracted materials gave very poor yields of methane, probably due to inactivation of the methanogen population as indicated by high VFA concentrations that were observed. This was most likely due to small amounts of residual chloroform even after extensive drying of the biomass post-extraction. This was confirmed by studies on the effects of various solvent extractions on AD performance (see Appendix A).

11) Dosing of calcium into the digestions enhanced methane production under some conditions. This was thought to be due to prevention of long chain fatty acid (LCFA) inhibition on cell surfaces.

12) Finally, the VFAs, ammonia nitrogen levels (TAN), and pH of the digestions remained within acceptable ranges (data in Appendix A). This suggests that there are no serious issues such as ammonia toxicity inherent to these digestions and that the C/N ratios of these feedstocks are not problematic when the correct digestion conditions are used.

In summary, the BMP assay phase of the project provided a critical opportunity to work out problems and find suitable conditions for good digestion of both whole cell and extracted biomass from all five species. Importantly, *no serious issues of ammonia toxicity, C/N ratios, or cell wall recalcitrance were encountered that could not be overcome through careful optimization*. This work put the project in position to move on to larger-scale continuous AD studies under Task C.

AD Scale-Up

Scale-up to continuous reactor conditions was critical to establish industry relevancy, as the conditions in these reactors are quite different from the small batch BMP reactors. Although we were ultimately successful in digesting all ten of algal biomass materials in BMPs, as described above, we chose to focus the scale-up work on the *N. salina* biomass from Solix BioSystems. This decision was based on the good performance of this material in BMP assays, the industrial relevance of this species, and the fact that we had ample supplies of both un-extracted ("NS1") and hexane extracted ("NS2") material from Solix. The SMP values of 0.56 and 0.38 L CH_4/g VS and VS reductions of 78% and 74% reported above for NS1 and NS2, respectively, were among the best observed. These results were encouraging that the stated goals for continuous digestion could be achieved, but scale-up can often pose un-anticipated challenges.

Sequencing Batch Reactors (SBRs) were employed for the AD scale-up studies, as shown in Figure 4. These reactors each have a working volume of 5 L and were mixed by impellers (100 rpm) for 10 minutes every 2 hours. The temperature was controlled at 35^{o}C by placement in a mesophilic chamber. Each cycle of the sequencing batch mode consisted of four stages: filling (feeding), reaction (with mixing, and comprising most of the time), settling, and discharge of supernatant. The hydraulic retention time (HRT) was controlled at 20 days. In order to provide enough seed to ensure successful start-up, inoculum-to-substrate ratios of 2.5 were used in both reactors. The two reactors were run in parallel, one with the un-extracted *N. salina* biomass (NS1) and the other with the hexane extracted material (NS2). OLR in both digesters ranged from 0.5-5 g VS L^{-1} d^{-1} and was increased step by step to the point of digester failure. Evaluation of system performance for each condition was carried out during pseudo steady state conditions, when biogas production, methane content and Chemical Oxygen Demand (COD) variations were less than 10 %.

Figure 4. Sequencing Batch Reactors used at WSU for scale-up studies. Apparent are impeller motors at the top of the units and multiple ports for adding and removing materials at various levels of the reactors.

Detailed results are presented in Appendix A for both NS1 and NS2 digestions. Both worked well (after some initial issues), and both readily surpassed the associated Milestone metrics for demonstrating stable biogas production over at least 30 days and reaching at least 50% conversion of incoming volatile organic carbon. In fact, both experiments proceeded for over *eight months*, and the OLRs were increased stepwise in order to demonstrate the highest loading rates that could be achieved before collapse of the digestion. The maximum OLR levels tested were 3.0 and 5.0 gVS/L/d for NS1 and NS2, respectively, which are in line with the upper end of conventional AD OLRs. In general, the productivities, methane concentrations, and degree of biodegradation shown for these digestions agreed well with the BMP observations, with the whole cell biomass again showing significantly higher methane yields. In addition to following biogas productivity (SMP) and VS destruction, measurements were made on the liquid effluent (after removal of solids by centrifugation) from each phase of the digestion for Total Ammonia Nitrogen (TAN), Total Kjeldahl Nitrogen (TKN), Total Phosphorus (TP), Total Inorganic Carbon (TIC), and alkalinity as reported in Tables 5 and 6 of Appendix A.

Because the use of lipid-extracted algal biomass is most process relevant, we focus here on the highlights of the NS2 digestion (hexane-extracted *N. salina* biomass from Solix BioSystems). The effluent from this digestion was furthermore used for the nutrient recycle studies of Task D, and the results of this digestion were used to inform the techno-economic modeling under Task E. Figure 5 shows the volumetric methane productivity over time for the NS2 SBR reactor. The OLR at each of the eight phases is indicated. The reactor ran for approximately 280 days before eventually crashing at an OLR of 5.0 gVS/L/d. Table 4 shows specifics of the data collected during each phase of the digestion. Not surprisingly, the SMP and VS destruction decreased as more material was forced through the reactor (*i.e.*, higher OLRs). The SMP ranged from 0.29-0.42 L CH_4/gVS, which is fairly broad but all within the range of reasonable AD productivities and consistent with previous BMP results. Note that at the two highest OLRs, the volumetric methane production rate is well above industry's standard acceptable level (see Appendix A). VS

destruction dropped below 50% only at OLRs greater than 2.5 gVS/L/d. The economic trade-off between digestion efficiency and loading rate has been modeled as discussed below.

As OLR increased, the concentration of nitrogen and phosphorus in the liquid effluent increased accordingly. Although there was some significant variation between the phases of the experiment, in general about 90% of the input nitrogen came through in the liquid fraction of the effluent, and about 30% of the input nitrogen came through as ammonia (TAN). Wide variations were seen for phosphorus as well, but it appears that a significantly smaller percentage of the input P came through in the liquid fraction (averaging around 50%). As a caveat, we should note that there is some uncertainty around these numbers due to issues of reactor leakage, and because of unknowns regarding the long-term accumulation of solids within the reactor it was not possible to completely close the mass balances for N and P. Note also that these numbers differ slightly from those previously reported due to refinements in the calculations.

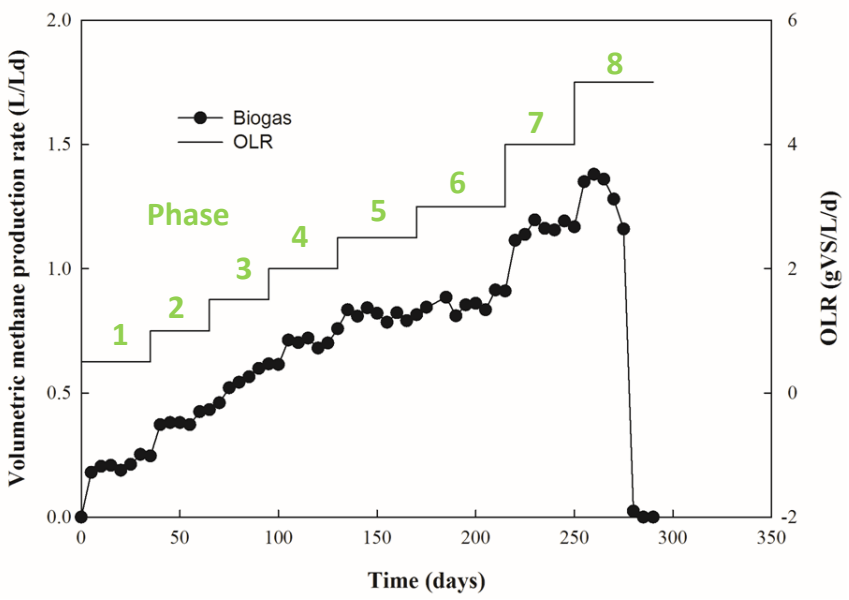

Figure 5. Results of NS2 (extracted *N. salina*) SBR digestion, showing methane volumetric production rate as a function of time (dots). Step-wise plot shows increasing OLR levels for each phase in gVS/L/d (on right-hand axis).

Table 4. Summary of results from continuous digestions of NS2 (extracted *N. salina*) biomass for the eight phases. Note trend of decreasing methane productivity (SMP) and solids destruction ("VS destr. %") as loading rate (OLR) increases. Measurements of TAN and TKN nitrogen in the liquid effluent and the fraction of input N those constitute are indicated; likewise TP phosphorus in the liquid fraction is shown. TKN and TP fractions in the solids fraction are calculated by subtraction. Values over 100% may be due to fluctuations in the accumulation and purging of solids from the reactor. Highlighted rows were the subjects of further study in Tasks D and E.

Phase	OLR gVS/L/d	SMP L/g VS	VS destr. %	TAN mg N/L in liquid	TKN mg N/L in liquid	TAN % of input N in liquid	TKN % of input N in liquid	TKN % of input in solids (calc.)	TP mg P/L in liquid	TP % of input P in liquid	TP % of input P in solids (calc.)
1	0.5	0.42	nd	nd	nd	nd	nd	nd	nd	nd	nd
2	1.0	0.40	72.6	387	678	31	54	46	95.8	38	62
3	1.5	0.39	72.8	466	1800	25	96	4	244	65	35
4	2.0	0.34	56.7	740	2700	30	108	<0	283	57	43
5	2.5	0.33	51.3	925	2670	30	86	14	178	29	71
6	3.0	0.29	48.7	1090	3490	29	93	7	287	38	62
7	4.0	0.29	46.7	1560	5300	31	106	<0	465	47	53
8	5.0	0.28	45.7	1660	5610	27	90	10	640	51	49

In summary, the continuous AD studies on algal biomass were quite successful and informative. Stable biogas production was maintained for long periods of time, high loading rates were achieved, and excellent biogas productivities were observed using both extracted and un-extracted *N. salina* biomass. An important benefit of the success of these studies is that it allowed us to stockpile effluent from these digesters for future experimentation such as the testing of nutrient recycle to algal growth systems under Task D. In addition, the results of these continuous studies provided more relevant real-world data for techno-economic and life cycle modeling of the AD component of algal biofuels production under Task E.

Nutrient Recycle Studies

Providing nitrogen and phosphorus to a productive algal culture is associated with significant economic and life cycle costs. Very little of these elements should end up in the extracted oil or the biogas. AD effluent, by contrast, is rich in both nitrogen and phosphorus, as shown in Table 4 above. However, few data are available on the bioavailability of these nutrients for algal growth or whether using AD effluent as a nutrient source might pose insurmountable problems such as inhibition, toxicity, or light occlusion. Task D of this project was designed to test this on a small scale. We focused our attention on nitrogen rather than phosphorus because of its significantly greater impact on the life cycle balance and techno-economics (see below).

The NS2 digester effluent is composed of both liquids and solids, which can be separated by centrifugation. Table 4 above shows the concentrations of ammonia (TAN), total nitrogen (TKN), and phosphorus (TP) in the liquid portions of the materials. This analysis was confined to the liquid phase after solids removal because particulates in samples will complicate the measurements, and because the direct addition of the solids to algal cultures could be problematic. There is some variability and uncertainty associated with the data for the different phases of the NS2 digestion, but overall it appears that about 90% of the input nitrogen and about 50% of the input phosphorus came through in the liquid fraction of the effluent. About one third of the nitrogen in the effluent was in the form of ammonia (TAN). The solids, then, contain a relatively small fraction of the input nitrogen but a relatively large fraction of the input phosphorus. Nitrate and nitrite were undetectable in the liquid effluent (data not shown). Presumably most of the non-TAN nitrogen is in the form of organic nitrogen compounds, including suspended AD cell debris (both from undigested algal cells and AD microbes) that was not pelleted by centrifugation. The loss of phosphorus to the solids fraction is not unprecedented and may be due to precipitation into insoluble particles as calcium and/or magnesium salts. There may also be differential degradation in AD of the cellular components that contain the preponderance of these elements; for example, the bulk of nitrogen is in proteins, which are relatively easily degraded. In any case, it is an important finding that most of the input nitrogen in the biomass fed to the digester is available in the liquid effluent, and close to a third of the input nitrogen is in the form of ammonia/ammonium, which can be easily utilized by many species of algae.

To test nutrient recycle empirically, we have focused on growth studies using *N. salina* strain CCMP1776 from the National Center for Marine Algae and Microbiota, as this was the strain employed by Solix for their production of the original biomass. Thus, this "closes the loop" on recycling nutrients in a hypothetical *N. salina* production facility. The first step was to confirm that this strain was amenable to growth on ammonium as the primary nitrogen source, as some species of algae can be selective about their preferred nitrogen source. Small-scale shake flask experiments were conducted using CCMP1776 grown in seawater-based media (f/2). This strain was shown to grow quite well with chemical ammonium (NH_4Cl) as the sole nitrogen source at moderate levels (1 mM), but higher levels (5 mM) proved to be quite inhibitory. The reason for this is unknown, but this is an important finding as it suggests that high concentrations of ammonium-rich effluent could prove toxic. Further *N. salina* growth curves were carried out with chemical nitrogen sources to identify the optimal ammonium concentration. As shown in Figure 6, there was a very strong concentration dependency. At a level of 1.5 mM NH_4Cl, the growth rate was approximately equal to the 5.0 mM nitrate control, (but in this case the final OD was much higher due to collapse of the nitrate cultures). Interestingly, any lower or higher level of ammonium gave poorer performance. These data show how critical it is to dial in the optimal ammonium concentration. Modest growth in the "no nitrogen" control flasks is probably due to low levels of nitrogen present in the seawater component of the f/2 growth media.

The next step was to conduct shake flask experiments on *N. salina* using the NS2 AD digester effluent as the sole nitrogen source. Both Phase 2 and Phase 5 materials were tested, with most experiments done with Phase 2. Solids were removed from the effluent by centrifugation. Effluent supernatant was added to nitrogen-free (phosphate replete) growth medium at various concentrations up to 16% (v/v), and duplicate shake flask cultures were grown. Growth data for these studies are shown in Figure 7. The results show that the effluent is able to support rapid growth of the cultures, with the two highest concentrations giving growth rates in excess of the positive control of 1.5 mM NH_4Cl. For comparison to Figure 6, the effluent concentrations of 8% and 16% in Figure 7 correspond to TAN

concentrations of approximately 2 mM and 4 mM, respectively. It is interesting that these higher ammonium concentrations in the context of the effluent were not inhibitory as they were for chemical ammonium. These data surpassed the metrics associated with the Milestone for this task, in that we were able to *completely* replace chemical nitrogen supplementation to support growth of microalgae with *no impact* on growth rate (and possibly an enhancement relative to cultures with equivalent chemical ammonium levels). At the risk of over-interpreting these qualitative experiments, it should also be noted that at 2% effluent (~0.55 mM TAN), the total nitrogen (TKN) provided by the effluent should be approximately 1.5 mM, yet the growth rate was substantially below the 1.5 mM NH_4Cl control. This would likely not be the case if all of the non-TAN nitrogen were bioavailable. In other words, the data are consistent with only the TAN nitrogen being readily utilized, but the complexities of such biological experiments make such conclusions risky.

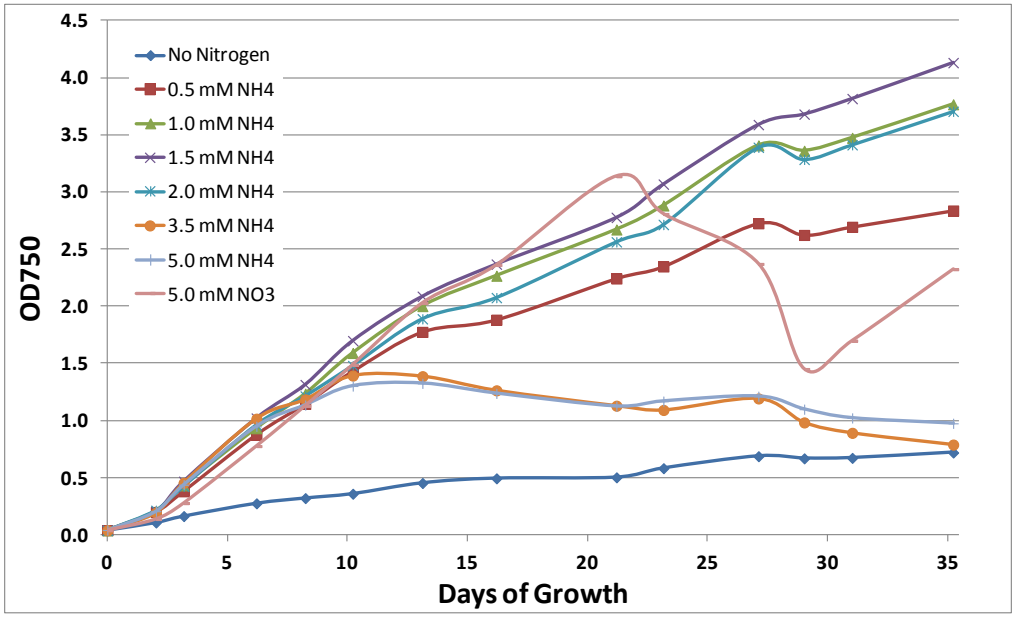

Figure 6. Growth of *N. salina* CCMP1776 at various ammonium concentrations. Each point represents the average of duplicate shake flask cultures. Best growth is observed at 1.5 mM NH_4Cl. 150 mL cultures were grown in 500mL flasks and shaken at 120rpm at 22°C with a 16:8 hour light:dark cycle.

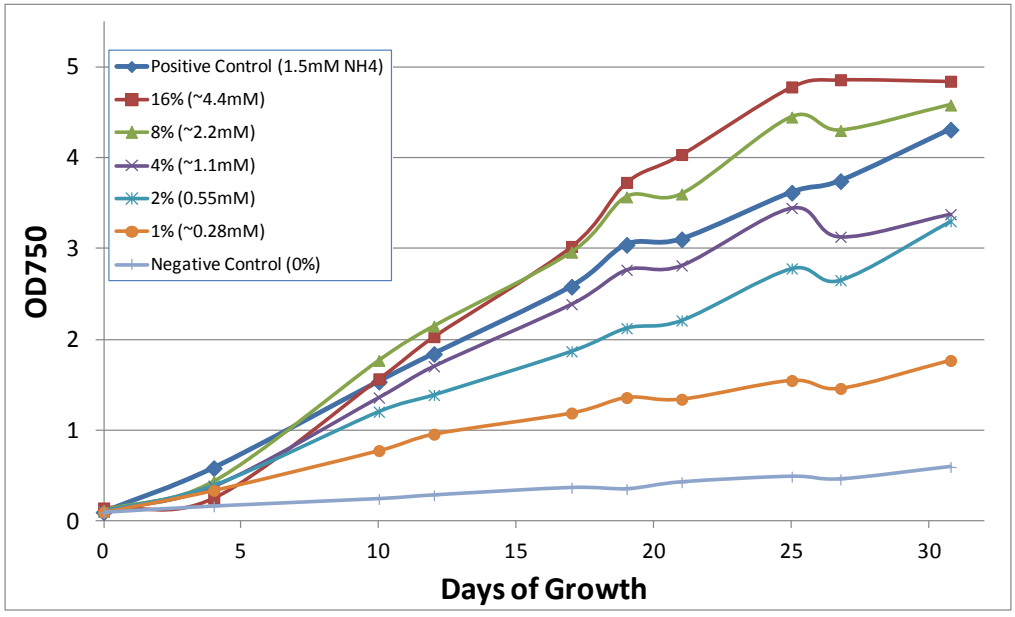

Figure 7. Growth of *N. salina* CCMP1776 at various effluent concentrations. Each point represents the average of duplicate shake flask cultures. Shake flask conditions were identical to Figure 6.

Additional sets of growth studies were carried out. In one set, growth curves were generated in 1 liter Roux bottles (see Milestone Report D.ML.5 for more details). This setup allowed for cultivation under higher light levels and with supplemental CO_2 sparging. Once again, AD effluent supported very active growth, demonstrating that effluent can substitute very well as a nitrogen source even under these conditions of higher light and CO_2 concentrations. A final set of shake flask studies was undertaken in an attempt to tease out the subtleties of nitrogen and phosphorus availability. This included comparing filter sterilized liquid effluent, whole effluent (liquids + solids), and the solids portion alone for their ability to replace the nitrogen component of the media. Unfortunately, the results of this experiment could not be used to draw meaningful quantitative conclusions. This was primarily due to very significant growth in the negative control, most likely due to excessive nitrogen in this batch of seawater-based f/2 medium. In addition, the triplicates in some cases did not match well enough to make quantitative conclusions (*i.e.*, the error bars were too large). Despite these problems, we can make some qualitative assessments about the data:

1) In all cases, addition of effluent resulted in enhanced growth relative to the control with chemical ammonia.
2) Whole effluent appeared to confer somewhat better growth than the filtered liquid fraction at the same TAN concentration. This suggests that some of the nutrients in the solids portion are bioavailable.
3) Using the solids fraction only (no liquid fraction) conferred quite good growth, again suggesting that at least some of the nutrients in the solids portion of the effluent are bioavailable.
4) An additional experiment to test whether whole effluent could replace phosphorus addition was inconclusive.

This experiment provides hints that nitrogen in the solids portion of AD effluent may be utilizable for algal growth, but further experimentation is clearly warranted. Of course, shake flasks are very different from outdoor ponds, and addition of whole effluent to pond cultures might pose other challenges related to the accumulation of solids. Further work should also examine the practicality and costs associated with methods to solubilize N and P in the AD solids fraction, perhaps through acid hydrolysis.

The liquid effluent was very dark colored, raising the question of whether using such materials as a nutrient source might block light and thus reduce algal photosynthesis. We therefore explored the opacity effects of adding AD effluent to algal cultures. As shown in Figure 8, at the level of effluent (either filtered or whole) that we are adding in these experiments, the effect on optical density over the region of photosynthetically active radiation (PAR) is minor compared to the optical density of the algal cells in a relatively dilute culture. Thus, we do not expect occlusion of light caused by addition of AD effluent to be a significant problem, as supported by the good growth rates observed under these conditions. However, we know very little about what compounds in the effluent are responsible for light absorption and the observed coloration, and it is possible that after multiple rounds of recycle such compounds could accumulate to the point of significantly blocking light penetration. Of course, replacement of water (blowdown) and natural biological, physical, or chemical pathways for removal of these compounds in the pond environment might help to ameliorate this effect.

In summary, we were successful in replacing 100% of the standard media chemical nitrogen source, with no negative impact on growth. In fact, in some cases the effluent was able to confer improved growth relative to the controls at similar TAN levels. This may be due to bioavailability of other nitrogen compounds within the effluent and/or additional beneficial nutrients within the effluent such as phosphorous. These findings are important in that we can now say with confidence that at least some portion (presumably the ammonium fraction) of nitrogen in AD liquid effluents can readily be recycled for algal growth. However, the fact that we are able to replace 100% of the required nitrogen for algal cultivation with AD effluent does not mean that can achieve 100% recycle of nitrogen. As shown above, only about 30% of the nitrogen input to the AD digester is available as ammonia/ammonium in the liquid effluent. Thus, achieving very high rates of nitrogen recycle will require mobilization and utilization of non-ammonia nitrogen compounds in the liquid effluent and also in the solids fraction. With current data, we cannot draw meaningful conclusions about the bioavailability of those other nitrogen fractions; future work should address this in more detail. *The most significant findings from our work on nutrient recycle are that we did not encounter some of the anticipated issues with using AD effluent as a nutrient source, such as toxicity or excessive occlusion of light*.

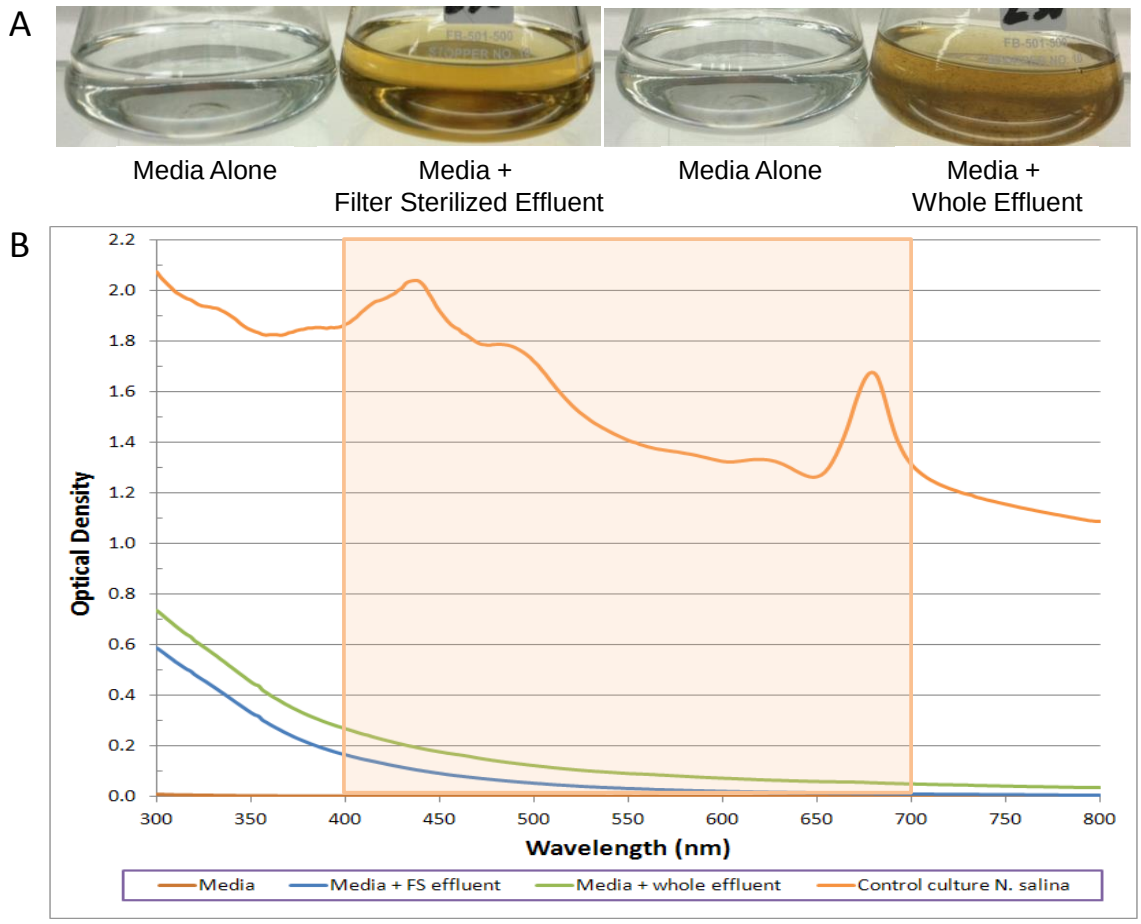

Figure 8. Opacity of effluent under conditions of growth studies. A, photos of growth flasks containing media with or without added effluent before inoculation. B, optical density *vs*. wavelength for growth media containing AD effluent as used in growth experiments (at 1.5 mM TAN levels), using either filter sterilized (FS) or whole effluent. The upper line indicates the optical density as a function of wavelength for a *N. salina* culture at just over 1 OD_{750} for comparison. Shaded region indicates PAR wavelengths from 400 to 700 nm.

Techno-Economic Analysis

The experimental work described above provided important information on algal AD parameters such as biogas yield, productivity, loading rates, retention time, effluent composition, etc. Task E was focused on using these findings to inform techno-economic analysis (TEA) and life cycle assessment (LCA) models surrounding the ALU pathway option. Since it has been deemed that LCA of algal biofuels falls more within the purview of Argonne National Laboratory (ANL), our approach regarding LCA has been to freely communicate our findings to Ed Frank of ANL for incorporation into their GREET models. The focus in this report is on the TEA modeling, examining the impact of our AD data and conducting sensitivity analyses surrounding those numbers. Overall, the biggest contributors of AD to the renewable diesel selling price are in the areas of nutrient recycle and heat/power generation, whereas carbon recycle is only a minor contributor (at least with the flue gas pipeline delivery modeled in NREL's baseline TEA process). High organic loading rates achieved in this project can significantly reduce AD capital costs and compensate for associated yield losses observed at higher loading rates. Modeling of various nutrient recycle fractions demonstrated the importance of these parameters and highlighted that nitrogen recycle plays a larger role in cost impacts than does phosphorous recycle. These TEA studies provide a rigorous basis for comparison of AD to competing pathways for utilization of extracted algal biomass. We present our findings below in answer to several questions regarding the impact of AD on the techno-economics of the ALU pathway.

What is the overall impact of AD on process economics?

AD has long been assumed in algal TEA models as a way to deal with spent material after lipid extraction. In conjunction with the harmonization effort, we have revisited the overall impact of AD on the process economics. Figure 9 shows the impact of removing various benefits of AD (both operating and capital cost impacts) on the renewable diesel (RD) selling price. The baseline value of $18.63/gal for the harmonized model for autotrophic pond production is based on many assumptions (see Davis *et al*., 2012) including fairly conservative assumptions around productivity (13 g/m^2/day algal biomass yield and 25% oil content). The heat and power benefits of AD, along with the nutrient recycle, are clearly the biggest contributors of AD to cost savings. The values for CO_2 recycle and the credit for sale of the AD effluent solids as fertilizer are clearly much smaller contributors; however, the former point regarding CO_2 recycle is specific to the baseline model assumption that bulk flue gas from an external source (*e.g*., power generation station) is delivered by pipeline to the algae facility. Therefore, a reduction in this flue gas makeup rate (due to CO_2 recycle) does not translate to a linear reduction in flue gas delivery costs due to economy of scale variations in pipeline capital cost. If purified CO_2 were instead utilized at a fixed material delivery price, the savings may be more pronounced as the trend would be linear with CO_2 recycle. Overall, the removal of the assumed AD process component in the harmonized model results in an addition of $1.81/gal to the base case, or about 10%. This includes removing the various benefits that translate to direct and implicit co-product credits (shown in Figure 9), as well as removing the capital costs for the AD and power generation equipment itself (whose cost impacts upon removal were subtracted from the cost increases for each explicit step shown in Figure 9). In other words, the $1.81/gal cost impact upon removal of AD would be the "delta" that must be made up for otherwise in a different co-product approach (such as animal feed), including the addition of drying equipment and other operations necessary to process the algal residue material into the final co-product. It should be noted that as improvements are made elsewhere in the process to reduce the $18.63 base case, such as increased cultivation productivity, the AD components may have a larger percentage impact on total price.

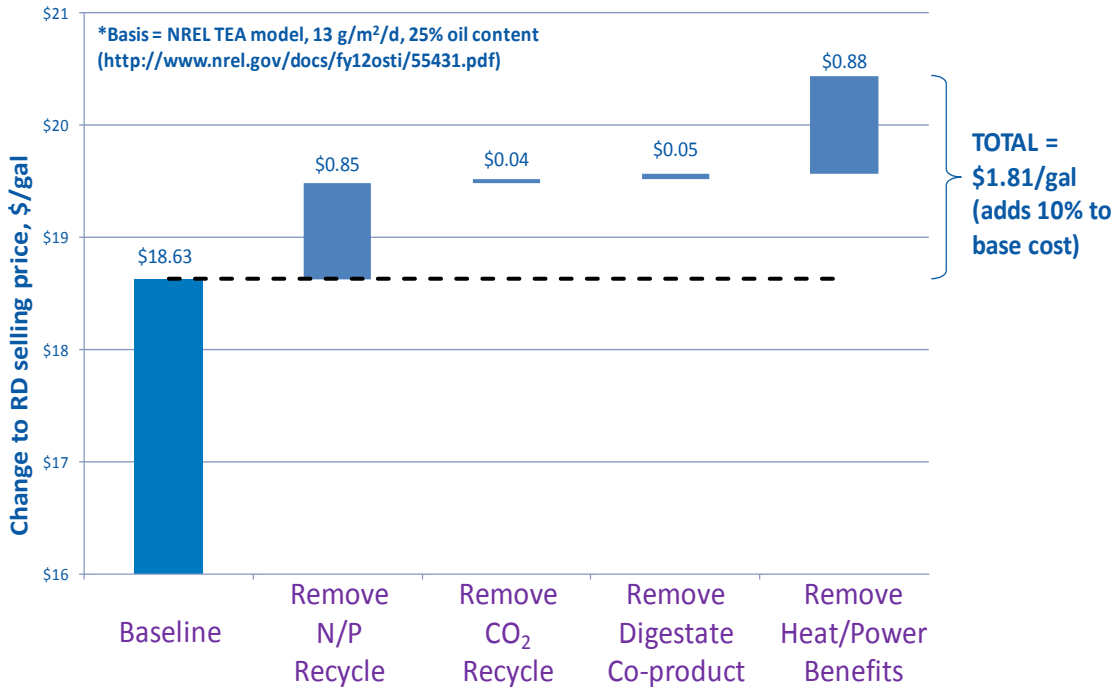

Figure 9. Overall TEA contributions of AD to RD selling price in harmonized model.

The modeling assumptions for AD parameters used in previous modeling have been based on limited literature data and work with other feedstocks. A primary purpose of this project was to provide real-world data over long-term trials for a variety of parameters such as biogas yields, digester loading rates, retention times, potential inhibitors, nitrogen and phosphorus recycle, etc. Previous digestion studies were generally less exhaustive and of shorter duration than our study, and few examined the digestion of lipid-extracted materials. In the end, however, our results were generally supportive of the previous assumptions used for TEA modeling. Table 5 shows a comparison of the values used in the harmonized model (Davis *et al*., 2012) versus the values demonstrated under this research project. Most importantly, our data on continuous digestion of lipid-extracted algal biomass support and validate the model's

assumed values for biogas yield, retention time, and organic loading rate (OLR). Nutrient recycle is the only area where values used in the harmonized model may be somewhat optimistic. There is still some uncertainty around the bioavailability of N and P in the effluent and the recycle to the cultivation step that can be achieved. However, our data (see Table 4) can be used to bracket the range of potential recycle. In Table 5, the "conservative" case assumes that only the amount of nitrogen measured as TAN (total ammonia nitrogen) in the liquid effluent is bioavailable (30% of input N in Phase 5) while only 20% of the input P is bioavailable (70% of the 29% in the liquid fraction for Phase 5). Alternatively, the "optimistic" case assumes that 77% of the input N (~90% of TKN in the Phase 5 liquid) and 25% of the input P (~86% of TP in the Phase 5 liquid) is bioavailable in the liquid effluent stream. (Note that these numbers were based on a previous iteration of the nutrient recovery calculations and that the Table 4 data now suggest a somewhat more optimistic case being conceivable.) The impacts of this spread in nutrient recycle values on the renewable diesel selling price are shown in Figure 10; only about a 3% difference in selling price is observed over the range tested. Sensitivities around nutrient recycle will be explored in more detail below.

Table 5. Comparison between assumed values for AD parameters from harmonized baseline model *vs.* values supported by data from this project.

Metric	Harmonization baseline	NREL/WSU-conservative	NREL/WSU-optimistic
Biogas yield (L CH_4/g TS)	0.30	0.30	
Retention time (days)	20	20	
Organic Loading Rate (g TS/L-day)	2.4	2.8	
% TS in feed	4.9%	5.6%	
Temperature (°C)	35	35	
% bioavailable N recovered in effluent	80%	30%	77%
% bioavailable P recovered in effluent	50%	20%	25%

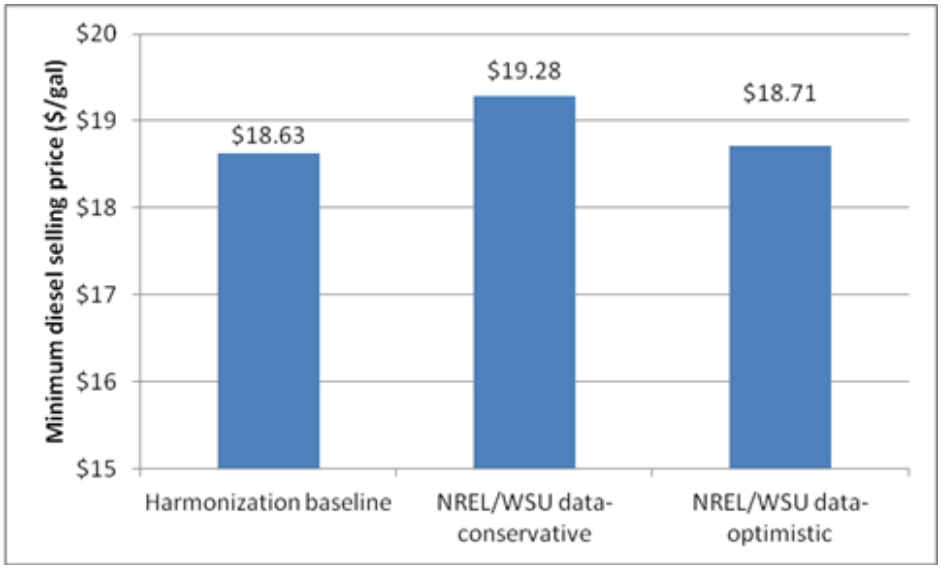

Figure 10. Comparisons of conservative and optimistic cases for nutrient recycle resulting from this project relative to the harmonized baseline (based on assumptions in Table 5).

What are some of the key sensitivities?

Again in conjunction with the harmonization effort, we examined some of the key sensitivities regarding the AD component of the process. Figure 11 shows changes in the renewable diesel selling price upon variation of four key parameters in the AD component of the model. All other assumptions are as shown and as described in Davis *et al*. (2012). Clearly varying the amount of nutrient recycle or the amount of methane produced from a given amount of total solids (TS) are the dominant variables. The volatile solids (VS) loading factor (or the OLR) is also a strong contributor because of the impact on digester size and resulting capital costs. Interestingly, the re-uptake by the algal culture of fixed carbon in the AD effluent is a very minor contributor; thus, this form of carbon recycle can safely be ignored in the modeling (and was not assumed).

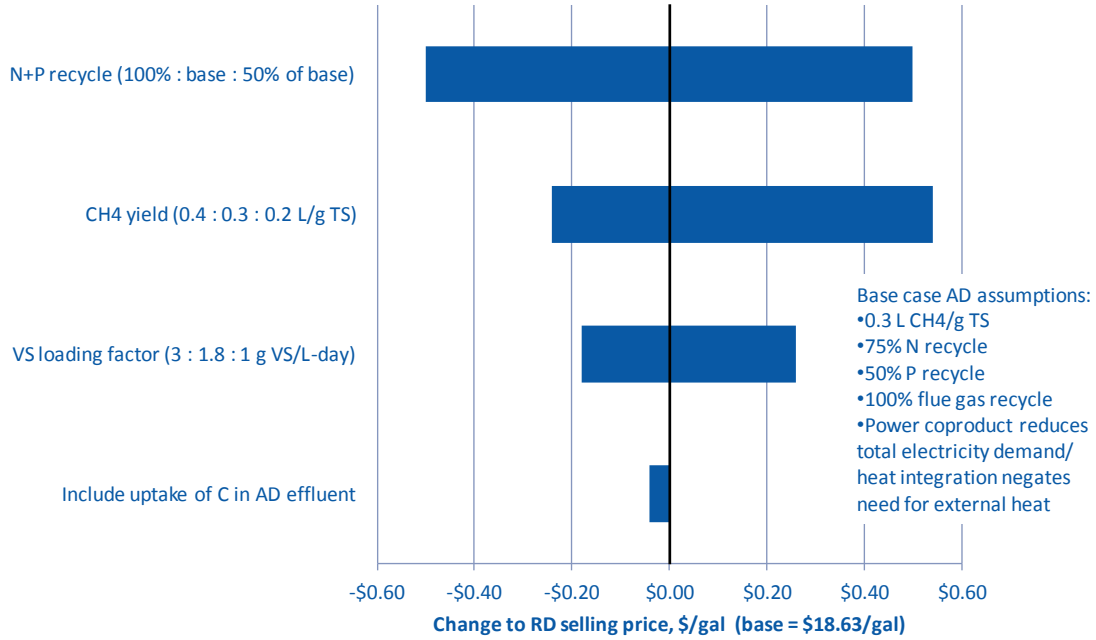

Figure 11. Tornado plot showing relative sensitivities to four central AD parameters.

What are the most significant contributors to AD capital costs?

Table 6 shows a breakdown of the costs for installed CAPEX across the various elements included in the AD portion of the model, as associated with the base $18.63/gal RD case. Clearly the final two items on the list dominate the cost spreadsheet.

Table 6. Cost breakdown of capital equipment related to AD.

Component	Fraction of Total	CAPEX Cost (in $18.63/gal Base Model)
AD feed cooler	0.0025	$34,560
Biogas emergency flare	0.0010	$13,581
Polymer addition system	0.0003	$3,833
AD feed pump	0.0068	$95,395
AD sludge pump	0.0027	$38,448
Centrifuge feed pump	0.0018	$25,220
Centrifuge	0.1910	$2,675,945
AD system (AD + biogas blowers)	0.7940	$11,126,537
		TOTAL: $14,013,518

What is the trade-off between loading rate and biogas yield?

One of the interesting findings from the AD scale-up work described above was that as the loading rate (OLR) increased in the NS2 digester, the yield of biogas (per unit of solids added) decreased somewhat (see Table 4). Although this trade-off was not unexpected, it is interesting to know the impacts on the economics. To put this another way, is it more cost-effective to try to maximize yield or minimize the size of the digester? To examine this, we modeled three scenarios of yield/OLR based on data from three phases of the NS2 continuous digestions. The modeled parameters are summarized in Table 7; OLR was varied by more than a factor of five, and the resulting biogas yields decreased by about 30% at the highest *vs*. lowest loading rates examined. As shown in the final row of Table 7, the modeled AD volume is inversely proportional to OLR.

Table 7. Three scenarios modeled to examine the trade-off between biogas yield and loading rate of solids in the digester, as highlighted, based on project data at the multi-liter scale. Note that numbers for biogas yield differ from Table 4 because these units are for total solids (TS) rather than volatile solids (VS) in the previous table.

Parameter	Scenario 1	Scenario 2	Scenario 3
Based on WSU NS2 Phase number	2	5	8
Biogas yield (L CH_4/g TS)	0.36	0.30	0.25
Retention time (days)	20	20	20
OLR (g TS/L-day)	1.1	2.8	5.6
% bioavailable N recovered in effluent (using harmonization baseline assumptions)	80%	80%	80%
% bioavailable P recovered in effluent (using harmonization baseline assumptions)	50%	50%	50%
Resulting total modeled AD volume	104 MM gal	41 MM gal	20 MM gal

The results of Aspen modeling using these values are shown in Figure 12. The downward trend in the renewable diesel selling price (blue line) indicates that, despite the losses in yield at higher loading rates, the fuel selling price benefits significantly. This is due to the lower capital costs associated with the reduced digester volumes required at high OLR. This is a useful finding and argues for emphasizing the optimization of loading rates in a commercial facility. At the same time, however, it would be important to understand the LCA tradeoffs in this scenario, as reduced biogas yields will negatively impact the overall GHG profile of the system.

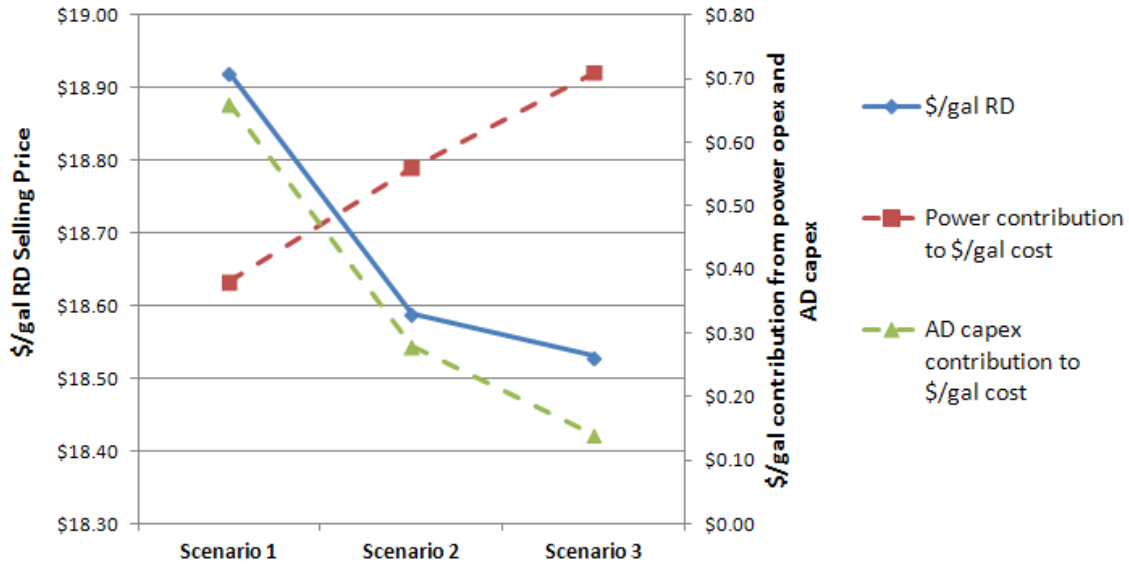

Figure 12. Aspen modeling results for the three scenarios of Table 7.

What is the sensitivity to nutrient recycle efficiency?

As shown above, the percentage of nutrients available for recycle is a significant factor in the economics of the AD process component. We wanted to explore further the implications of various recycle scenarios. To this end, we tested eight scenarios examining various levels of N and P recycle, as shown in Table 8. The results are plotted in Figure 13. The red bar indicates the base case harmonized renewable diesel selling price of $18.63/gal and assumes the harmonized 80%-50% availability of N and P, respectively. The second scenario shows the impact of no nutrient recycle from AD, and the third scenario shows essentially complete recycle of both (95%-95%), thus delineating the outer limits. Scenarios 4 and 5 show the impact of full recycle of only P or N, respectively. This comparison demonstrates that N recycle has a much greater impact on process economics than P recycle. This is encouraging in light of the fact that P recycle appears to be more challenging than N recycle relative to the baseline targets, as in many cases more than half of the phosphorous partitioned into the solids digestate phase which is not currently assumed in the baseline model to be recycled to the ponds. Scenarios 6 and 7 describe the "conservative" and "optimistic" boundaries based on the project data, as described above, thus suggesting that we have narrowed the possible range substantially (to less than a $0.50 difference between the high and low values). Note that the optimistic recycle case (scenario 7) is quite similar to the baseline assumptions and resulting cost, with the conservative case roughly 3% higher ($0.48/gal) on a final cost basis. Finally, the 8th scenario shows the outcome using the 20%-20% metrics from the Milestone language (see Abstract), which our data show to be quite conservative.

Table 8. Eight scenarios modeled to examine potential levels of nutrient availability for recycle.

Scenario #	N available in effluent	P available in effluent	Description
1	80%	50%	Baseline using harmonized assumptions
2	0%	0%	No recycle of N or P
3	95%	95%	Essentially complete recycle using whole effluent
4	0%	95%	Recycle P only
5	95%	0%	Recycle N only
6	30%	20%	NREL/WSU conservative
7	77%	25%	NREL/WSU optimistic
8	20%	20%	Milestone metrics

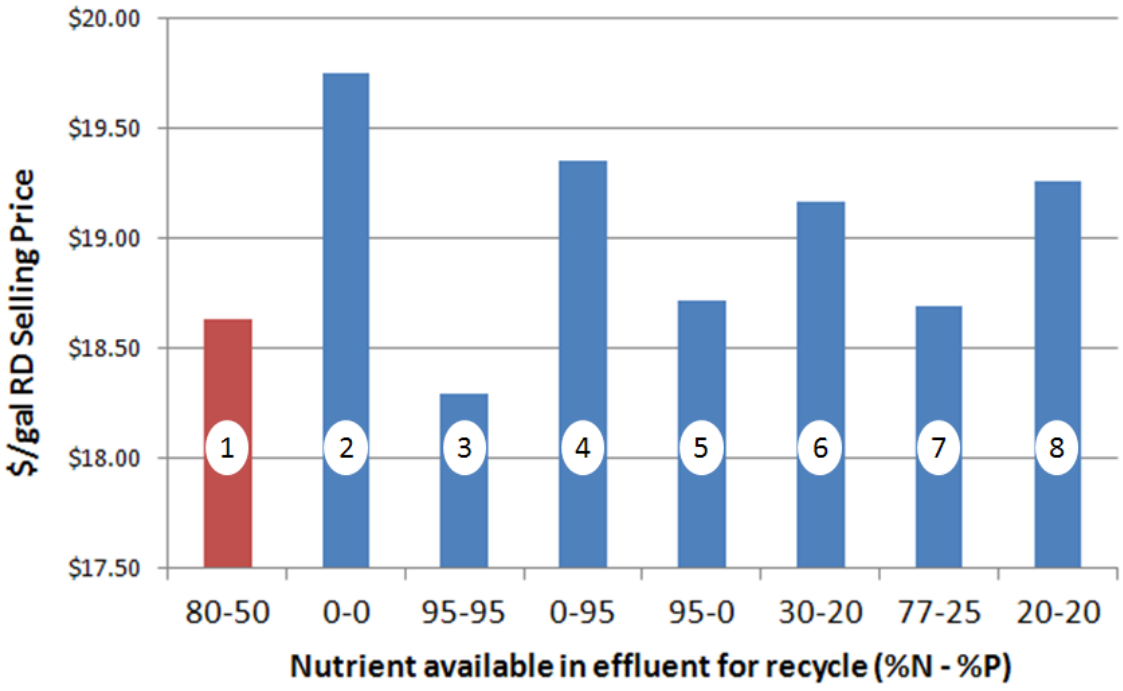

Figure 13. Sensitivity around nutrient recycle as modeled for the eight scenarios of Table 8.

What is the best use of the effluent solids fraction?

First, it should be noted that the fractions of nitrogen and phosphorus that end up in the effluent solids (sludge/digestate) varies between samples tested, and there is still some uncertainty in our studies due to incomplete mass balance closures. However, it appears that a small portion of the N (on the order of 10%) and more than half of the P end up in the solids fraction. Thus, disposition of this material is an important consideration. The general assumption has been that this sludge material would be sold based on its value as fertilizer for application to cropland. As noted previously, the value of this material is quite low. In the harmonization baseline the digestate fertilizer co-product credit equates to $0.05/gal RD (removing this credit would increase the RD price from $18.63 to $18.68/gal). The basis for this calculation was an assumed 20% N partitioning into the digestate (based on earlier numbers), of which 40% is assumed to be bioavailable and is the fraction used to set the fertilizer price.

It would be preferable to return the digestate to the algal growth system. Recycling of the whole effluent (liquids and digestate together) back to the ponds results in a RD price of $18.22/gal. This is after also removing the centrifuge capital cost (20% of total AD system cost), and assuming 95% total N and P recycle. Thus, under these assumptions, this scenario could provide a credit of $0.46/gal (relative to $18.68/gal with no co-product as per above) *vs*. the $0.05/gal credit for digestate sold as fertilizer. Thus, if possible, recycling of this material to the algal growth system would be advantageous. Preliminary studies in shake flasks have so far been inconclusive, but the solids settle very rapidly and don't appear to be significantly resolubilized on a useful timescale. Thus, further research is warranted on chemical or biological methods to mobilize the nutrients in this material for enhanced recycle. Also, the benefits of using the digestate for algal growth rather than land application may extend beyond TEA. The life cycle cost of land application of this material may be very significant, particularly because of the release of N_2O, which is a powerful greenhouse gas (Frank *et al*., 2011).

How significant is carbon recycle?

The lipid extracted algae sent to AD (Figure 1) can contain more than half of the carbon fixed by the growing algal culture. Combustion of the AD biogas stream in a turbine provides the opportunity to recycle this carbon, in the form of flue gas, to the cultivation system. In the model, roughly 30% of the carbon is recycled in the flue gas from the AD turbine, thus reducing CO_2 import by 30%. At a fixed size pipeline for off-site flue gas delivery, this reduces power demand for off-site flue gas transport by roughly 700 KW, which translates to a savings of $447,000/year (1% of all operating expenses, or 0.3% of allocated $/gal selling price contribution, or a $0.05/gal impact). The cost impact of this recycle is fairly marginal, as demonstrated in the waterfall plot of Figure 9 above, with only a $0.04/gal impact from flue gas recycle. This is due in part to the way that makeup CO_2 is costed in the model, as it is assumed to be delivered as flue gas *via* pipeline transport from a nearby power plant (including capital costs for flue gas pipelines and blowers, plus operating cost for blower power). The flue gas pipelines are quite large (four separate 1-mile pipelines each 6 ft diameter), so the cost impact for removing CO_2 recycle is to either increase pipeline diameter to accommodate a higher makeup flow or to increase blower power to transport more flue gas through the same size pipeline. Because of the pipeline delivery assumption, a fractional change in makeup CO_2 rate does not result in the same fractional change in delivery cost, as would be the case if scrubbed pure CO_2 were purchased as a material expense. It should be noted that CO_2 recycle is a complicated issue, and the assumptions currently utilized in the model are only "feasibility-level" estimates. Further work is warranted on understanding and optimizing both off-site and recycle CO_2 design/cost estimates. This is currently being done through a subcontract with an engineering firm to refine these estimates in greater detail.

In addition to the carbon that ends up in the flue gas, a significant fraction is present in the AD effluent, both in organic and inorganic forms. Recycling of carbon in the effluent is not assumed in the TEA. Based on the harmonized model base case, carbon in the effluent equates to roughly 50% of carbon in the turbine flue gas. Thus, the small savings noted above with flue gas carbon recycle would be even smaller when considering recycle of carbon in the effluent. Although not specifically addressed in this project, there may be other impacts of introducing this fixed carbon to outdoor pond systems, such as promoting the growth of contaminants.

How much biopower can be produced?

The biogas generated *via* AD contains substantial amounts of energy, with a heating value for the baseline scenario of almost 60 MW. This gas stream has the potential to provide a large fraction of the heat and power requirements for the entire facility. The ratio of electricity (biopower) to heat generated depends upon the efficiency of the generation

equipment. The baseline Aspen model assumes the use of gas turbines, but alternatives such as reciprocating engines or solid oxide fuel cells (SOFCs) are worth exploring. SOFCs offer the best electrical efficiency, at around 43- 47% (see references EPA1 and FCE1), although several commercially available units would have to be combined to meet the capacity required for this application. Reciprocating engines offer around 40% electrical efficiency (EPA2), still much better than the ~30% (EPA3) efficiency of available gas turbines. A preliminary, first-pass estimate for the consequences of these alternatives on net power demand are shown in Table 9.

Table 9. Comparison of three generation scenarios for biopower production using AD biogas.

Scenario	Biogas Heating Value, kW	Power Generated, kW	Compressor Power Demand, kW	Net Efficiency for Power Generation and Recompression, HHV Basis	Net Facility Power Demand, kW
Gas Turbine	59,517	14,342	0	24.1%	8,623
Reciprocating Engine	59,517	23,807	5993	29.9%	5,151
SOFC	59,517	27,973	5993	36.9%	985

The following points should be noted regarding Table 9:

- The electrical efficiency of gas turbines at this capacity is typically around 30%, assuming a discharge pressure of 0 psig (1 atm). In this application the discharge pressure is 5 psig to move the flue gas through the recycle piping and overcome pressure losses due to sparging into the ponds, which results in a reduced electrical efficiency of 24.1%.
- Reciprocating gas engines and SOFCs discharge the flue gas at 0 psig, therefore a compressor must be added immediately after the power generation unit to increase the flue gas pressure to 5 psig for recycle purposes (see Compressor Power Demand column). This therefore decreases the Net Efficiency column values below the nominal efficiencies stated above.
- The Net Facility Power Demand is the amount of power that must be imported/purchased from the grid to operate the facility. For the SOFC scenario, the preliminary estimate for this value is less than 5% of the total power used by the facility and is thus approaching a net-zero electrical power operation.
- A side-effect of improved electrical efficiency is less waste heat, which in the current model is used to raise high-pressure and low-pressure steam to satisfy facility heating demands. The difference in waste heat produced from a reciprocating gas engine versus a gas turbine is not likely to affect steam production.

How does AD compare to animal feed as an alternative co-product?

Although perhaps outside the scope of this project, it is interesting to compare the value of biogas from AD as a co-product to alternative co-product scenarios. In particular, the use of lipid extracted algae (LEA) for animal feed has often been proposed. This scenario has the potential to tap higher co-product values, but the cost of drying the material and the lost nutrients (N and P can no longer be recycled directly at the algal growth facility) must be considered. Figure 14 shows a comparison of the AD and animal feed co-product scenarios. These calculations were not based on the $18.63/gal RD base case but rather on a future case assuming 30 g/m^2/day and 50% lipid content (and with pond liners assumed), and the costs are shown for raw oil rather than RD (in 2011 dollars). The result is clear, however, that even at dried LEA prices for animal feed of $100 or $300/tonne, the economics still look worse than the AD scenario, while also requiring considerably more net power and nutrients and drastically more natural gas, all of which have important detrimental implications on LCA. The LEA price of $500/tonne favors the animal feed route. While quite preliminary, this cursory analysis suggests the impacts on demand for natural gas, nutrients, and power can only be overcome by what may be viewed as an aggressive price for LEA as animal feed (though not unreasonable for higher-value feed markets or for fish feed possibilities).

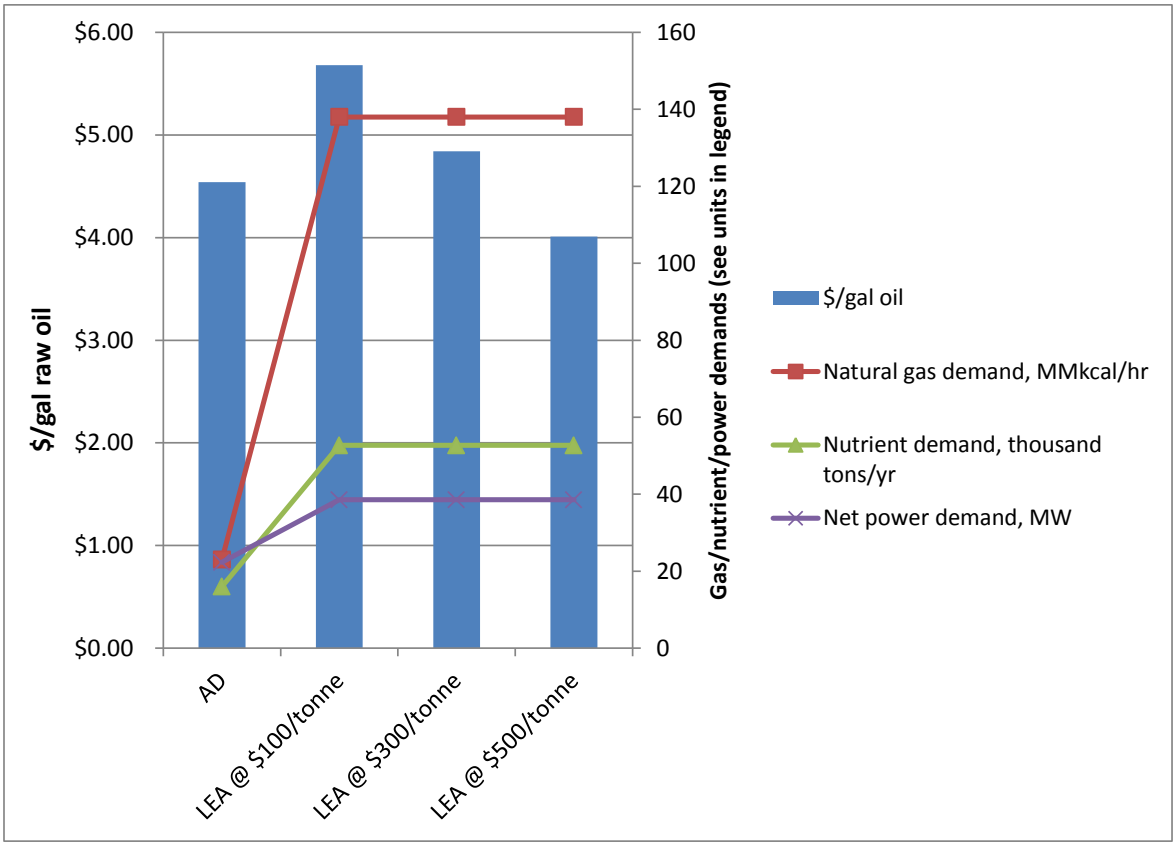

Figure 14. Comparison of AD to an alternative co-product scenario of using lipid-extracted algae (LEA) for animal feed at various market values. Lines show increased demands for natural gas, nutrients, and power in the animal feed scenario relative to AD.

We have presented a number of insightful findings related to TEA modeling of the AD component of the proposed ALU process. This project has successfully validated the baseline assumptions around biogas yield, retention times and loading rates that were used in previous TEA modeling. Although uncertainties still exist around the full extent of nutrient recycle possible, the data suggest that previous assumptions for recycle rates may have been somewhat optimistic, albeit not drastically. We have now expanded on the techno-economics surrounding the AD component of the harmonized Aspen model to include sensitivities around methane yield, organic loading rates, and various nutrient recycle scenarios in light of data generated within this project. Removal of AD from the harmonized baseline case results in a ~10% increase in the base-case renewable diesel selling price, with the biggest contributors in the areas of nutrient recycle and heat/power generation. Carbon recycle is only a minor contributor. Although biogas yield is important, decreased digester capital costs could more than compensate for reduced yields at high organic loading rates. Modeling of various nutrient recycle rates indicated that nitrogen recycle predominates in the TEA relative to phosphorus recycle and allowed us to test a range of outcomes, including the specific metrics associated with the Milestone. The TEA carried out suggests the importance of further research in several areas, most notably in mobilization of nutrients in the AD sludge for algal growth (*vs.* land application as fertilizer) and efficient use of biogas for generating electrical power. This work provides a rigorous basis for comparison of AD to alternative competing pathways for utilization of extracted algal biomass and alternative co-products, and preliminary results regarding animal feed show AD to be a superior option at moderate feed prices. The results from this project are also proving valuable to Argonne National Laboratory in support of their LCA modeling of algal biofuels processes.

Discussion

This project achieved its overall goals. We have demonstrated AD using five disparate microalgal feedstocks, both for extracted and un-extracted materials, with good rates and yields of biogas production. Scale-up to multi-liter

digesters was achieved for the industrially-relevant strain *Nannochloropsis salina*. The results of these digestions (yields, loading rates achieved, retention times, etc.) generally supported our original modeling assumptions, and the anticipated issues (*e.g.*, ammonia toxicity, C/N ratios, and cell wall recalcitrance) were either not encountered or were overcome through careful optimization. We have also demonstrated that the liquid effluent from algal AD can serve as a superior nitrogen source for re-growth of the original strain, with no apparent negative impacts on growth. These results provide important data to the algal biofuels industry and raise confidence around the feasibility of this process component for an algal production facility. The data also provide a useful basis for comparison to competing pathways for utilization of spent algae. Finally, the transfer of information from this project to the greater scientific and industrial community is an ongoing effort. This research has been presented at multiple venues as oral and poster presentations, including high-profile presentations by Craig Frear at the 2012 Algal Biomass Summit and the 2013 American Society of Agricultural and Biological Engineers (ASABE) international meeting. In addition, at least two manuscripts are in preparation and will be submitted in the coming months to peer reviewed journals. The findings have already found and will continue to find their way into TEA and LCA publications and reports by NREL and ANL.

Various challenges remain, and further research is warranted. Although this work provides optimism that AD can be optimized for many different species of algae, each algal feedstock will be different and some may pose more severe challenges. Scale-up of some species may be more difficult than others, and even for the *N. salina* feedstock tested, moving to commercial scales and potentially other reactor configurations will require further optimization. Commercial feedstocks could also pose new problems that were not encountered in this project, such as carryover of solvent or high salt levels from wet extraction, lower lipid and/or lower C/N ratios, etc. Achieving complete recycle of nutrients, to include non-ammonia nitrogen in the liquid fraction and both nitrogen and phosphorus in the digestate, will require further research to reveal methods for mobilization of those components. In addition, while it is infinitely scalable, biogas/biopower is still a relatively low value co-product, and it would be desirable to find alternative uses for lipid extracted algae that create greater value. Overall, however, AD has proven to be a compelling and robust option for recapturing energy, carbon, and nutrients from spent algal biomass and may continue to be the baseline option for some time to come.

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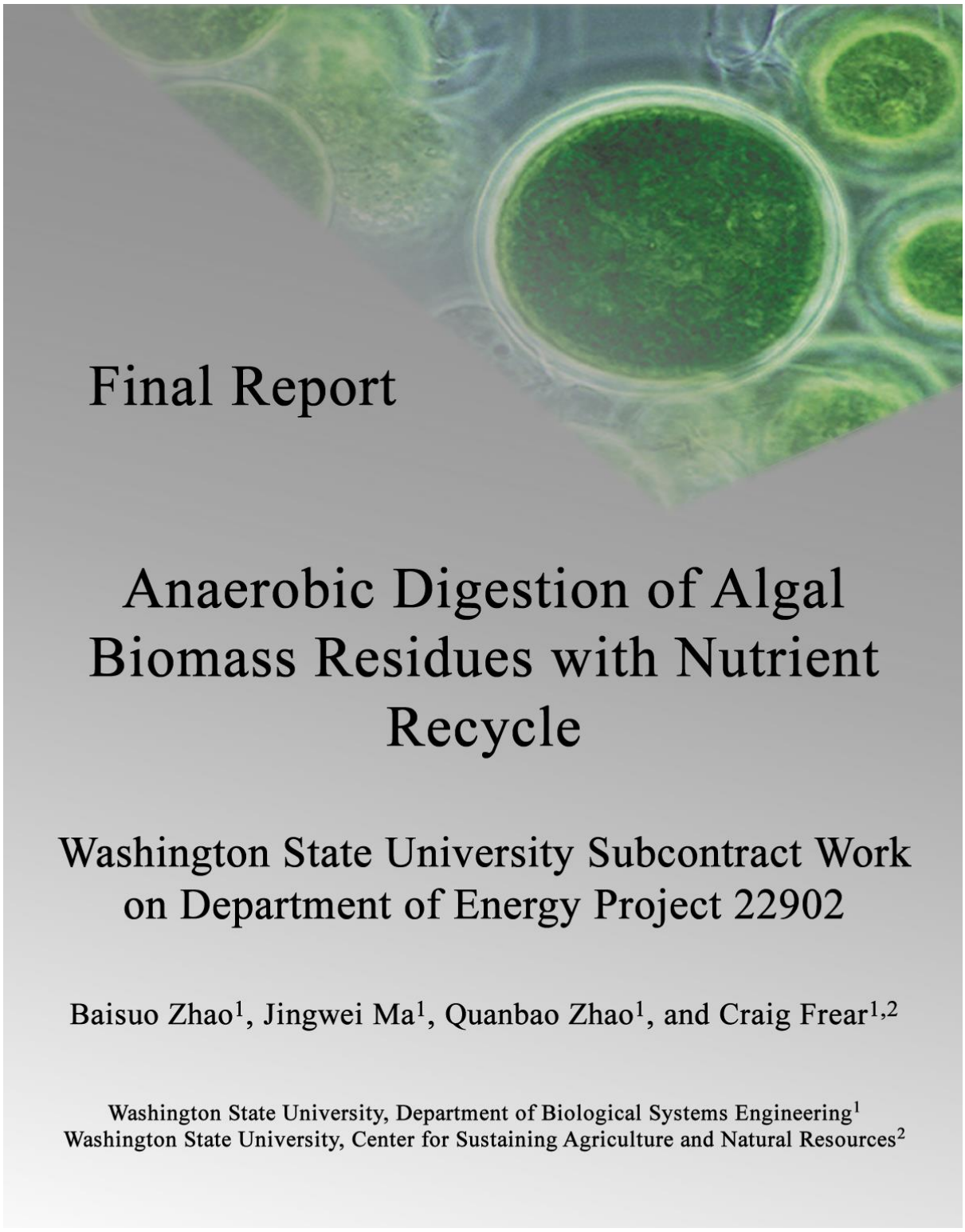
Appendix A: WSU Subcontract Final Report



Final Report

Anaerobic Digestion of Algal Biomass Residues with Nutrient Recycle

Washington State University Subcontract Work on Department of Energy Project 22902

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Anaerobic Digestion of Algal Biomass Residues with Nutrient Recycle

Background

Microalgae are currently considered as a renewable source of liquid and gaseous biofuels and a practical technology for the capture of carbon dioxide (Riebesell et al., 1993; Sialve et al., 2009). Microalgae represent one of the most sustainable and promising of biofuel feedstock, demonstrating particularly high growth rates (Scott et al., 2010) as well as an ability to thrive in harsh environments such as seawater (salinity ~35 g L^{-1}), alkaline lakes (pH ≥8.5), non-potable industrial wastewater, and arid and barren land areas—both which potentially allow for production while not directly competing with fresh water and arable land (Chi et al., 2011; Clarens et al., 2010; McGinn et al., 2011; Wijffels & Barbosa, 2010). Microalgae, and their entrained lipids, can offer several different types of biofuel and bioenergy production options including *trans*-esterified biodiesel (Chisti, 2007; Scott et al., 2010), fermented bioethanol (Bush & Hall, 2006), photo-biological hydrogen (Ghirardi et al., 2000; Melis & Happe, 2001), hydrocarbon biofuels for drop-in replacements of gasoline, diesel, and jet fuel (Jones & Mayfield, 2012; Regalbuto, 2009), and anaerobically generated methane (Sialve et al., 2009; Uellendahl & Ahring, 2010).

While the biofuel potential of microalgae is more focused, at both research and commercial levels, on production of drop-in fuels, production of bio-methane from either whole cell or algal residue has been regarded with general interest. From a whole-cell perspective, microalgae blooms cultivated in open ponds or waterways, often for wastewater treatment and environmental protection purposes, are typically low in lipid content, warranting a lower-value use and simpler processing approach representative of anaerobic digestion (AD) (Sialve et al., 2009). From a residue perspective, algal bio-refineries are anticipated to produce algal biomass composed of ≥ 50% lipid content by dry weight (Scott et al., 2010). As a result, as much as 50% of all cultivated solids will be residue, requiring disposal, particularly if value-added approaches for the use of residual proteins, polysaccharides, and other chemicals cannot find cost effective separation technologies as well as suitably large and viable markets (Chakraborty et al., 2012; Miao et al., 2012). Both of these materials can be viewed as attractive feedstock for the production of energy and/or compressed fuels as well as the recovery of associated nutrients, mainly nitrogen and phosphorous. Therefore, the potential of AD using low-lipid whole cells or lipid-extracted residual biomass can offer significant benefits for reducing an algal bio-refinery's cost and environmental impacts (Davis et al., 2011; Delrue et al., 2012; Sialve et al., 2009).

A review of microalgae AD research shows a wide range of production values, on the range of 0.09−0.54 L CH_4 g^{-1} volatile solids (VS) (Nallathambi Gunaseelan, 1997; Park & Li, 2012; Sialve et al., 2009; Uellendahl & Ahring, 2010). Such a wide range is non-preferred for modeling of costs and benefits, which is essential to algal bio-refinery development (Zamalloa et al., 2011). While the large range is due notably to strong species dependence and varying lipid concentrations within the biomass (Mussgnug et al., 2010; Zamalloa et al., 2011), it has also been proposed that the range, and particularly low values in some studies, is

due in part to concerns related to biological inaccessibility to whole cells with intact membranes/walls (Sialve et al., 2009) as well as inhibitory conditions and agents experienced during digestion (Lakaniemi et al., 2012). Inhibitory conditions include low C/N ratios (Brune et al., 2009; Yen & Brune, 2007) and high salinity (Lakaniemi et al., 2012) while inhibitory agents include product inhibitors such as ammonia (Brune et al., 2009; Sialve et al., 2009) and long-chain fatty acids (LCFA) (Frigon et al., 2013; Park & Li, 2012). Several studies have focused on overcoming low C/N ratios as well as comparatively low methane productivities by practicing co-digestion (Samson & Leduy, 1982; Yen & Brune, 2007), reporting doubling of methane yield and productivity when *Spirulina* was co-digested with wastewater sludge and aquaculture algal sludge was digested with waste paper.

While the above few references have studied individual aspects of algal AD, either whole cell or extracted residue, for individual species, no comprehensive study across numerous industrial species has been completed, allowing for more accurate detailing of maximum methane productivity capabilities and understanding of factors resulting in the reported low and wide range of results.

Goal/Objectives

While the larger project is multi-institutional and provides for multiple, integrated research objectives, two key objectives are at the heart of the WSU subcontract. The first objective was to complete extensive biological methane potential (BMP) studies on delivered algal biomass, whole cell and extracted residue, and with various degrees of biomass pretreatment. The second objective was to translate BMP data and capabilities into continuous digestion operation for determination of system capabilities upon scale up to a 5-L reactor size.

During the course of experimentation, a few important observations were made which ultimately altered part of the original focus of the sub-project. First, it became clear that particular solvent extraction methods resulted in sustained inhibitory conditions for the digestion, meaning that several of the planned pre-treatment conditions to be experimentally studied could not be conducted as available biomass for these studies were contaminated with the inhibitory solvent. Fortunately, it was found that under the conditions evaluated (algal biomass received as dried and then partly ground to release resulting clumped biomass), pretreatment did not appear to be a necessary step, as effective digestion occurred in a timely manner—assuming that adequate loading and inoculum to substrate ratios were maintained. As a result the emphasis on pre-treatment of cellular biomass prior to AD was not included as a project objective. Throughout the course of the sub-project, regular communication, in the form of monthly calls and quarterly data releases, were conducted while also providing partners with needed models and effluent so as to conduct additional project objectives.

Materials/Methods

<u>Whole cell microalgae and lipid-extracted microalgae residue</u>

Five model microalgae strains, similar to those generated in pilot and commercial algal fuel production facilities, were selected for their span of diversity as well as specific tolerable

growth conditions, lipid and protein contents, and other distinct physiological features. Each strain was delivered and studied in both whole cell and extracted residue form, generating ten specific algal biomass treatments (Table 1) that were studied throughout the project. Growth and extraction methods varied by strain and source, amongst them, *Chlorella vulgaris* UTEX 395 (C) and *Phaeodactylum tricornutum* CCMP 632 (P) were cultivated by the National Renewable Energy Laboratory (NREL); *Nannochloropsis* sp. (N), *Nannochloropsis salina* (NS), and *Nanofrustulum* sp. (NF) were kindly provided by Seambiotic, Solix Biosystems, and Cellana, respectively. N and NS belong to *Eustigmatophyceae*, P and NF to *Bacillariophyceae*, and C to *Chlorophyceae*. Replete and nitrogen deplete conditions were used to grow C using a suspended polyethylene bag system while P was grown in a 250-liter raceway pond with 5% carbon dioxide sparge and natural daylight (Laurens et al., 2012b) also under replete conditions. Outdoor production systems were used to grow N1 (raceway), NS1 (photo-bioreactor), and NF1 (raceway), with limited information on growth conditions available due to proprietary relations. All microalgae biomass were harvested and centrifuged at room temperature. Subsequently, the biomass were dried and frozen at -20ºC prior to lipid extraction or for AD treatment.

Extraction Methods and Evaluation

While original experimental designs for production and evaluation of all lipid-extracted species were to utilize Soxhlet reactors via Bligh-Dyer extraction chemistry involving a 2:1 v/v chloroform/methanol solvent ratio, subsequent BMP studies showed extensive biological inhibition, apparently as a result of this methodology. To evaluate this hypothesis, five different solvent systems (chloroform/methanol (2:1 v/v) (control), hexane/isopropanol (3:2 v/v), dichloroethane/methanol (1:1 v/v), dichloroethane/ethanol (1:1 v/v), and acetone/dichloromethane (1:1 v/v)), all widely used in industry (Lee et al., 1998) were evaluated for possible BMP inhibition using whole cell N1 biomass. N1 were immersed into the above organic solvents (i.e. 1.5 g microalgae biomass/10 ml solvent), respectively. Then, those solvents were evaporated in the hood at room temperature and dried at 105ºC until constant weight was achieved, upon which BMP trials were then conducted. Subsequent recognition of the chloroform/methanol inhibition and no similar inhibition involving 3:2 mixture of hexane/isopropanol led the authors to utilize this extraction methodology in a Soxhlet set up (Laurens et al., 2012a) for lipid-extracted C, N and P biomass (as defined above) residues, with the exception being the NF2 grown by Cellana, which utilized a methyl pentane solvent that showed no inhibitory effects. The authors note that with a hexane/isopropanol solvent system extraction, fatty acids did remain in the residue due to incomplete extraction (as discussed in Laurens et al. (2012a)).

BMP Assay and Performance Monitoring

As all whole-cell microalgae or lipid-extracted microalgae residues received from industry and project partners arrived as compacted freeze dried solids, biomass were milled through a 1 mm screen using a direct-drive cyclone sample mill (Cole Parmer, Verner Hills, IL, US) so as to reduce the level of compaction and reduce particle size. Seed inoculum with total solids (TS) of 18.0 g/L and volatile solids (VS) of 13.4 gL^{-1} was obtained from the Pullman, WA,

US Wastewater Treatment Plant (WWTP) primary anaerobic digester treating activated sludge at 35ºC. The inoculums were transferred in sterile N_2-sparged bottles, kept at room temperature during transportation, and then stored at 4ºC until use. During BMP assays, biomass treatments were inoculated with seed and placed into 250 ml flasks that served as bioreactors for the BMP studies. No additional external nutrients/trace elements were added to the BMP bottles as it was assumed that basic nutrient requirements for anaerobic microorganisms were provided by the wastewater-based inoculum (Labatut et al., 2011). The working volume in each BMP reactor was 200 ml with the rest serving as headspace. Cultures were neutralized with acids and bases to pH 6.95-7.05, flushed with N_2 gas for 15 min to induce anaerobic condition, and incubated in a 16-cell automated Challenger AER System (Fayetteville AR) maintained at 35±1 ºC and mixed continuously with a magnetic stirrer set to 200 rpm. Two sets were run for each treatment, one for total biogas (i.e. carbon dioxide and methane) and another for methane via use of biogas scrubbing tubes composed of pellets of sodium hydroxide and color indicator. Each set was run in triplicate and BMP runs were conducted over a 20-40 day time period depending upon rate at which near complete production of methane was achieved.

In a primary experiment for determination of optimal seed concentration to ensure effective AD, inoculation of NS1 and NS2, as test biomass, occurred across five-inoculum to substrate (I/S) ratios—0.1, 0.5, 1.0, 1.5, and 2.0. This was achieved by varying the seed inoculum concentration ranging, from 1 to 20 g VS L^{-1} while maintaining a constant microalgae substrate concentration of 10 g VS L^{-1} liquid. Subsequent analysis of data from this primary experiment determined that I/S ratio of 1.0 was suitable for effective AD of all biomass treatments—subsequently all future BMP biomass studies utilized this I/S ratio.

Recognizing potential long-chain fatty acid (LCFA) inhibition during BMP trials, a side experiment was conducted in regard to further elucidating the extent to which LCFA was playing a potential role in inhibition as well as potential methods for reduction of its impact. It is known that, precipitation with divalent cations can be an effect way of preventing LCFAs from upsetting an anaerobic digestion system (Hanaki et al., 1981; Koster, 1987). As a result a study was completed to investigate the effect of adding Ca^{2+} so as to prevent free LCFA that is excreted from the algal biomass from accumulation onto the methanogen surface, thereby reducing the negative impact to activity of methanogens and methane production. Both the whole cell NS1, and the lipid-extracted NS2 were tested with different dosage of calcium. Calcium was dosed at 0, 0.5, 1.0 and 2.0 times of LCFA (in mole ratio). As in other experiments, the I/S ratio was 1.0 for all experiments.

BMP performance indicators were calculated from raw biogas and methane data. Specific indicators include VS reduction percentage (VS %); specific methane productivity (SMP; L CH_4 g VS fed^{-1}); total methane productivity (TMP; L CH_4 g VS^{-1}) both through experimental calculation (VS %) and theoretical calculation (Sialve et al., 2009); 95% methane production time and maximum methane production rate (days to achieve 95% of total realized methane production; L CH_4 L^{-1} d^{-1}; both calculated using modified Gompertz equation as the

described by Zwietering et al. (1990); and first order hydrolysis constant (K_h) as described by Angelidaki et al. (2009). Chemical performance indicators, such as biological product inhibitors are discussed below in analytical methods section.

Continuous Digestion Trials

After completion of BMP trials, a continuous digestion objective was completed so as to ascertain if BMP performance standards could be maintained upon scale up to a continuous digestion at a scale of 5-L. As NS1 and NS2 were the most abundant available biomass as well as biomass most reflective to desired commercial algal refinery lipid concentrations, only these two biomass treatments were evaluated. As earlier BMP trials indicated the importance of a high I/S ratio, it was decided that the continuous digestions would be completed in reactors capable of accumulating and maintaining high biomass concentrations, namely a sequencing batch reactor (SBR). Two identical digesters (64 cm in height and 10 cm in diameter) with working volume of 5 L were operated as SBR at hydraulic retention time (HRT) of 20 days. Each digester was mixed with a separate impeller driven by a respective motor at 100 rpm. Intermittent mixing was carried out with 10 min in every 2 h. Milled algal biomass and WWTF anaerobic sludge, as described earlier in BMP methods, were introduced to each digester at 1:1 volume ratio when experiments started. Digesters were then placed in a mesophilic chamber (35°C) and operated in SBR mode, which consisted of 4 stages: filling, reaction, settling and discharging in one cycle. Organic loading rate (OLR) in both digesters ranging from 0.5-5 g VS L^{-1} d^{-1} was increased step by step until digester failure.

As calculation of VS from a SBR reactor can be more complicated (potential for undigested algal biomass residue in the retained biomass) than simple influent and effluent subtraction characteristic of more common complete mix reactors, the following VS reduction calculation was completed.

$$R_i = \frac{S_{i-1} \cdot (V - V_e) + C_i - S_i \cdot (V - V_e) - S_{e,i} \cdot V_e}{C_i}$$

where, R_i is VS reduction rate in i th cycle; S_{i-1} is mixed liquor VS in i-1 th cycle; S_i is mixed liquor VS in i th cycle; C_i is the amount of VS added at the beginning of the i th cycle; $S_{e,i}$ is effluent VS at the end of the i th cycle; V is digester volume; V_e is discharged volume.

Analytical Methods

Total Solids (TS) and VS were measured using standard analytical methods (APHA, 2011). Elemental analysis for carbon (C), hydrogen (H), oxygen (O), nitrogen (N), and sulfur (S) were performed per standard methods as described in (Pella, 1990) using a Leco CHN-O-S analyzer (St. Joseph, MI, US). Lipids were analyzed as fatty acid methyl esters after a one step acid catalyzed *in situ trans*-esterification reaction using a GC-FID (Agilent 6890N) equipped with an HP-5 ms capillary column (30 m×0.25 mm id×0.25 µm) according to the procedure of Laurens et al. (2012a). Protein were calculated from elemental N content

(Lourenço et al., 2004). Carbohydrates were determined by H_2SO_4 acid hydrolysis followed by HPLC measurement of monosaccharides (Laurens et al., 2012b).

Gas composition, not determined directly through alkaline scrubbing, was determined by a Varian 3800 GC-TCD (Palo Alto, CA, US) fitted with a Restek (Bellefonte, PA, US) shincarbon column (2×1/16 inch) using the method by Wen et al. (2007). The concentrations of volatile fatty acids (VFA), including acetate, propionate, butyrate, and valerate, in the effluent were determined by a gas chromatograph (Shimadzu Corp., GC-2014, Japan) equipped with a flame ionization detector and a 30 cmX0.25 mmX0.25 um capillary column (HP-INNOWax, Agilent Technologies, Palo Alto, CA, US). The liquor samples were first centrifuged at 12,000 rpm for 5 min, and were then acidified with 1% formic acid and filtrated through 0.22 mm membrane and finally measured for free acids. The temperatures of the injector and detector were 250 and 300^{o}C, respectively. The initial temperature of oven was 70^{o}C for 3 min followed with a ramp of 15^{o}C/min to final temperature of 230^{o}C for 3 min. Helium was used as carrier gas with a flow rate of 0.93 mL/min at a split ratio of 40:1. Alkalinity, pH, and Ripley ratio values were analyzed using a Mettler Toledo T50A Automatic Titrater (Schwerzenbach, Switzerland) according to standard methods (APHA, 2011). Total Kieldahl nitrogen (TKN) and total ammonia nitrogen (TAN) were both evaluated using a Tecator 2300 Kjeltec Analyzer (Eden Prairie, MN, US) using standard methods (APHA, 2011). Total phosphate (TP) level was assessed by PhosVer 3 Acid Persulfate digestion (Hach, Loveland, CO, US).

Samples were also analyzed for micro-metal content. Samples were first digested with a CEM SP-D microwave digestion system (CEM, Buckingham, England). Samples (100 mg sample (+/- 5 mg)) were loaded into a 35 ml quartz digestion vessel. To this vessel 4 ml of 30% reagent grade hydrogen peroxide was added and allowed to react with the sample for 10-20 hours. 25-35 minutes prior to digestion 6ml of concentrated (69-71%) reagent grade nitric acid was added to each reaction vessel. Digested samples were rinsed into freshly washed 100 ml volumetric flasks and diluted. 1 ml of an internal standard solution containing 10 ppm Li-6, Sc, Ge, Y, In, Tb, and Bi (Accustandard, Environmental Internal Standard Mix, New Haven, CT, US) was added to each sample to improve the accuracy of later analysis. Calibration samples were made from a commercially available stock solution (Accustandard Environmental Calibration Standard, New Haven CT, US). Calibration points included 0, 0.1, 0.5, 2, 5, 20, 50 and 100 ppm for Na, K, Mg, Ca, and Fe and 0, 1, 5, 20, 50, 200, 500, 1000 ppb for all other elements. Diluted samples were analyzed with an Agilent 7500cx ICP-MS (Santa Clara, CA, US) equipped with an octopole collision/reaction cell.

Results and Discussion

Characteristics of whole microalgae and their biomass residues

In order to evaluate the distribution of microalgae characteristics on their conversion to methane production potential, various inherent parameters were carefully investigated as shown in Table 2. Ash contents were significantly different ranging from approximately 8 to 45%, with the diatom NF1/NF2 providing the high end of this ash range, presumably to the

presence of silicon. Regardless, within all biomass, the presence of notable ash implied that abundant mineral elements for anaerobic microbial nutrition would be offered during the AD process. In addition, quite high concentrations of N, S as well as P (evaluated in the effluent) also imply suitable nutrient supply for anaerobic microbial nutrition during AD processing. Major non-mineral compositions were C (27.45−56.20%), H (4.23−8.76%), O (23.41−29.33%), N (2.90−7.77%), and S (0.55−1.29%) for whole cell microalgae while C (20.19−47.80%), H (2.93−6.90%), O (27.82−34.00%), N (2.93−8.15%), and S (0.76−1.29%) for lipid-extracted microalgae, respectively. The often wide ranges are due to the noted ranges in the aforementioned ash as well as lipid, protein and carbohydrate concentrations, with lipid-extraction and bulk removal of carbon during the extraction leading primarily to the shifts in percentages seen between whole cell and extracted residue.

Table 1 reports analyses on metals with results indicating all tested algal species had considerable sodium and potassium (0.5-3%), due to their culturing conditions. NF species had a higher level of calcium, magnesium and iron compared to others while C species were rich in copper and zinc and P1 had a higher level of manganese. In general, the concentration of the metals within all of the algal biomass was not high enough to inhibit the AD process, while still supplying more than adequate supply of necessary micro-metal nutrients for biomass maintenance and growth (Soares et al., 2012).

Table 1: Metal analysis of whole cell and lipid extracted microalgae biomass

mg/g	**C1**	**C2**	**N1**	**N2**	**NS1**	**NS2**	**NF1**	**NF2**	**P1**	**P2**
Na	10.2	5.7	N/A	N/A	16.3	10.3	14.9	12.5	32.2	11.7
Mg	8.2	5.0	6.6	7.1	9.9	6.3	10.4	24.2	9.1	10.7
K	10.9	15.3	14.1	15.8	17.6	13.6	13.2	9.6	22.6	20.6
Ca	2.2	4.2	3.3	4.1	2.7	1.9	18.4	98.4	29.2	3.7
Fe	0.5	0.1	0.4	0.4	0.6	0.5	1.3	1.5	0.9	0.4
μ*g/g*	**C1**	**C2**	**N1**	**N2**	**NS1**	**NS2**	**NF1**	**NF2**	**P1**	**P2**
Al	40.8	BDL	209.3	266.6	166.7	186.5	127.3	215.1	297.8	484.6
Cr	7.7	BDL	3.5	3.9	2.8	2.9	2.8	6.7	22.5	17.3
Mn	29.5	75.6	13.9	16.8	9.5	71.9	97.8	66.4	194.6	54
Co	2.0	6.9	BDL	BDL	2.2	1.7	1.5	0.6	1.2	0.9
Ni	1.8	BDL	BDL	BDL	1.5	4.8	4.9	5.4	4.0	10.5
Cu	794.9	143.4	10.2	14.1	32.3	26.4	4.6	4.0	23.2	9.9
Zn	107.1	111.3	90.6	114.5	61.8	48.4	32.9	25.4	94.4	91.9
Ba	1.4	0.7	4.1	5.8	108.3	62.1	2.6	11.4	6.8	12.2

BDL: below detection limit; Values measured as single replicates (n=1).

C/N ratios for whole cell biomass ranged from 6.8−14.7 with a generalized lowering of this range in lipid-extracted residues, producing a range from 5.51-8.46. Both these ranges, and in particular the lipid-extracted, are well below optimal C/N ratios of 20-30 preferred for both aerobic and anaerobic treatment of organic wastes (Parkin & Owen, 1986). Such low ratios have been implicated as harbingers of product inhibition resulting from the anaerobic conversion of protein N to ammonia N—a known inhibitor to the AD process both in its ionic

and, in particular, its free form (Koster & Lettinga, 1984). BMP effluent analysis showed TAN levels ranging from 228-1,316 mg N L^{-1} with the higher range of values arising from lipid-extracted digestion (Table 3), however this entire range is well below threshold TAN inhibition indicators of 1,700-2,000 mg N L^{-1} reported byKoster and Lettinga (1984). Frear et al. (2011) showed similar lack of TAN inhibition during stable digestion of manure at a C/N ratio of 11, even while producing higher effluent TAN concentrations (2,600 mg N L^{-1})—presumably a result of bacterial acclimation to these higher TAN levels (Angelidaki & Ahring, 1994; Calli et al., 2005).

Table 2: Algal species characterization

% DW	C1	C2	N1	N2	NS1	NS2	NF1	NF2	P1	P2
Algal species	*C.vulgaris* UTEX 395	*C.vulgaris* UTEX 395	*Nanno-chloropsis* sp.	*Nanno-chloropsis* sp.	*Nannochloropsis salina*	*Nannochloropsis salina*	*Nanofrustulum* sp.	*Nanofrustulum* sp.	*P. tricornutum* CCMP 632	*P. tricornutum* CCMP 632
Morphology	Green algae	Green algae	Eustigmatophyte	Eustigmatophyte	Eustigmatophyte	Eustigmatophyte	Diatom	Diatom	Diatom	Diatom
Saline	Estuarine	Estuarine	Marine	Marine	Marine	Marine	Marine	Marine	Marine	Marine
Extraction	Whole Cell	Hexane	Whole Cell	Hexane	Whole Cell	Hexane	Whole Cell	Pentane	Whole Cell	Hexane
Source	Suspended	Suspended	Raceway	Raceway	PBR	PBR	Raceway	Raceway	GH Pond	GH Pond
Ash	11.23	7.70	11.71	18.08	7.04	10.31	44.82	51.15	20.92	17.00
VS/TS	88.77	92.30	88.29	81.92	92.96	89.69	45.18	48.85	79.08	83.00
Carbon	52.81	44.90	52.84	43.47	56.20	47.80	27.45	20.19	44.12	38.62
Hydrogen	6.13	5.03	6.00	5.13	8.76	6.90	4.23	2.93	5.14	5.70
Oxygen	29.33	27.82	23.41	34.00	25.63	31.80	29.31	33.96	28.30	28.07
Nitrogen	7.77	8.15	7.00	6.84	3.78	5.65	2.90	2.93	6.43	6.80
Sulfur	0.72	0.79	0.73	0.80	0.55	0.77	0.96	0.76	1.29	1.28
Lipid-FAME	9.81	2.83	10.65	3.03	37.16	11.82	12.95	2.55	7.61	6.12
Protein N	35.13	38.96	34.03	32.70	17.21	26.72	12.52	8.70	26.53	32.50
Total Carbs	16.94	12.15	7.64	9.56	11.52	17.04	8.97	11.01	18.95	16.14
Glucose	5.29	ND	4.15	ND	7.95	11.68	6.42	8.12	2.92	ND
Unknown	28.04	38.36	35.97	36.63	27.07	34.10	20.74	26.59	25.99	28.24
C/N Ratio	6.80	5.51	7.55	6.36	14.87	8.46	9.47	6.89	6.86	5.68

PBR—Photobioreactor; GH—Greenhouse; DW—Dry Weight; FAME—Fatty Acid Methyl Ester; C/N—Carbon to Nitrogen Ratio; ND—Not determined. Values measured as single replicates (n=1).

Of interesting note is the lipid, protein and carbohydrate compositional analysis of the biomass. Lipid contents in the whole cell microalgae ranged from 7.61 to 37.61%, indicating that despite the commercial interest of these strains as well as the attempts to accumulate lipids through control of growth conditions, in general, the lipid concentrations were relatively low, with even NS1, at 37.61%, somewhat below industry targets. In addition, attempts at lipid extraction resulted in only moderate removal percentages, ranging from 19.6-80.3% removal when including the P2 outlier and 68.2-80.3% when excluding its presence. The meaning being that a considerable fraction (>20%) of lipids remains within the residual biomass, contributing extensively to AD biogas production as well as potential LCFA inhibition during digestion. While, biomass and extraction processing were not done to scale or truly representative of existing or future industry capabilities, these present numbers offer credence to previous discussions for the role AD can play as a commercial-ready processing technology for whole-cell microalgae to less valued bio-methane (Sialve et al, 2009), not to mention its role in the more discussed option as processor of residual biomass that still contains a large share of the original energy-rich biomass.

Impact of extraction solvent on subsequent AD

Chloroform/methanol is regarded as the excellent solvent for lipid extraction from microalgae biomass (Bligh & Dyer, 1959; Lee et al., 1998), however early BMP tests showed evidence of extensive process inhibition present only within the chloroform/methanol extracted residues. For example, using N1/N2 as the test biomass system, under an organic loading rate (OLR) of 8.5 g VS L^{-1} liquid and approximate 10% v/v seed inoculum at 35ºC, the methane yield from N2 biomass residues extracted using chloroform/methanol mixture was only 0.03 L g^{-1} digested VS, which was extremely low in comparison to the N1 whole cell microalgae methane production rate of 0.42 L g^{-1} digested VS. Subsequent analysis of the N2 AD effluent showed a comparatively large VFA concentration of 3.23 g L^{-1}, well above that noted in the N1 AD effluent (non-detectable). A hypothesis from these results is that the chloroform/methanol mixture, while extremely volatile and presumably removed from the treated biomass, somehow retained an inhibitory effect, which strongly inhibited the methanogen population (extremely low methane production, which presumably could have been hydrogen gas that was not scrubbed from the alkaline tubes) while allowing for sustained activity within the hydrolyzing and acidifying bacteria (elevation of effluent VFA concentrations).

In order to verify this chloroform/methanol effect and hypothesis as well as its possible implications to other industry-reliant solvent mixture extracts, a comparative study was completed and summarized in Figure 1. The chloroform/methanol solvent mixture resulted in near complete methane production inhibition while all other tested solvent mixtures resulted in similar non-inhibited production. This data and hypothesis explanation is not without literature-review merit. Chloroform severely inhibited both acid fermentation and methane production at high concentrations (Hu & Chen, 2007). However, methanogens were sensitive to chloroform at very low concentration, selectively inhibiting methanogenic activity while not affecting hydrolysis and the fermentation process (Hu & Chen, 2007). Chloroform reportedly inhibited the production of methane from both H_2/CO_2 and acetate forms of methanogens (Chidthaisong & Conrad, 2000). With respect to this particular microalgae AD observation, Ehimen et al. (2009) reported a low methane production rate from the algal biomass after

chloroform/methanol lipid-extraction. It was their assertion that repression of methanogenesis was attributed to the inhibitory effect of chloroform that remained bound to the residual microalgae biomass.

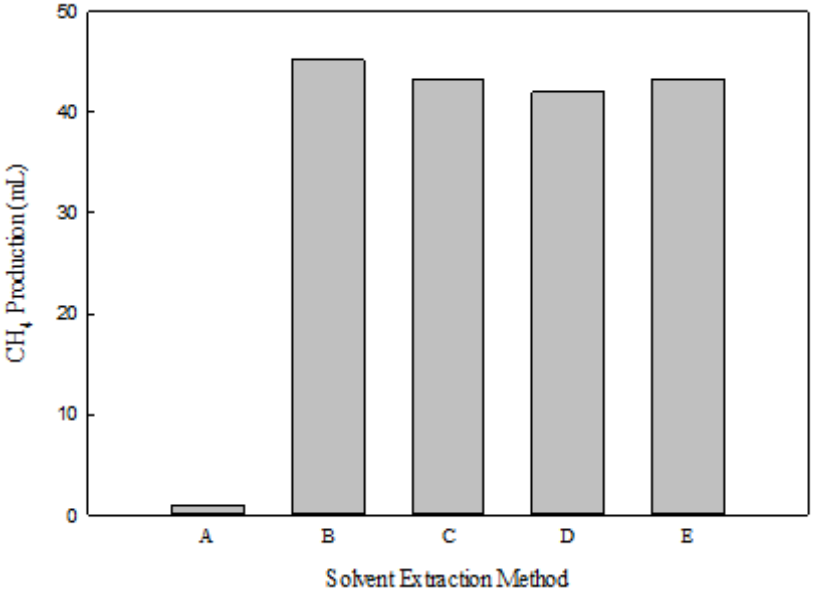

Figure 1: Methane production during digestion of N1 treated with different solvents A: Chloroform/methanol (2:1); B: Hexane/isopropanol (3:2); C: Dichloroethane/methanol (1:1); D: Dichloroethane/ethanol (1:1); E: Acetone/Dichloroethane (1:1)

Inoculum to Substrate Ratio (I/S) Impact on Digestion

While reviewing algal digestion literature, it became clear that many of the studies used a variety of BMP protocols, specifically in regard to the source and concentration of inoculums, although for many of the studies insufficient detail was contained within the papers to completely ascertain the protocols used. Therefore as a first step in development of the BMP study, an initial analysis on the effect of I/S ratio was developed as previous literature has shown the importance of I/S ratio in determination of SMP, particularly in regard to recalcitrant material prone to inhibitory agents as microalgae might be with its aforementioned low C/N ratio, presence of LCFA, and cellular membrane/wall macromolecular matrices (Alzate et al., 2012; González-Fernández & García-Encina, 2009).

Table 3 and Figure 2 are a summary of the I/S study utilizing both NS1 whole cell and NS2 lipid extracted biomass. As shown in Table 3 and Figure 2, the maximum biogas production, VS reduction, and methane content were reached at the I/S ratio of 1.0 for NS1 while at the I/S ratio of 0.5 for NS2. Meanwhile, the total VFA in their respective effluent were only 146 and 82 mg L^{-1}, both of which are emblematic of low concentrations seen in effective AD treatment. When the I/S ratios were increased greater than 1.0, the VFA could not be detected regardless of whole microalgae and lipid-extracted biomass residues, although overall biogas production showed only slight to no improvement due to that additional degradation. The ratio also had some impact on the composition of biogas. For example, the produced methane from NS1, most likely comprising some hydrogen, accounting for 40.78% at the I/S ratio of 0.1 while 65.22% at

the I/S ratio of 1.0. Thus while, little improvement in biogas production was seen as the I/S ratio was raised above 1.0, there most likely was a greater conversion to methane as indicated by the reduced VFA concentrations, presumably due to an enriched and more robust methanogenic community, capable of converting both the acetate and hydrogen forms to methane.

Table 3: BMP of NS1/NS2 at various inoculums to substrate ratios

Algae biomass			NS1					NS2		
Ratio (I VS/S VS)	0.1	0.5	1.0	1.5	2.0	0.1	0.5	1.0	1.5	2.0
Biogas (L Biogas)	0.10	0.65	0.84	0.83	0.83	0.17	0.50	0.50	0.50	0.51
Effluent pH	5.80	6.72	6.96	7.06	7.10	6.29	6.94	7.11	7.19	7.20
Total VFA (mg/L)	916.8	592.8	145.7	ND	ND	821.8	81.5	ND	ND	ND
VS Reduction (%)	19.55	53.45	60.10	60.01	75.38	31.50	59.61	62.56	62.80	63.41
Methane (%) at 30 d	40.78	67.32	65.22	64.20	64.05	47.04	63.98	62.33	62.58	62.94

I/S—Inoculum to Substrate Ratio; ND not detected by GC; data reported as mean of triplicate runs (n=3)

The threshold I/S ratio of 1.0 is consistent with literature. Hashimoto (1989) determined that a minimum ratio of 0.5 was required for straw digestion at concentrations of 10–40 g VS L^{-1}. Furthermore, Hashimoto showed that maximum methane production rates were achieved when I/S ratios reached 2.0. Owen et al. (1979) as well as Chynoweth et al. (1993) showed similar results and suggested I/S ratios of 1.0 and 2.0, respectively.

An explanation for the observed trend in I/S ratio as well as the varying impact on whole cell versus lipid-extracted biomass involves known mechanisms for LCFA inhibition in anaerobic cultures. While lipids are rapidly hydrolyzed to LCFA and glycerol during AD, subsequent further degradation, via beta-oxidation, to acetate and hydrogen is less rapid and often delayed, not allowing for attainment of the true biogas potential contained within lipids. Primary reasons for this delay rest on the toxicity of the LCFA intermediate (Angelidaki & Ahring, 1992; Hanaki et al., 1981; Palatsi et al., 2009), with studies reporting scum layer production with associated biomass washout (Hwu et al, 1997) as well as microbial membrane transportation inhibition (Hwu et al, 1998), all due to the tendency for LCFA to form adsorptive layers around the microbial surfaces. Degree to which LCFA is experienced and therefore presumably to which the adsorptive mechanism is elicited depends upon specific surface area, carbon chain length, degree of saturation (Salminen & Rintala, 2002)and most importantly, type of microbe, with methanogens reported to be more susceptible to LCFA inhibition as compared to acidogens (Mykhaylovin et al., 2005; Pereira et al., 2003).

The above summary of known LCFA interaction with AD biology fits in nicely with the BMP curves summarized in Figure 2. As noted, for NS1 a minimum I/S ratio of 1.0 was required to achieve stable digestion, while for NS2, the required I/S ratio was only 0.5. Notably, NS2 has considerably less lipids and therefore LCFA due to the extraction process, thus not exposing the microbes to as high a concentration of surface attaching chemicals. Conversely, the higher concentrations of LCFA in NS1 presumably induced a

greater degree of surface adhesion and therefore microbial inhibition, requiring a greater inoculums concentration to overcome this phenomenon. The conclusion is that in order for effective digestion of whole cell or lipid extracted microalgae biomass to occur without disruption a suitably large inoculums concentration is required, particularly if the remaining lipid concentration is large. This conclusion has implications to future scale-up as reactor designs will need to ensure effective biomass accumulation, perhaps requiring biomass retaining reactors such as anaerobic sequencing batch reactors (ASBR), anaerobic sludge bed reactors (ASBR), and hybrid reactors, as opposed to more traditional continuous stirred tank reactors (CSTR).

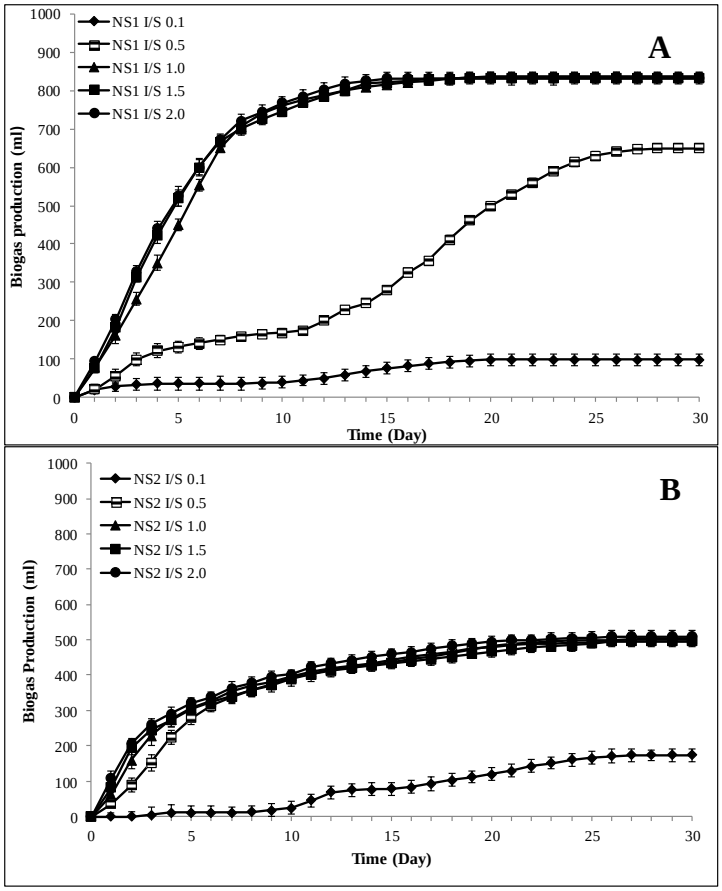

Figure 2: Biogas production curves for digestion of (A) NS1 and (B) NS2 at various I/S ratios

Additional LCFA studies with calcium dosing

Although the previous data showed stable and effective digestion at I/S ratio of 1.0, as can be seen from Figure 3, it was still possible to raise the level of biogas production by dosing the system with calcium. The hypothesis behind the response is that the introduction of suitable concentrations of calcium allowed for a calcium-LCFA substrate that relieved the bacterial biomass of the aforementioned cell-surface inhibition, thus further solidifying the LCFA surface inhibition conclusion and generating a potential chemical means to further reduce its negative implications. The effect of calcium dosage concentration appears to solidify this explanation as the lower 0.5X dosage still showed some degree of inhibition before 200 hours, while 1.0X and 2.0X dosage showed no signs.

Analysis of FAME results (Figure 5) indicates that for NS1, LCFA degradation is faster at the beginning with no addition of calcium, with introduction of the calcium-LCFA substrate not only reducing latter stage methanogenic cellular surface inhibition, but also delaying overall degradation up to that final methanogenic step. Interestingly, for NS2, the calcium dosage had no significant effect on methane production or the degradation of the LCFA (Figure 4 and 6). This might due to the low LCFA concentration, which has little effect on binding methanogens at the high I/S ratio utilized.

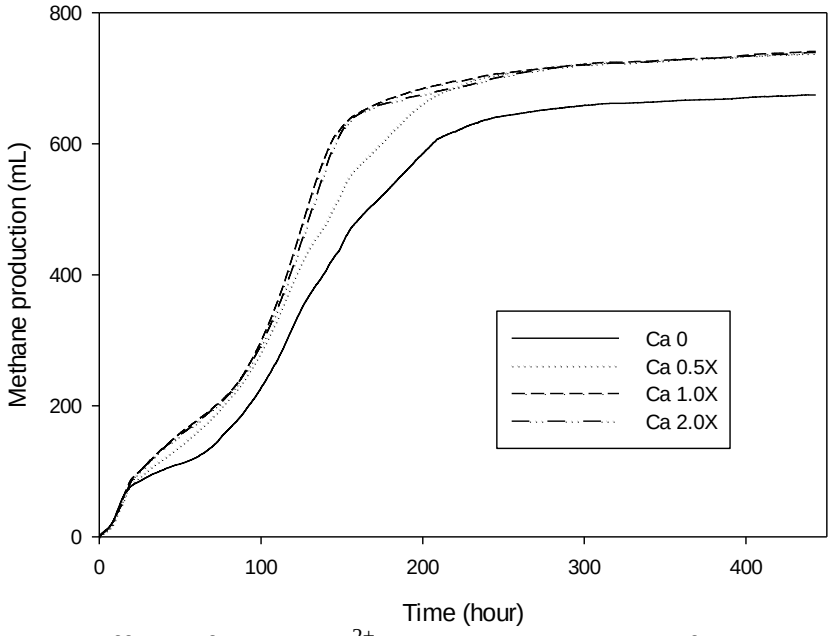

Figure 3: Effects of dosing Ca^{2+} on methane production from NS1 biomass

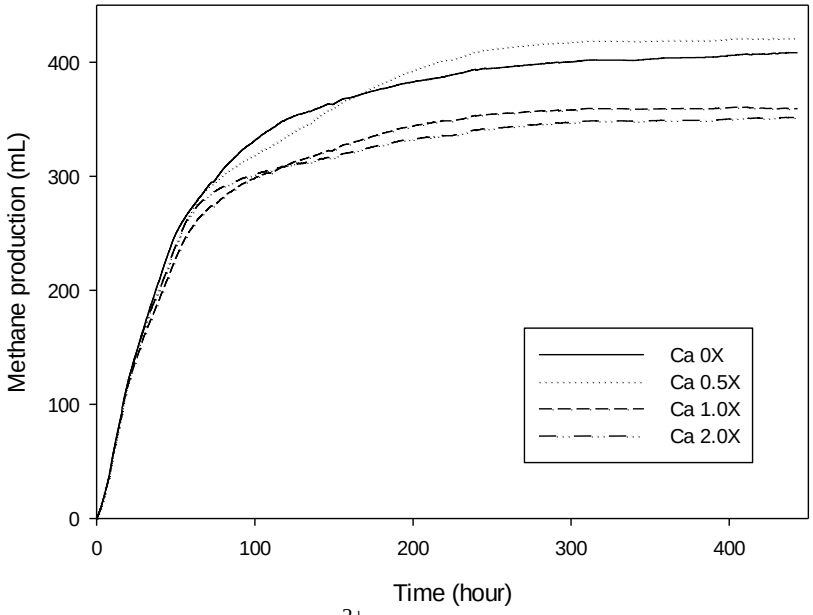

Figure 4: Effects of dosing Ca^{2+} on methane production from NS2 biomass

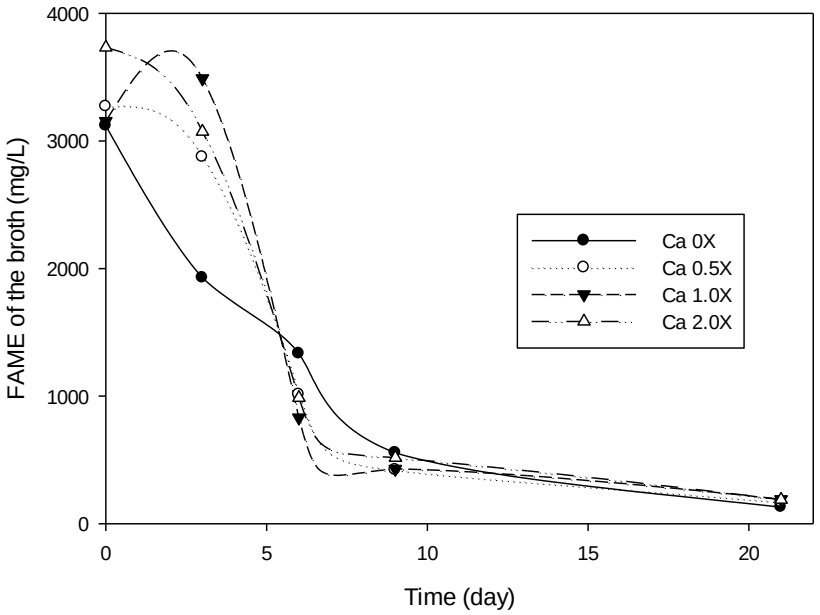

Figure 5: Profile of FAME in the broth during anaerobic digestion of NS1

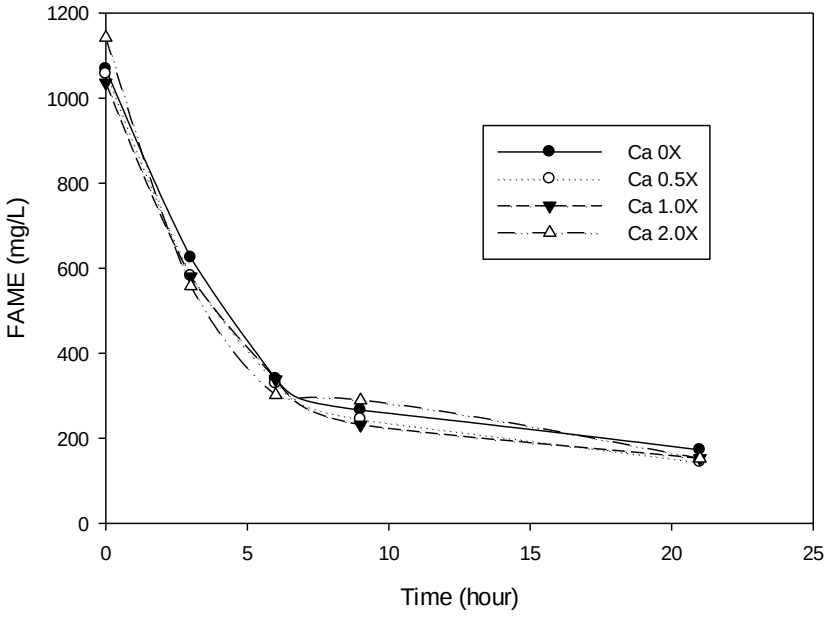

Figure 6: Profile of FAME in the broth during anaerobic digestion of NS2

BMP and performance indicators

Table 4 and Figures 8-9 summarize the data from BMP evaluation of the ten different microalgae biomass. Figure 8 biogas curves show that use of an I/S ratio or 1.0 ensured effective digestion with normal biogas production over time. Effluent VFA and pH values confirm the effective digestion, as near neutral pH as well as very low levels of VFA indicate effective transformation of organics all the way through to methane. Low levels of TAN also indicate that ammonia inhibition from an assumed too low C/N ratio

was not a problem. Overall organic conversion ranged from 59.33-78.50 as a measure of %VS reduction. Notably this range was relatively small, showing that all of the biomass could have a relatively similar percentage of total organic material converted to methane during the 20-30 day digestion, although important variations did exist. For example, each lipid-extracted form resulted in lowered VS reduction as compared to its whole cell companion. This is presumably due to lipid-extracted material being composed of a greater fraction of non-biodegradable or recalcitrant organic material such as lignin, crystalline cellulose, etc.

Table 4. BMP and performance indicators for studied biomass

Algae biomass	C1	C2	N1	N2	NF1	NF2	NS1	NS2	P1	P2
Biogas (L Biogas)	5.28	5.31	5.36	5.90	6.93	4.37	8.36	5.11	5.20	4.89
CH_4 Production (L CH_4)	3.37	3.14	3.57	3.99	5.07	3.04	5.57	3.83	3.37	3.39
CH_4 Fraction (%)	63.82	59.13	66.60	67.62	73.16	69.56	66.63	74.95	64.81	69.32
95% CH_4 Production (D) [1]	9.75	12.04	5.58	13.94	11.36	9.89	12.71	7.84	13.49	9.70
Max CH_4 (L CH_4/L D) [2]	0.046	0.037	0.087	0.037	0.072	0.056	0.074	0.054	0.040	0.050
1^o Hydrolysis (K_h) [3]	0.22	0.19	0.41	0.17	0.22	0.31	0.16	0.24	0.16	0.23
Effluent pH	7.17	7.50	7.16	7.05	7.16	7.10	6.94	6.94	7.28	7.45
TAN Effluent (mg N/L)	401	865	458	1316	322	509	228	326	613	690
Total VFA Effluent (mg/L)	250	177	ND	ND	ND	118	151	155	190	55
VS Reduction (%)	66.06	64.21	65.90	64.41	76.41	59.33	78.50	73.83	70.60	60.20
SMP (L CH_4/g VS fed)	0.337	0.314	0.357	0.399	0.507	0.304	0.557	0.383	0.337	0.339
TMPE (L CH_4/g VS d)	0.510	0.489	0.542	0.619	0.663	0.512	0.710	0.519	0.477	0.563
TMPT (L CH_4/g VS) [4]	0.604	0.552	0.682	0.531	0.882	0.457	0.749	0.598	0.629	0.580
Degradation (%)	55.76	60.19	52.36	75.09	57.47	66.49	74.33	64.06	53.54	58.48

95% CH_4 Production—Days in which 95% of total CH_4 production is realized; SMP—Specific methane productivity; TMPE—Total methane potential from experimental calculation from VS destruction percentage; TMPT—Total methane potential from theoretical calculation; ND—Non Detected; data reported as mean of triplicates (n=3).

[1, 2] Calculation using modified Gompertz equation as the described by Zwietering et al. (1990)

[3] Calculation as described by Angelidaki et al. (2009).

[4] Theoretical calculations as described by Sialve et al. (2009)

Analysis of the biogas production over time as well as the 95% methane production parameter shows that even 30+ day digestion led to little significant increases in appreciable biogas production and therefore organic degradation, with most biomass achieving 95% of realized potential by no later than day 15. This indicates that as with animal manures, extended digestion time for minimal return on degradation of recalcitrant organics, has diminishing economic returns (Frear et al, 2011). While pre-treatments, not studied in this project could make recalcitrant material more accessible to anaerobic degradation (Mussgnug et al., 2010; Sialve et al., 2009; Zamalloa et al., 2011), the already high %VS reductions also show that from a cost perspective this might not be

economically rewarded, unless an extremely high price for methane energy off-takes and/or necessary biomass solids reduction is preferred.

Unlike literature representing a wide range of maximum methane potentials, this study showed that while important differences did exist, the overall range among all biomass, both whole cell and lipid-extracted was quite small, ranging from 0.304-0.557 L CH_4 g VS^{-1}. For comparison purposes, this SMP range is higher than that of dairy manure (0.21-0.24 L CH_4 g VS^{-1} (Frear et al., 2011)), similar to that of food scraps/green waste now utilized in municipal digesters (0.35-0.55 L CH_4 g VS^{-1} (Palatsi et al., 2009)), but below that of pure lipids and/or fats, oils, and greases (FOG) (0.90-1.0 L CH_4 g VS^{-1}; (Møller et al., 2004).

As might be expected from known high SMP for lipids and FOG, the most important variable to this microalgae SMP spread was lipid content. Figure 7 details the linear relationship that was found between ash-free lipid dry weight percentage and the resulting SMP of the ten evaluated microalgae biomass. As each of the biomass had varying percentages of ash as well as lipid, simple subtraction of the non-biodegradable ash allowed for this comparison between lipids and SMP. While not a perfect relationship (R^2 = 0.814), it is clear that one of the most important parameters dictating ultimate SMP was the whole cell or residual biomass lipid content. Presumably, as algal refineries come closer to reality, lipid-extraction processes will become more effective than done in this study, leading estimated SMP to be a bit lower within the developed range, but notably small in range for modeling purposes. More importantly, as BMP studies on microalgae biomass can be costly and time-consuming, such narrowing of the SMP range with a suitable linear equation for relationship to remaining lipid content can be important.

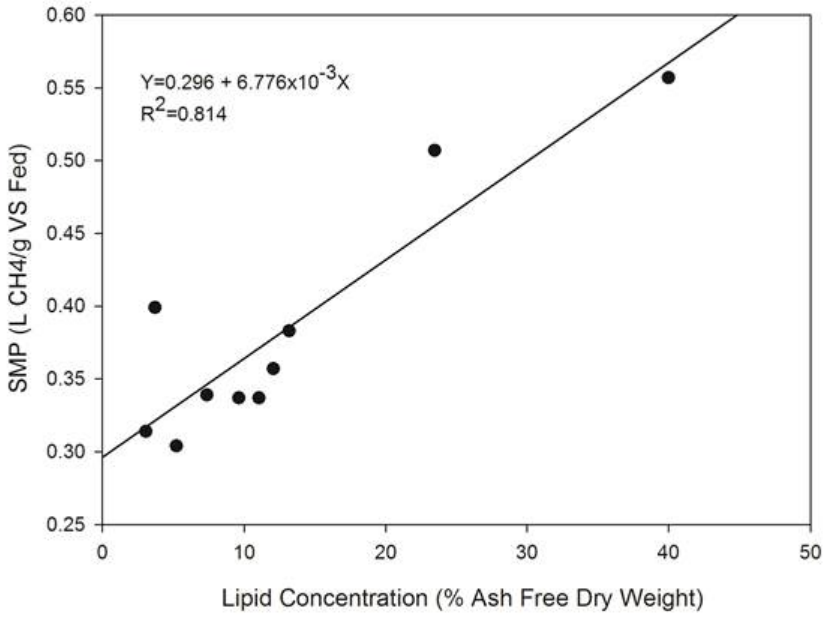

Figure 7: Relationship between ash-free lipid content and SMP for microalgae biomass

Theoretical methane potentials (TMPT) were calculated from the elemental characterization of the microalgae biomass and compared to the generated SMP for

determination of biodegradability factors. Biodegradability factors ranged from 52.36-75.09% with greater percentage biodegradability in general found within the lipid-extracted biomass, which is opposite to the findings related to VS% and SMP. While VS% and SMP reduced upon lipid-extraction, presumably due to larger percentage of more recalcitrant organic material, the biodegradability ratio increased, presumably due to more efficient degradation of available type of organic material—perhaps a legacy of the aforementioned LCFA inhibition and its reduced intensity with lower lipid and LCFA content.

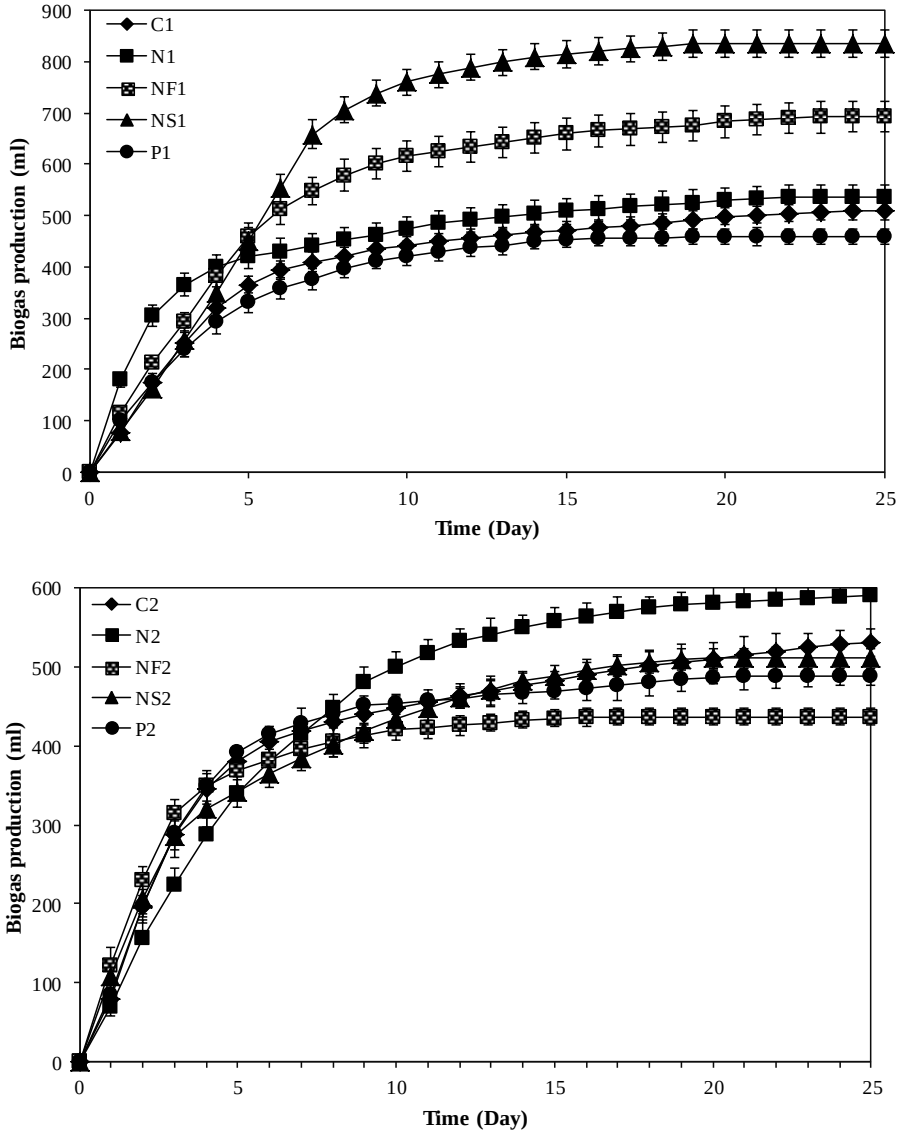

Figure 8: Biogas production curves from both lipid-extracted and whole cell biomass

While it is hard to clearly identify notable trends or strengths and weaknesses to digestion as a factor of species, it is clear that under the parameters of this digestion protocol (high I/S ratio and partial milling of dried biomass), all species and biomass tested resulted in rather efficient digestion times (95% rate between 5.58-13.94 days) requiring no more than industry standard 20 days while also degrading an appreciable percentage of available organics (59.33-78.50. These are all contrary to some arguments that low C/N

ratio, poor accessibility to cellular contents, low overall residual organic content, etc. would not allow for very effective, delayed or inhibited digestion. Most notable is the reduced SMP range as compared to literature, showing upon analysis the importance of proper BMP protocol development, particularly in regard to I/S ratio, for effective comparisons.

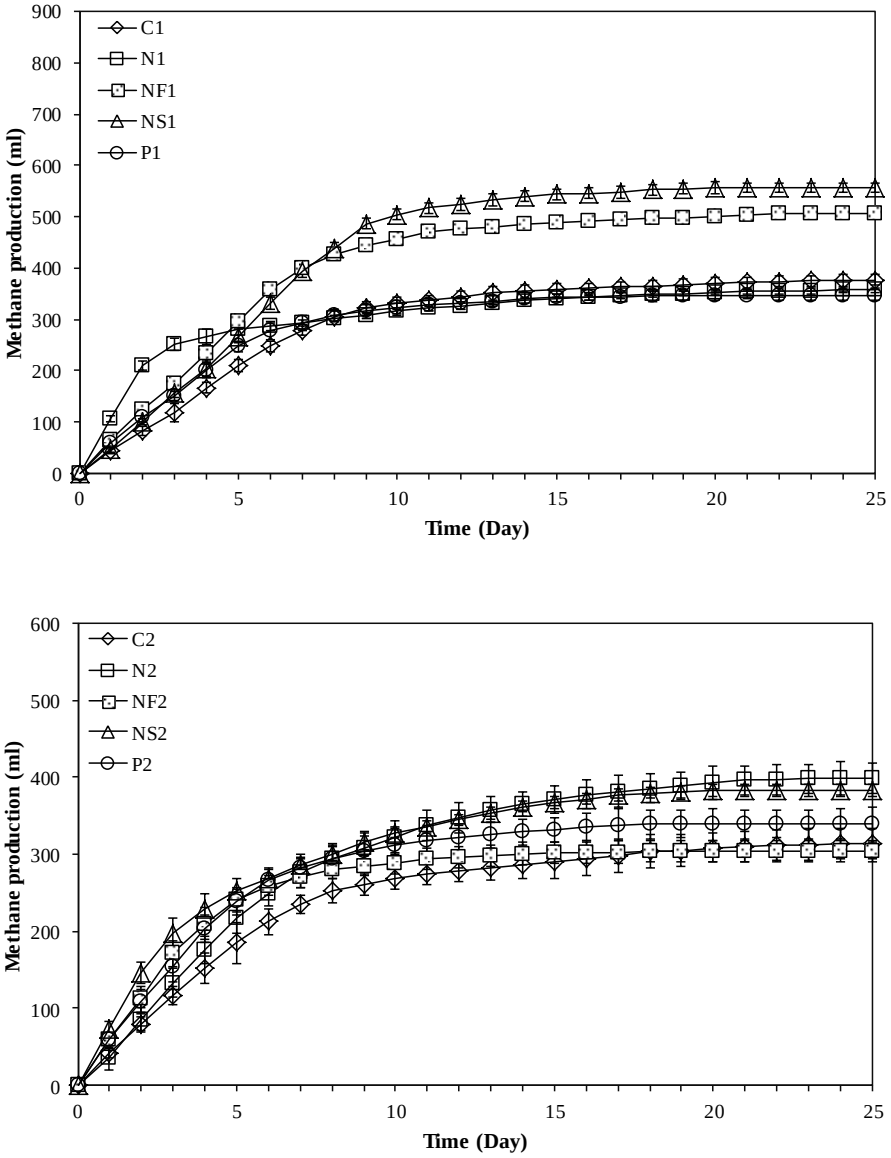

Figure 9: Methane production curves from both lipid-extracted and whole cell biomass

Of the five species tested, a non-statistical analysis, of that data shows that NS in particular appeared to outperform all other species in regard to most BMP performance parameters, both for its whole-cell and lipid-extracted forms, although that might have more to do with lipid content than any species or cellular-form aspect. Clearly, though, any possible further comparison in regard to performance based on species will require a study with comparatively similar growth, harvest, lipid content and lipid extraction practices, something that was not available in this study. In addition, it will be important

to utilize imaging analyses to better determine the role cellular membranes/walls and harvesting/extraction processes have on overall digestion and access to organic content. This is particularly true for this study as the moderate level of milling as pretreatment as well as the methods for harvesting that resulted in dried biomass could have had extensive impact on the membrane/wall structure and significantly impacting the rate/degree of AD that could conceivably be achieved in a commercial operation not using these techniques. In addition, use of image analysis might also offer more explanations as to why species and whole cell versus lipid-extracted experienced such wide shifts in 95% methane achievement rate as well as maximum methane production rate.

Continuous Digestion

From Tables 5-6 and Figures 10-11, it can be seen that both digesters reached desired productivities, methane concentrations, and degree of biodegradability determined earlier by BMP tests at lower scale and OLR. The NS1 digester showed higher SMP ranging from 0.59-0.65 L CH_4 g VS^{-1} (BMP mean of 0.557 L CH_4 g VS^{-1}), while the NS2 digester showed a lower SMP ranging from 0.29-0.42 L CH_4 g VS^{-1} (BMP mean of 0.383 L CH_4 g VS^{-1}). VS reduction percentages at lower OLR were also on par with earlier BMP trials, while percentages decreased at higher OLR tested due to accumulation of undigested algae residue. Results showed that the OLR in NS2 digester could reach up to 5 g VS L^{-1} d^{-1}, while the NS1 digester failed at an OLR of 3.0 g VS L^{-1} d^{-1}. This compares to typical wastewater treatment plants digesting primary sludge at mesophilic temperatures with OLR ranges of 1-3 L CH_4 g VS^{-1} (Rittmann & McCarty, 2001)—so from a scale-up perspective, these OLR are suitable for commercial application. At these two maximum OLR, both NS1 and NS2 could achieve a volumetric methane production rate (VMP) of 1.40 m^3 CH_4 m^{-3} d^{-1}, which for comparison purposes, most commercial sludge/manure digesters are deemed effective if their VMP is near 1.0 m^3 CH_4 m^{-3} d^{-1}; again pointing to commercial viability.

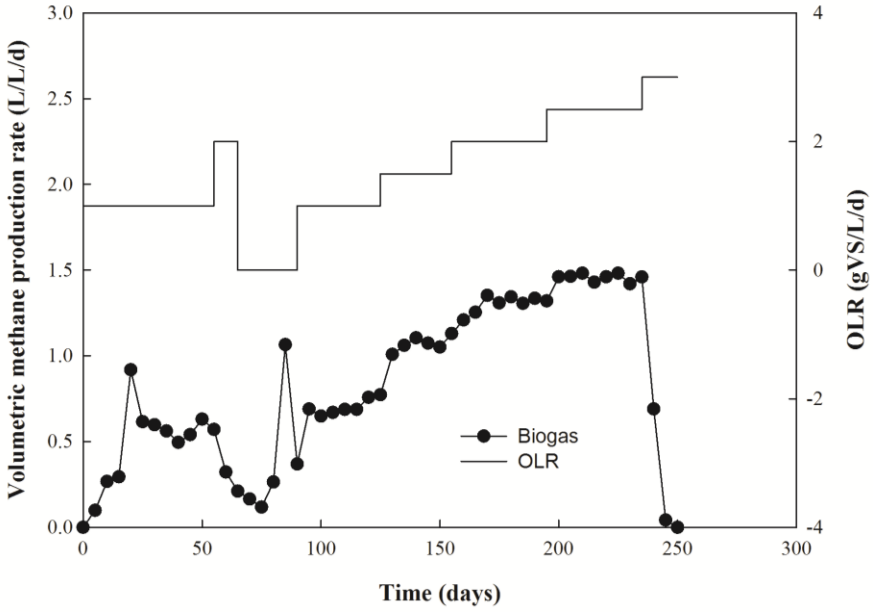

Figure 10: Methane production from NS1 digester at different OLR

Table 5: Performance of NS1digester

Parameter	OLR g VS/L/d	VMP L/L/d	SMP L/g VS	VS % %	TAN mgN/L	TKN mgN/L	TP mgP/L	TIC mg/L	Alkalinity mg/L
Phase 1	1	0.65	0.65	72.9	140.4	585.6	133.6	153.5	1228.0
Phase 2	1.5	0.99	0.66	63.0	165.7	1053.7	193.2	171.3	1367.2
Phase 3	2	1.24	0.62	54.5	566.9	2158.8	347.5	447.3	3394.9
Phase 4	2.5	1.47	0.59	53.8	633.5	2547.2	414.0	475.5	3771.9

Reasons for the decreased viability of NS1 at higher OLR as compared to NS2 is attributed to LCFA inhibition with LCFA accumulating within the digester and attaching to the retained biomass, reducing bacterial performance until ultimately the digester failed. As the ultimate preferred use for AD within the algal biorefinery concept is to treat lipid-extracted microalgae, the excellent results with NS2 is encouraging, but the noted impact of LCFA on ultimate OLR of NS1 should be useful to both those potentially digesting whole cell microalgae.

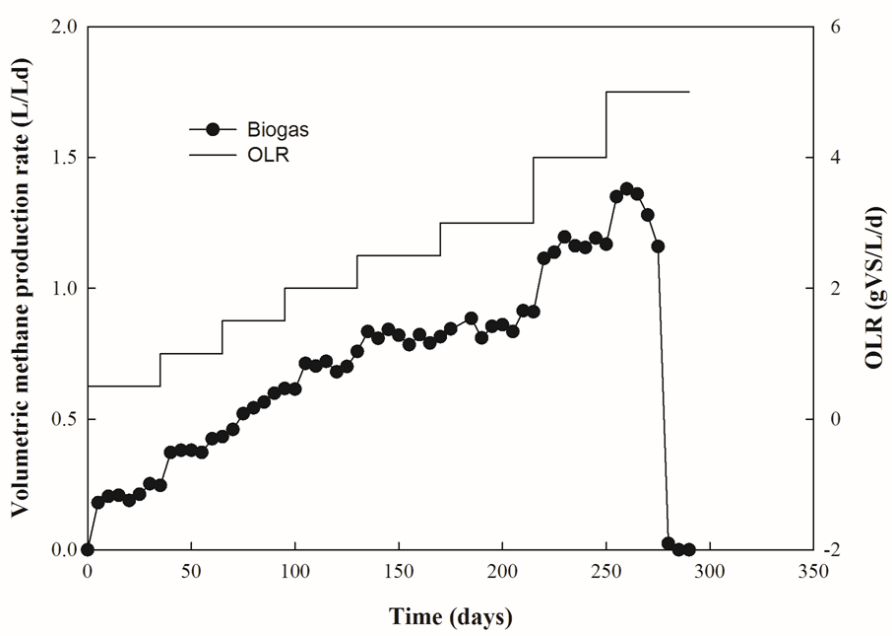

Figure 11: Methane production from NS2 digester at different OLR

As OLR increased, naturally effluent nutrient concentrations increased, with NS1 ultimately achieving a TAN of 634 mg N L^{-1}, a TKN of 2,547 mg L^{-1}, a TP of 414 mg L^{-1}, and total alkalinity of 3,772 mg L^{-1} at OLR of 2.5 g VS L^{-1} d^{-1}. Similarly, NS2 at its highest OLR of 5.0 g VS L^{-1} d^{-1} achieved concentrations of 1,655, 5,613, 640, and 11,148 for TAN, TKN, TP and alkalinity respectively. Importantly, even at this high OLR, TAN effluent is below threshold inhibition levels (Koster and Lettinga, 1984) while total N, total P and alkalinity all supply valuable N, ammonia form of N (~30% of TKN as TAN),

valuable P, and inorganic sourcing of C as well as buffering capability particularly useful for algal cultivation if effluent were to be recycled back to growth stages within the refinery. Tests are now on-going at NREL for growth of microalgae utilizing these effluent with concentrated nutrients.

Table 6: Performance of NS2 digester

Parameter	OLR g VS/L/d	VMP L/L/d	SMP L/g VS	VS % %	TAN mgN/L	TKN mgN/L	TP mgP/L	TIC mg/L	Alkalinity mg/L
Phase 1	0.5	0.21	0.42	-	-	-	-	-	-
Phase 2	1	0.40	0.40	72.6	387.1	678.1	95.8	259.8	1854.3
Phase 3	1.5	0.58	0.39	72.8	466.3	1801.0	244.2	459.2	3353.5
Phase 4	2	0.68	0.34	56.7	739.6	2695.6	282.9	709.8	4025.1
Phase 5	2.5	0.82	0.33	51.3	924.8	2673.9	178.5	788.8	4916.6
Phase 6	3	0.86	0.29	48.7	1093.4	3486.5	286.6	850.8	5800.5
Phase 7	4	1.16	0.29	46.7	1563.3	5300.8	465.1	1084.9	8666.8
Phase 8	5	1.40	0.28	45.7	1655.3	5612.9	640.0	1049.0	11148.1

Conclusion

Different from a review of literature, the SMP and other AD performance parameters for the five industrial strains evaluated were within a much tighter range. It is believed that control of a viable I/S ratio (1.0 VS/VS) during BMP and continuous digestion was instrumental in overcoming LCFA inhibition and providing for a tighter and more effective range of SMP (0.304-0.557 L CH_4 g VS^{-1}) and VS reduction (59.33-78.50%). SMP appeared to not so much be related to species but more to LCFA content within the biomass, with a linear relationship between SMP and ash-free lipid content being developed for easier, less time-consuming determination of approximate SMP for particular biomass strains grown.

Experiments at lower I/S ratios, high OLR, and with calcium doping highlighted the vulnerability the microalgae AD process has to LCFA if controls are not in place. In the case of continuous digestion a high I/S ratio was accomplished through use of SBR processing, allowing for a much higher biomass concentration. Previous concerns related to C/N ratio, ammonia toxicity, reduced methane percentages, and poor access to organic material via cellular membranes/walls were not noted when proper digestion controls were in place. All biomass digested well within industry standard 20 days, with most achieving 95% methane accumulation prior to day 15. Methane content ranged from 60-75% while effluent TAN and VFA levels were quite low, indicating effective, complete digestion with little concern of product inhibition, despite all samples having C/N ratios well below that ideally preferred. Throughout all experiments, a certain degree of milling of dried biomass was utilized, thus bringing into question the role the drying and milling processes had in easing AD conditions as compared to more realistic commercial environments where no drying or milling will most likely take place. Further study on the role of this pretreatment to cellular structures via image processing is warranted. Another observation noted in regard to commercial harvest is the use of extraction solvent mixture, with studies determining that chloroform/methanol mixtures were extremely

inhibitory to methanogenic bacteria, requiring use of different solvent mixtures if AD is to be a critical component of the biorefinery approach.

Scale up to continuous digesters showed general maintenance of previously identified BMP capabilities. The NS1 digester showed higher SMP ranging from 0.59-0.65 L CH_4 g VS^{-1}, while the NS2 digester showed a lower SMP ranging from 0.29-0.42 L CH_4 g VS^{-1}. VS reduction percentages at lower OLR were also on par with earlier BMP trials, while percentages decreased at higher OLR tested due to accumulation of undigested algae residue. Results showed that the OLR in NS2 digester could reach up to 5 g VS L^{-1} d^{-1}, while the NS1 digester failed at an OLR of 3.0 g VS L^{-1} d^{-1}. At these two maximum ORL, both NS1 and NS2 could achieve a VMP of 1.40 m^3 CH_4 m^{-3} d^{-1}, which for comparison purposes, most commercial sludge/manure digesters are deemed effective if their VMP near 1.0 m^3 CH_4 m^{-3} d^{-1}; pointing to commercial viability. Effluent nutrient concentrations were notably high at higher OLR, allowing for potentially important economic benefits upon recycle of these nutrients to the growth ponds. Effluents and their characteristics were made available to NREL for such recycle/growth studies.

Deliverables

Anticipated deliverables from the sub-contract research on this project are as follows:

Refereed Journal Articles

1. Zhao, B., Ma, J., Zhao, Q., Laurens, L., Jarvis, E., Frear, C. (In development) Anaerobic digestion of whole and lipid-extracted microalgae from five industrial strains—Determination of important methane and nutrient information. Applied Energy.
2. Ma, J., Zhao, Q., Laurens, L., Jarvis, E., Frear, C. (In development) Continuous anaerobic digestion of whole cell and lipid-extracted microalgae biomass in sequencing batch reactors—Methane and nutrient production with microbial population shifts. Bioresource Technology.
3. Zhao, Q., Yu, L., Ma, J., Laurens, L., Jarvis, E., Frear, C. (In development) Kinetic model for long-chain fatty acid (LCFA) degradation in microalgae, both whole cell and lipid-extracted. Bioresource Technology.

Conference Presentations

1. Frear, C., Zhao, B., Zhao, Q., Ma, J., Pienkos, P., Laurens, L., Sweeney, N., Davis, R., Nagle, N., Jarvis, E. 2012. Anaerobic digestion of algal biomass residues with nutrient recycle. Algae Biomass Summit, September 24th-27th, 2012, Denver, Colorado, USA.
2. Frear, C., Zhao, B., Zhao, Q., Ma, J., Pienkos, P., Laurens, L., Sweeney, N., Davis, R., Nagle, N., Jarvis, E. 2013. Anaerobic Digestion of Whole and Lipid-Extracted Algal Biomass from Four Industrial Strains--Determination of Important Methane and Nutrient Information. ASABE National Conference, July 22th-24th, 2013, Kansas City, MO, USA.
3. Ma, J., Zhao, Q., Laurens, L., Jarvis E., Nagle, N., Frear, C. Anaerobic digestion of whole and lipid-extracted algal biomass—Continuous digestion in sequencing batch reactors. Algae Biomass Summit, September 30th-October 3rd, 2013, Orlando FL, USA.

Next Steps

As with any project, lessons learned from its conclusion lead researchers to new hypotheses and potential engineering solutions as well as identification to where limitations of the existing study reside. Below is a brief highlight of a few areas WSU believes further and additional study is warranted, either using continuing available funding or via access to new extramural grants to be applied for and hopefully awarded. It is hoped that all potential avenues of research could be done in partnership with NREL as it is the belief of WSU that the multi-institutional and collaborative work completed in this study was extremely useful and beneficial to all involved.

1. *Image analysis*—A follow up study involving dried as well as wet, concentrated biomass across species, with or without physical pre-treatment, as was done in this study, should be completed with image analysis at the core of determining the impact these harvest, pretreatment and AD treatments have on cellular membrane and wall structure. Design should allow for both qualitative as well as quantitative conclusions with results having strong ties to cellular biochemistry. This study could be easily done as a follow up using existing potential funds or as a more complete separate study.
2. *Nutrient staging that integrates specific AD/nutrient recycle effluent outcomes with desired growth conditions*—intriguing research continues in regard to specific idealized growth conditions across seeding, growth and N replete/deplete conditions. Each of these conditions requires particular pH/buffer and carbon/nutrient concentrations, particularly if a sequence of heterotrophic to mixtrophic/phototrophic growth is to be completed between seeding and growth. AD effluents in combination with developed nutrient recovery technologies can supply a range of effluents that could be ideal for each of these growth segments. WSU is interested in partnering with NREL and other industry partners in ascertaining how/best these range of suitable effluents could be produced and then utilized by growth facilities for performance optimization.
3. *Unique use of AD for simultaneous lipid extraction, residual biomass digestion and targeted, phased nutrient recycle*—without going to far into the intellectual merit and potential property, WSU has identified a unique set of engineering approaches and conditions that could potentially maximize the role of AD to much more than residual biomass to methane treatment. WSU is interested in partnering with NREL on proof of concept work related to this new technological approach and is now putting together a white paper for team review.

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