1
2
3
4 **Anaerobic digestion of lipid-extracted *Auxenochlorella protothecoides* biomass for**
5
6 **methane generation and nutrient recovery**
7

8
9 Authors: Pavlo Bohutskyi^{1*}, Ben Ketter², Steven Chow¹, Kameron Adams¹, Michael J.
10
11 Betenbaugh², F. C. Thomas Allnutt^{3,4}, Edward J. Bouwer¹
12
13

14 Affiliations:

15
16 ¹ Department of Geography & Environmental Engineering, Johns Hopkins University,
17
18 3400 North Charles Street, Baltimore, MD 21218-2686 USA
19
20

21 ² Department of Chemical & Biomolecular Engineering, Johns Hopkins University, 3400
22
23 North Charles Street, Baltimore, MD 21218-2686 USA
24
25

26 ³ Phycal Inc., 51 Alpha Park, Highland Heights, OH 44143 USA
27

28 ⁴ Current Address: BrioBiotech, P.O. Box 26, Glenelg, MD 21737 USA
29
30

31 * Corresponding author, email: bohutskyi@jhu.edu, phone: (443) 794-1780
32
33
34
35
36
37
38
39
40
41
42
43
44

45 **Abstract**
46

47 This study evaluated methane production and nutrient recovery from industrially produced,
48
49 lipid extracted, algal biomass (LEA) of *Auxenochlorella protothecoides* using semi-
50
51 continuous anaerobic digestion (AD) at different organic loading rates (OLRs) and
52
53 hydraulic retention times (HRTs). It was shown, that AD can improve biofuel production
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 efficiency and sustainability, especially for scaled processes, through up to 30% increase in
5
6 energy generation (up to 0.25 L of methane per g of LEA volatile solids) and partial
7
8 nutrient recovery and recycling. The nutrient recycling with the AD effluent may reduce
9
10 the cost of the supplied fertilizers by up to 45%. However, methane production was limited
11
12 to nearly 50% of theoretical maxima potentially due to biomass recalcitrance and inhibition
13
14 effects from the residual solvent in the LEA. Therefore, further AD optimization is
15
16 required to maximize methane yield and nutrient recovery as well as investigation and
17
18 elimination of inhibition from solvent residues.
19
20
21
22

23 **Keywords:** biogas production; inhibition by solvent residues; integrated biodiesel and
24
25 methane production; nitrogen and phosphorus recycling; trace element recovery; process
26
27 sustainability
28
29
30

31 **1. Introduction**

32
33 The recent expansion of interest in renewable and sustainable microalgal biofuels is driven
34
35 by rising energy demand and higher prices coupled with society's growing awareness of
36
37 exhaust gas emissions associated with the world's continued overreliance on fossil fuels.
38
39 The use of microalgal biomass for feedstocks for fuel production has several key
40
41 advantages: 1) photosynthetic productivities higher than terrestrial plants, 2) possible
42
43 cultivation on non-arable lands, 3) entire organism available for conversion, and 4) easy
44
45 integration with industrial CO₂ generation point sources for productive reuse ([Schenk et al.,
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65](#) 2008). However, several technological and economic hurdles need to be managed in order
66
67 to demonstrate market viability as an efficient and sustainable industrial algal biofuel
68
69 process.

Considerable attention has been given to converting the lipid fraction of algal biomass into biodiesel or green diesel. These processes have demonstrated production of liquid drop-in fuels for motor vehicles and jets from the lipid fraction. However, these processes only utilize the lipid fraction of algal biomass. The residual biomass after lipid extraction is composed mostly of carbohydrate and protein fractions and can represent 30-70% of the ash free dry weight (AFDW) of the overall dry biomass harvested ([Bohutskyi & Bouwer, 2013](#)). Recent progress demonstrated the potential to minimize the amount of lipid extracted algal residues (LEA) generated by inducing the cell lipid content up to 50-70% by applying environmental stress (nutrient starvation or exposure to extreme salt content, pH, temperature, radiation, or inhibiting chemicals) ([Gao et al., 2013](#)), providing an excess of organic carbon substrate (*e.g.*, sugar) to employ heterotrophic growth conditions ([Bohutskyi et al., 2014b](#)), or modifying the lipid metabolic pathways ([Yu et al., 2011](#)). Unfortunately, these methods can either compromise the algal growth rate, require supplementation by expensive glucose, or are still in the development stage. Additionally, certain types of algal strains, especially those that grow competitively in open ponds or even wastewater, have low lipid contents (*e.g.*, 5-20% AFDW) and might not be easily replaced with the lipid-rich microalgae species due to competition and/or contamination under open pond culturing conditions. Therefore, it is critical to develop biofuel production technologies that make use of the entire algal biomass (*e.g.*, lipid and LEA) to maximize energy productivity and pass the threshold for algal biofuels to be cost competitive. Some of the options are to produce high value co-products (self-limiting markets when algal biofuels are at commercial scale) or conversion of LEA into additional fuel. Anaerobic digestion (AD) is an established, scalable technology that can be used to transform LEA or

1 whole algal biomass into the biofuel methane ([Bohutskyi & Bouwer, 2013](#)). Furthermore,
2
3
4
5
6 the preliminary studies under batch conditions using the biomethane potential procedure
7
8
9 (anaerobic medium amended with nutrients, vitamins, pH and redox buffers) performed in
10
11 our laboratories as well as other laboratories demonstrated that the use of AD for
12
13 conversion of LEA residues was possible ([Bohutskyi et al., 2014b](#); [Hernandez et al., 2014](#);
14
15 [Zhao et al., 2014](#)). In addition, integration of AD in the algae-to-biofuel process provides
16
17 the potential for recycling part of the nutrients to further reduce the overall costs of the
18
19 biofuel production process ([Bohutskyi et al., 2014c](#)). The investigation of LEA anaerobic
20
21 digestion in continuous bioreactors at conditions more similar to industrial application is a
22
23 critical step in scaling up this technology.
24
25
26
27

28 Reuse and recycling most of the macro- and micronutrients required for algal growth
29
30 need to be achieved in order to assure sustainability of the algal biofuels and reduce
31
32 production costs ([Pate et al., 2011](#)). Failure in developing efficient nutrient recycling
33
34 technology during algal biofuel production will put the industry in competition with
35
36 agriculture for nutrients (*e.g.*, nitrogen and phosphorus). Generation of 30 billion gallons
37
38 per year (BGY) of algal biofuel equivalent or nearly 30% of the US liquid fuel
39
40 consumption without nutrient recycling would require 130-320% and 60-150% of the
41
42 current nitrogen and phosphorus fertilizer consumption in the U.S., respectively; the ranges
43
44 depend on the algal biomass lipid content ([Pate et al., 2011](#)). Moreover, augmented algal
45
46 production requires supplying essential trace elements such as Mg, Ca, Mn, Co, and Mo.
47
48 Importantly, some of these elements are naturally released from degraded biomass into the
49
50 aqueous phase during the AD process. Previously, it was demonstrated that nitrogen,
51
52 phosphorus and several trace elements can be partially recovered from algae and LEA with
53
54
55
56
57
58
59
60
61
62
63
64
65

AD effluent for recycling to the cultivation stage ([Bohutskyi et al., 2014c](#)). However, no data are available on what fraction of the essential macronutrients and trace elements assimilated in algal cells can be recovered from the AD effluent and also what the impact of the AD operational parameters will have on the recovery efficiency. To address these issues, a semi-continuous AD system was implemented in order to determine the methane production from the industrially produced *Auxenochlorella protothecoides* lipid-extracted residues. The amount of nutrients available for recycling was measured and compared to the total nutrient content in LEA. In addition, the current study evaluated the impact of the organic loading rate (OLR) and hydraulic retention time (HRT) on methane production and the amount of recovered macro- and micronutrients. It is believed that these data provide vital insights for the development of an economical and sustainable microalgal biofuel industry.

2. Materials and methods

2.1. Microalgal biomass

The industrially produced lipid extracted biomass (LEA) of *Auxenochlorella* (formerly *Chlorella*) *protothecoides* was used as substrate for semi-continuous anaerobic digestion in the current study. The LEA was generously provided by Phycal Inc. from its subpilot-scale cultivation facility. The *A. protothecoides* biomass was generated using a sequential phototrophic-heterotrophic growth system. The alga was cultivated in an open-pond under photoautotrophic conditions followed by cultivation in the fermenter under heterotrophic conditions to boost lipid content and then dewatered using centrifugation ([Bohutskyi et al., 2014b](#)). The lipid fraction of the biomass was extracted (see description below) and the slurry of whole algae (WA) and LEA were stored at -80°C until use.

2.2. Lipid extraction procedure

The lipid fraction of the biomass was extracted using acetone followed by hexane as extraction solvents according to the following procedure: 1 L of 300 g/L biomass was transferred in a 4L plastic container, mixed with 1 L of acetone and the pH of the mixture was adjusted to pH 7 using 10 M NaOH solution. Next the biomass-acetone mix was processed three times in the Labstar LS1 Bead Mill machine using the following conditions: 617.5 mL of 0.6-0.8 mm beads, agitator speed at 2800 rpm, pump speed at 85 rpm. Then the DI water was added to the processed slurry with 0.5 to 1 ratio, the solution was mixed, incubated in a water bath at 65°C for 20 min and centrifuged in 50 mL tubes (containing 30 mL of solution) at 4,000 rpm for 10 min (Beckman J21C; 6 L rotor). Next, 5 mL of hexane was carefully added into the centrifuge tube containing the biomass and hexane was pumped back and forth several times without disturbing the liquid inside the 10 ml glass pipette. The hexane level containing lipids was transferred into a preweighed 15 mL glass tube. This step was repeated two more times. The combined hexane phase was transferred into a Zanntek Analytical Evaporator adjusted to 85°C and nitrogen flow rate 1.0 SCFH (1 ft³/hr). The lipid content was determined by subtracting the mass of the empty vial from the mass of the vial after hexane evaporation.

2.3. Anaerobic digestion system description and operation

The AD experiments were carried out in a semi-continuous stirred tank reactor using 3 L spinner flasks (Bellco Glass, Inc., Vineland, NJ, USA) containing 2 L of the anaerobic culture. The bioreactors were equipped with four sampling ports for solution withdrawal, feeding, biogas collection and sampling for analysis of methane content. The AD systems were operated in a controlled environmental chamber at $35 \pm 1^\circ\text{C}$ and continuously mixed

with a variable-speed overhead drive motor (Bellco Glass) that rotated an acrylic shaft with polyethylene 12x15 cm (HxD) impellers at ~20 rpm. The volume of the produced biogas was measured using wet-tip gas meters (Wet Tip Gas Meter Co., Nashville, TN, USA) equipped with a HOBO Pendant[®] G Data Logger (Onset Computer Corporation, Bourne, MA, USA). Anaerobic digester-treated sewage sludge (obtained from the Back River Wastewater Treatment Plant, Baltimore, MD, USA) was used as the starter inoculum. The digesters were fed with algal biomass for about 3 months to “washout” sewage sludge residues prior to starting data and sample collection for this study. The anaerobic bioreactor was operated at four different regimes applied sequentially (Table 1). First, the bioreactor was operated at an organic loading rate (OLR) of 1.0 gVS/L-day and hydraulic retention time (HRT) of 20 days (Run 1). The second run was at an OLR 2.0 gVS/L-day and HRT of 20 days (Run 2). The third run was at an OLR 1.0 gVS/L-day and HRT at 40 days (Run 3). The final run was at an OLR 1.5 gVS/L-day and HRT of 40 days (Run 4). The AD feedstock solutions were prepared in 2 L batches by diluting either the frozen WA paste or LEA with Milli-Q[®] water to obtain the desired VS (volatile solid) content. The LEA solution was homogenized for 10 minutes using an Oster[®] 10-Speed blender equipped with a 25 mm radius blade at 27,000 rpm (A. J. Oster Corp., Warwick, CT, USA). The feedstock solutions were stored at 4°C and pre-heated to ~30°C by microwaving for approximately 1 minute prior to usage (Emerson, St. Louis, MO, USA). The manual withdrawal of digested algae and addition of the algal feedstock was done once a day using a 60 mL syringe. The collected digestate samples were stored in a -20°C freezer until use. For nutrient analysis, the samples were thawed at room temperature, then centrifuged in 50 mL tubes at 4,200 rpm (4,000×g) and 4°C for 5 min. The supernatant was

separated, filtered through a 0.22 µm nylon syringe filter and used to measure nutrient concentration. The data points were generated from digestate samples collected on different days (2-4 days) at the end of every run and reported as the average with standard deviation provided.

2.4. Estimation of energy production from algal biomass

Theoretical methane yield was estimated from the algal biomass' elemental composition achieved in this study using the modified Buswell formula (Equation 1) ([Buswell & Mueller, 1952](#)):

where: c , h , o , n and s are determined from the biomass elemental composition and stoichiometric coefficients x and y are calculated as: $x = (4c + h - 2o - 3n - 2s) / 8$ and $y = (4c - h - 2o + 3n + 2s) / 4$

The energy yield from algal biomass was calculated using specific energy 39.5 MJ/kg and 37.1 MJ/m³ for triacylglycerols (TAGs) and methane respectively. The detailed calculations are shown in Supplementary data (Appendix A).

2.5. Calculation of nutrient costs and requirement for scaled up process

To make these calculations the following techniques and assumptions were applied. The nutrients required for biodiesel production were estimated based on the elemental analysis of whole *A. protothecoides* biomass or based on part of the supplied nutrients assimilated by algae during cultivation. The nutrients remaining in the spent growth medium were assumed to be all recycled to the cultivation system. Similarly, the fertilizers used for production of glucose provided for algal growth at heterotrophic conditions were left out of the current calculations. The content of biodiesel-convertible lipids in biomass was

assumed equal to content of neutral lipids or TAGs with volumetric density 0.92 kg per liter ([Pate et al., 2011](#)). The bulk prices for chemical fertilizer were obtained using the [www.alibaba.com](#) sales service web portal. Then, the required amount of nutrients was estimated for algal bioenergy production that would replace 10% of the liquid fuel consumption in the U.S. or 29 billion gallons per year (BGY) by algal biofuels ([USEIA, 2013](#)). Finally, this calculated fertilizer requirement was compared to the agricultural nutrient consumption reported in the U.S. in 2011 ([USGS, 2014](#)) to estimate the impact of nutrient recycling on sustainability of algal biofuel production. The detailed calculations are shown in Supplementary data (Appendix B).

2.6. Analytical techniques

Algal biomass was analyzed to determine total solids (TS) and volatile solids (VS) according to the *Standard Methods for the Examination of Water and Wastewater* ([Eaton & Franson, 2005](#)). Carbohydrate content was determined by the phenol-sulfuric acid method using glucose as the standard. Crude protein was estimated by multiplication of total nitrogen in the LEA by a correction factor of 6.25. The content of C, H, O and N of freeze-dried WA and LEA was determined by a subcontractor laboratory (Micro Analysis, Inc., Wilmington, DE, USA) using a %CHN Analyzer. The content of trace elements in biomass samples was measured using microwave-assisted digestion (CEM Mars Xpress, Matthews, NC, USA) in concentrated nitric acid (TraceMetal™ Grade, Fisher Chemical) followed by analysis on an Elan® DRC™ II ICP-MS (PerkinElmer/Sciex, Concord, ON, Canada). The detailed description of the procedure has been published previously ([Bohutskyi et al., 2014b](#)).

Hach Kits (Hach USA, Loveland, CO, USA) were used to measure the concentrations of total nitrogen (N_{tot}), ammonia (NH_3), phosphate (PO_4^{-3}), and total phosphorus (P_{tot}) in the filtered supernatant of the AD effluent. The content of trace elements was measured using 1 mL of the sample spiked with 1 mL of concentrated HNO_3 for microwave-assisted digestion. The metals content of the digestate was subsequently analyzed with an ICP-MS system.

Biogas analysis. Biogas composition was analyzed using a Shimadzu GC equipped with the Hayes Q 80/100 column and thermal conductivity detector (TCD). Helium was used as the carrier gas at 2 bar pressure. The Inj/Det temperature and column current were 130°C and 80 mA, respectively. A 250 μL sample of biogas was obtained using a Hamilton SampleLock™ syringe and injected into the GC. The GC-TCD was calibrated by injecting 50, 150, 150, 200 and 250 μL of N_2 , CH_4 and CO_2 analytical standard gases (Supelco-Sigma Aldrich, St. Louis, MO, USA).

3. Results and Discussion

3.1. Algal biomass characterization and theoretical methane yield

The compositions of WA and LEA are shown in Table 2. The fraction of ash is below 10% for both WA and LEA. The concentrations of all parameters, except volatile solids (VS) and hydrogen, were higher in the LEA than in WA. *A. protothecoides* biomass generated by a sequential phototrophic-heterotrophic process has very high lipid content of up to 60%. Lipids have very low ash content and abundant hydrogen. Therefore, extraction of lipids causes an increase in ash and reduction in hydrogen concentration in the LEA. In contrast, the concentrations of all other elements increased substantially in the LEA vs. WA as the result of lipid extraction. Lipids, more specifically neutral lipids, have low

1
2
3
4 contents of O, N, P and S. Moreover, these nutrients and trace elements are usually
5
6 components of polar or charged compounds or present in the biomass in ionic forms. Most
7
8 of them do not co-extract with the lipid fraction and therefore become more concentrated
9
10 in the LEA. Therefore, it is not surprising that the content of most trace metals and
11
12 metalloids in LEA is nearly 2.8-5 times higher compared to the original WA. The elevation
13
14 in these elements is slightly higher than that anticipated (up to 2.5 times) due solely to
15
16 removal of lipids. These unexpectedly higher values may be the result of contamination
17
18 with metals during the bead milling and acetone/hexane extraction processes. In support of
19
20 this explanation is the concentrations of Zn and Ni increased in the biomass approximately
21
22 thirty times in comparison to the levels in the WA.
23
24
25
26
27

28 The theoretically calculated maxima of biogas and methane yields are shown in
29
30 Table 3. As shown earlier, the type of algal biomass and substrate composition has a major
31
32 impact on the methane produced during anaerobic digestion ([Bohutskyi et al., 2014a](#)). The
33
34 WA, containing high concentrations of energy-rich lipids, had higher biogas potential and
35
36 methane content. However, the LEA was more favorable for anaerobic digestion due to
37
38 higher protein content and therefore a lower C:N ratio. In contrast, the anaerobic digestion
39
40 of the lipid-rich WA may be limited in nitrogen content since the optimal carbon to
41
42 nitrogen ratio for AD is in the range from 15:1 to 25:1 ([Eder & Heinz, 2006](#)).
43
44
45
46
47

48 **3.2. Effect of organic loading rate (OLR) and hydraulic retention time (HRT)**

49 **on anaerobic digestion of lipid extracted algal residues (LEA)**

50
51
52

53 The *A. protothecoides* LEA was subjected to AD in a semi-continuous completely mixed
54
55 bioreactor. The efficiency of conversion was investigated at four different operational
56
57 regimes applied sequentially by varying the OLR and hydraulic retention times (HRTs)
58
59
60
61
62
63
64
65

(Table 1). First, the bioreactor was operated at an OLR 1.0 gVS/L-day and HRT 20 days. Second, the OLR was increased to 2.0 gVS/L-day while maintaining the same HRT of 20 days. Third, the HRT was extended to 40 days but the OLR was reduced to 1.0 gVS/L-day. Finally the OLR was increased to 1.5 gVS/L-day for an HRT of 40 days. The volumetric and specific biogas production rates at these different operational parameters are shown in Figures 1 A and 1 B, respectively. The AD process was stable for at least 20-30 days in a row under certain conditions. However, in every run except the run with OLR 1.0 gVS/L-day and HRT 40 days, the conversion process was disrupted at some point. The reduction in biogas production rate was observed together with a drop in pH from about neutral (pH 7.0-7.2) to acidic values (pH 4.7-5.5). Interestingly, pH neutralization by NaOH addition effectively restored the process under the OLR 1.0 gVS/L-day, HRT 20 day treatment but it was not beneficial under the OLR 2.0 gVS/L-day, HRT 20 day treatment. Various factors can cause instability of the AD process, including fluctuation in operational parameters (temperature, pH, OLR, redox potential or gas leakage) or the presence of inhibitory compounds (ammonia, sulfide, organic compounds, light or heavy metals) ([Chen et al., 2008](#)). The presence of solvent residues with the wet algal biomass feed to the bioreactor seems to be the most probable reason for process interruptions in the current study. The recovery of the anaerobic organisms at OLR 1.0 gVS/L-day, HRT 20 days and robust performance at OLR 1.0 gVS/L-day, HRT 40 days could be explained either by acclimation of the anaerobic organisms or partial degradation of the solvent residues in the bioreactor. However, greater inhibition of the anaerobes was observed at the higher OLR 2.0 gVS/L-day and associated higher solvent dose. While certain anaerobic organisms can degrade both alkanes (such as n-hexane ([Aeckersberg et al., 1991](#))) and ketones (such as

1 acetone ([Zhang et al., 2011](#))) so that they may be degraded by anaerobic organisms, it has
2
3
4
5
6
7 been shown that both alkanes and ketones can be toxic to AD microbial consortia,
8
9 especially to acetoclastic methanogens, and inhibit biogas production ([Hayward & Lau,](#)
10
11 [1989](#); [Playne & Smith, 1983](#); [Zhang et al., 2011](#)). Therefore, the solvent toxicity levels
12
13
14 need to be understood or its residues removed from LEA in order to avoid or mitigate
15
16 negative impact on methane production. Preliminary LEA drying could be one of the
17
18 methods. Zhao *et al.* reported the toxicity effect after utilizing chloroform/methanol (2:1,
19
20 v/v) for extraction but no inhibition when hexane/isopropanol (3:2, v/v) or
21
22 acetone/dichloromethane (1:1, v/v) were used. They utilized LEA subjected to air and oven
23
24 drying prior to AD ([Zhao et al., 2014](#)). The oven drying process could effectively remove
25
26 or reduce significant amounts of residual solvent but needs to be done in a cost effective
27
28 manner to be relevant to production of algal biofuels. Even more beneficial from an
29
30 economical and environmental prospective could be application of the alternative extraction
31
32 methods that do not utilize solvents such as reverse micelle or supercritical CO₂ extraction
33
34 techniques. In addition, processing of microalgae at supercritical condition may increase
35
36 biodegradability and methane production through disintegration of the algal cell wall and
37
38 hydrolysis of biomass polymers into compounds readily available for anaerobic bacteria
39
40 ([Hernandez et al., 2014](#)).
41
42
43
44
45
46
47

48 The average biogas and methane production rates are shown in Figure 2. While
49
50 increasing the HRT from 20 to 40 days at an OLR of 1.0 gVS/L-day slightly improved the
51
52 specific biogas and methane production rates (Figure 2 A), the volumetric gas production
53
54 rate at HRT 20 and an OLR 1.0 gVS/L-day is more than 50% higher compared to other
55
56 runs (Figure 2 B). Interestingly, raising the OLR from 1.0 to 1.5 gVS/L-day with HRT 40
57
58
59
60
61
62
63
64
65

1
2
3
4 days did not enhance the volumetric gas production rate but substantially decreased
5
6 specific gas production rate. The average methane content in biogas was 50-52% at all
7
8 operational parameters except an OLR of 1.5 gVS/L-day and HRT 40 when methane
9
10 content decreased to 47%. This relatively low methane content is slightly lower but in
11
12 agreement with the theoretically predicted value (Table 3) and reflects rich carbohydrate
13
14 content in the LEA. As was shown before, carbohydrates have the lowest methane
15
16 potential compared to proteins and lipids ([Bohutskyi et al., 2014a](#)). The observed biogas
17
18 and methane yields were nearly 45-55% lower than the theoretically calculated values
19
20 (Table 4). The highest ratio was detected for the OLR 1.0 gVS/L-day and lowest for the
21
22 OLR of 1.5 gVS/L-day (both at HRT 40). Interestingly, VSR rates (Table 4) followed the
23
24 same trend but were about 15% higher than the ratio of observed to theoretical biogas
25
26 production rates. An explanation for this difference is that the Buswell empirical formula
27
28 (Equation 1) may overestimate the methane and biogas potential because it does not
29
30 account for organic carbon assimilated into microbial cells. Additionally, it may be
31
32 evidence of minor gas leakage. In general, the measured specific methane production 0.18-
33
34 0.25 L/gVS is similar or slightly lower than the previously reported methane yields, which
35
36 were in the range 0.2-0.35 L/gVS ([Bohutskyi et al., 2014b](#); [Hernandez et al., 2014](#); [Zhao et al., 2014](#)).
37
38 However, the higher values were collected by applying biomethane potential
39
40 procedure using defined anaerobic medium amended with nutrients, vitamins, pH, redox
41
42 buffers and providing long residence time at batch conditions.
43
44
45
46
47
48
49
50
51
52

53 Comparison of the maximum theoretical amount of energy that can be produced to
54
55 actual production of energy as biodiesel and methane achieved in the current study as well
56
57 as that of a projected optimistic scenario is shown in Figure 3. First, the important
58
59
60
61
62
63
64
65

1 observation is that conversion of whole biomass into methane alone through AD is more
2
3
4 energy favorable based on the theoretical maximum than production of biodiesel alone.
5
6
7 The projected benefit for methane over biodiesel is 43%, 9% and 20% higher for
8
9 theoretical maximum, observed and optimistic energy production scenarios, respectively.
10
11
12 AD converts all fractions of biomass (lipids, proteins, and carbohydrates) into methane and
13
14 minimizes generation of residues. However, an integrated biodiesel and methane process
15
16 generates the highest amount of energy with an increase of 46%, 31% and 40% in total
17
18 energy production over biodiesel in theoretical maximum, observed and optimistic
19
20 scenarios, respectively. Moreover, production of liquid fuel is more advantageous from an
21
22 economic point of view. Another important observation from these data is that the
23
24 observed energy recovered is substantially lower than the theoretical maximum. The
25
26 theoretical maximum scenario presumes conversion of all lipids into biofuel. However,
27
28 only the easily convertible TAGs fraction was processed into biodiesel under the current
29
30 scenario. The decrease in energy yield for AD is even more dramatic and caused mostly by
31
32 incomplete conversion of algal biomass into biogas. As discussed earlier, the
33
34 biodegradability may be limited by cell wall and algal protein recalcitrance. The methane
35
36 production was significantly lower than maximum predicted methane yield (Table 4). Both
37
38 these factors caused 40% and 32% reductions, respectively, in energy yield under the
39
40 current scenario. Future research (included in the optimistic prospective scenario) should
41
42 focus on optimization of technology to convert all lipid fractions (including polar lipids
43
44 and waxes) into fatty acid methyl esters to maximize fuel yield. In addition, the AD
45
46 efficiency and methane yield have to be improved from observed 50% to at least 80%.
47
48
49 While our lab showed previously that thermal and thermo-chemical pretreatments can
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 increase methane yield from *Nannochloropsis* biomass ([Bohutskyi et al., 2014a](#)), it would
5
6 be beneficial to develop less energy consuming and more environmentally friendly
7
8 pretreatment methods. Finally, it was shown that triacylglycerol by-products could be used
9
10 as AD substrate enhancing energy yield from algal biomass even further ([Siles Lopez et](#)
11
12 [al., 2009](#)). Implementation of these advances could allow increasing the energy production
13
14 by nearly 30% from 20 MJ/kgVS achieved in the current study to 26 MJ/kgVS if all lipid
15
16 fractions are processed into biodiesel and LEA together with other by-products are
17
18 effectively converted into methane through anaerobic digestion.
19
20
21
22

23 **3.3. Nutrient recovery and availability for recycling**

24
25 The concentration of the elements in the liquid fraction of the AD effluent was determined
26
27 in order to determine the fraction released from the LEA which became available for
28
29 recycling to the algal cultivation stage. The content of soluble macro- and micronutrients at
30
31 different operational conditions is shown in Figure 4.
32
33

34
35 The concentration of the soluble nitrogen increased with increasing the OLR from 1
36
37 to 2 gVS/L-day during Run 2 (Figures 4 A). Most of the soluble nitrogen was measured as
38
39 ammonia. Nitrogenous compounds, mostly proteins, present in the LEA were biologically
40
41 converted by anaerobic microorganisms into biogas and soluble ammonia nitrogen.
42
43 Additionally, the extension of the HRT from 20 to 40 days resulted in minor increases in
44
45 the concentration of soluble nitrogen reflecting the enhancement of biological degradation
46
47 of proteins with increasing HRT. These data are in agreement with the biogas and methane
48
49 production discussed in the previous section. However, increasing the OLR from 1 to 1.5
50
51 gVS/L-day at HRT 40 days (Run 4) didn't result in further elevation of the soluble
52
53 nitrogen concentration, possibly due to process inhibition considered earlier. The soluble
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 nitrogen represented only up to 40% of the nitrogen supplied with LEA (Figure 5 A). The
5
6 rest of the nitrogen was likely trapped in recalcitrant organic matter, assimilated by the
7
8 anaerobic microorganisms or precipitated as struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$). It was reported
9
10 earlier that a significant fraction of the *Chlorella*'s cell wall may be composed of
11
12 nitrogenous bio-polymers and extraordinary robust sporopollenin components as part of
13
14 trilaminar wall structure ([Atkinson et al., 1972](#); [Burczyk et al., 1999](#)). Moreover, Takeda
15
16 showed that a chitin-protein complex, resistant to enzymatic digestion (cellulase, chitinase,
17
18 and glucosylase) and acid hydrolysis, made up to 40% of the cell wall in some *Chlorella*
19
20 *protothecoides* species ([Conte & Pore, 1973](#)). Similarly, degradation of nearly 30% of the
21
22 particulate protein was observed during anaerobic digestion of bacterial biomass ([Bougrier](#)
23
24 [et al., 2007](#)). The precipitation as struvite is most likely only responsible for a small
25
26 amount of ammonia loss since the pH ~7 is not favorable for struvite formation ([Nelson et](#)
27
28 [al., 2003](#)). Therefore, the presence of these bio-polymers might be partially responsible for
29
30 relatively low level of nitrogen recovery.
31
32

33
34 The concentration of soluble phosphorus increased along with increasing of OLR and
35
36 HRT (Figure 4 B). Orthophosphate represented the major fraction of the soluble
37
38 phosphorus. However, the fraction of orthophosphate in total soluble P was reduced at
39
40 higher OLRs and especially during Run 4. The phosphorus in LEA feedstock is present
41
42 mostly as orthophosphate and organic phosphorus compounds such as phosphorylated
43
44 proteins, phospholipids, phosphosaccharides (*e.g.* ATP and phosphoglycans) and nucleic
45
46 acids. Organic P is mineralized to orthophosphate during anaerobic digestion and might be
47
48 precipitated as metal phosphates or struvite as discussed above. Interestingly, the amount
49
50 of P released from the LEA that remained soluble varied from 30 to 60% of total LEA
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

phosphorus. The soluble fraction increased when more of the P is present in the form of organic phosphorus with lower reactivity compared to orthophosphate. These values are somewhat higher than the previously reported value for soluble orthophosphate fraction, nearly 20% at similar phosphorus load for AD treating sewage sludge ([Carliell & Wheatley, 1997](#)). This difference might be due to a lower concentration of phosphorus and metals in LEA.

The remaining macro- and micronutrients could be classified into several groups based on the concentration response pattern to changes in the AD operation conditions. The first and largest group includes Mn, Zn, Fe, Co, Ni, Cr, As, Tl, Cd, Pb, Ag, In and Cs. The soluble concentration of these elements didn't vary and remained either constant or changed insignificantly at all operational conditions, potentially due to limited solubility of these elements in the aqueous phase. The only exception was a spike in the concentration of Zn, Co, Ni, Cr, Cd, Pb, and Ag at an OLR 1.5 gVS/L-day and HRT 40 days. One possible reason is the accumulation of the organic compounds in the soluble phase due to inhibition of the mineralization process. The decrease in pH value indicates a rising content of short chain fatty acids (SCFA) because of methanogen inhibition (discussed in previous subsection). Additionally, the amount of polyphosphates in the medium increased too (Figure 4 B). Both, SCFA and polyphosphates are soluble and positively charged. They may serve as organic ligands stabilizing positively charged metal ions in the solution. A similar elevation in Cr, Zn, Ni and Co concentration in the inhibited AD bioreactor with the buildup of polyphosphate content was reported earlier ([Carliell-Marquet & Wheatley, 2002](#)). The second group of elements includes Mg, Ca, S, B, Rb and Sr. The concentration of these elements increased with increasing OLR. Interestingly, the extension of the HRT

1
2
3
4 from 20 to 40 days negatively impacted the soluble concentrations of S and Sr. One
5
6 possible explanation could be their slower precipitation kinetics compared to the elements
7
8 from the first group. Finally, a third group is comprised of Mo, Cr, Ni, Be, Ba, and V.
9
10 Their soluble concentrations decreased as the OLR and HRT were increased. Most metals
11
12 precipitate as Fe/Mn oxides and sulfides ([Dabrowska, 2011](#)) leading to stronger
13
14 precipitation when more Fe/Mn and sulfides are present in the AD. In addition, adsorption
15
16 and co-precipitation on newly generated sulfide precipitates may be an important
17
18 mechanism for limiting the solubility of other elements.
19
20
21
22

23
24 Only a few elements, including Mg, Ca and S, in addition to N and P showed a strong
25
26 potential for recycling with the liquid fraction of the AD effluent. Their soluble fraction
27
28 varied between 20 and 50% depending on the operational conditions. These elements are
29
30 essential for microalgae. Along with other functions magnesium serves as a stabilization
31
32 and coordination factor in various enzymes and light-harvesting pigments including
33
34 chlorophyll, it is a mediator in many signaling pathways and involved in gene transcription
35
36 and translation of genes ([Wilkinson et al., 1990](#)). Sulfur is a component of many amino
37
38 acids and one of the key parts in electron transport within the photosynthetic machinery
39
40 ([Haehnel, 1984](#)). Calcium is vital for regulation of metabolism through a variety of Ca^{2+} -
41
42 binding proteins ([Allan & Trewavas, 1987](#)). The soluble fractions of Mn and Fe were
43
44 between 10-25% and 4-15%, respectively. Manganese is another trace element especially
45
46 important to photosynthetic organisms, such as microalgae, since it is a component of the
47
48 water splitting polynuclear cluster in photosystem II ([Raven et al., 1999](#)). Only up to 5% of
49
50 Cu, Zn, Mo and Co could be recovered from the LEA with digestate for potential
51
52 recycling. However, these elements are also essential for augmented algal growth. Copper
53
54
55
56
57
58
59
60
61
62
63
64
65

is part of the photosynthetic and mitochondrial electron transport chain and zinc serves as a co-factor in various metalloenzymes, such as in carbonic anhydrase necessary for efficient hydration of dissolved CO₂ in solution ([Raven et al., 1999](#)). Molybdenum and cobalt are also important co-factors or parts of essential organometallic compounds such as nitrate reductase ([Fernandez & Cardenas, 1981](#)) and vitamin B₁₂ ([Banerjee & Ragsdale, 2003](#)), respectively. It would be beneficial to increase the soluble fraction of these elements in the AD effluent for more efficient recovery. Importantly, up to 30-50% of the potentially toxic elements, including As, Tl, Cd, Pb, Ba, Be and Cs, remain soluble at certain operating conditions. In contrast to nutrients discussed previously, it would be advantageous to reduce their soluble fraction in the AD effluent since there is a potential for a build up in their concentrations in the algal production system if continuous recycling is routinely used. Moreover, further studies on the potential release of these pollutants into the environment with the solid fraction of the AD effluent would be highly relevant for a more complete understanding of the environmental sustainability of emerging algal biofuel technologies.

3.4. Economic and sustainability effects from the nutrient recycling

The economical importance of the nutrient recycling from AD effluent and its significance for process sustainability were predicted by estimating their amounts and costs per gallon of the generated biodiesel. Then, the nutrient amounts for scaling algal biofuel production to 10% of liquid fuel consumption in the U.S. (29 billion gallons per year, BGY) were estimated in order to evaluate the impact of nutrient recycling on sustainability of algal biofuel production. These calculations account only for nutrients supplied and assimilated by microalgae during the cultivation process leaving out unutilized nutrients remaining in

1
2
3
4 the spent growth medium or used for algal growth during heterotrophic conditions with
5 glucose. The first scenario without nutrient recycling was compared to the second scenario
6
7 that included nutrient recovery through recycling of the AD effluent after LEA conversion.
8
9

10
11 The costs of essential macro- and micronutrients per gallon of the produced algal
12 biodiesel are shown in Figure 6 A. Even without recycling the total nutrient cost represents
13
14 \$0.165 per gallon of algal biodiesel or 4-5.5% of the desired biofuel price of \$3-4 per
15
16 gallon. Remarkably, the costs of nitrogen, phosphorus and potassium (\$0.089, \$0.034 and
17
18 \$0.03 per gallon respectively) represent the major fraction of the overall nutrient cost in
19
20 algal biomass during cultivation. The contribution from all other nutrients is slightly higher
21
22 than \$0.01 per gallon. However, scaling algal biofuel production to 10% of the U.S. liquid
23
24 fuel consumption is a challenge since it would require enormous amounts of fertilizers,
25
26 comparable to the current consumption of these elements for the total agricultural needs in
27
28 the U.S. (Figure 6 B). The demand for nitrogen, phosphorus, potassium and magnesium
29
30 fertilizers would increase by nearly 15-25% compared to their current use in agriculture.
31
32 Significant elevation of the demand for fertilizers like nitrogen, phosphorus and potassium
33
34 can boost their price and therefore the prices for all agricultural products, including food
35
36 and even produced biodiesel. Notably, recycling of AD effluent could potentially reduce
37
38 the nutrient expenditure by 45% to \$0.092 per gallon of algal biodiesel. But most
39
40 importantly, implementation of nutrient recycling could decrease the requirement for
41
42 nitrogen, phosphorus, potassium and magnesium fertilizers for algal cultivation to nearly
43
44 6-16% of current consumption of these elements for agricultural needs in the U.S.
45
46 (Figure 6 B). Further optimization of AD process and nutrient recovery from AD effluent
47
48 could further enhance process efficiency and sustainability and the associated costs.
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Conclusions

The methane production from lipid-extracted algal residues (LEA) was up to 0.25 L/gVS increasing the energy yield from algal biomass by more than 30%. Additionally, up to 40-60% of N and P, 30-60% of Mg, Ca, and S, and 15-25% of Mn and Fe present in the LEA could be available for recycling with AD effluent. As N, P and K represent the main cost of nutrients supplied to a cultivation system, their recovery from LEA and recycling can reduce fertilizer costs by 45% and improve the process sustainability especially for a scaled-up process.

Acknowledgements

This project was financially supported by the U.S. NSF CBET Program (Grant No.1236691 to JHU), U.S. DOE CCS Program (Grant No.DE-FE0001888 to Phycal) and The Bureau of Education and Cultural Affairs of U.S. Department of State through an International Fulbright Science and Technology Award to Pavlo Bohutskyi. The authors are also grateful to Dr. George Oyler and Julian Rosenberg (Clean Green Chesapeake) for providing bioreactor equipment for the current study.

References

- [1] Aeckersberg, F., Bak, F., Widdel, F. 1991. Anaerobic oxidation of saturated hydrocarbons to CO₂ by a new type of sulfate-reducing bacterium. *Arch. Microbiol.*, 156 (1991), 5-14.
- [2] Allan, E.F., Trewavas, A.J. 1987. The role of calcium in metabolic control. in: *The biochemistry of plants*, (Eds.) P.K. Stumpf, E.E. Conn, Vol. 12, Academic Press. San Diego, USA.
- [3] Atkinson, A.W., Gunning, B.E.S., John, P.C.L. 1972. Sporopollenin in the cell wall of *Chlorella* and other algae: Ultrastructure, chemistry, and incorporation of ¹⁴C-acetate, studied in synchronous cultures. *Planta*, 107 (1972), 1-32.
- [4] Banerjee, R., Ragsdale, S.W. 2003. The many faces of vitamin B₁₂: catalysis by cobalamin-dependent enzymes. *Annu. Rev. Biochem.*, 72 (2003), 209-247.
- [5] Bohutskyi, P., Betenbaugh, M.J., Bouwer, E.J. 2014a. The effects of alternative pretreatment strategies on anaerobic digestion and methane production from different algal strains. *Bioresour. Technol.*, 155 (2014), 366-372.
- [6] Bohutskyi, P., Bouwer, E. 2013. Biogas Production from Algae and Cyanobacteria Through Anaerobic Digestion: A Review, Analysis, and Research Needs. in: *Advanced Biofuels and Bioproducts*, (Ed.) J.W. Lee, Springer New York, pp. 873-975.
- [7] Bohutskyi, P., Kula, T., Kessler, B., Hong, Y., Bouwer, E., Betenbaugh, M., Allnutt, F.C.T. 2014b. Mixed Trophic State Production Process for Microalgal Biomass with High Lipid Content for Generating Biodiesel and Biogas. *Bioenergy Res.*, 7 (2014), 1174-1185.
- [8] Bohutskyi, P., Liu, K., Kessler, B., Kula, T., Hong, Y., Bouwer, E., Betenbaugh, M., Allnutt, F.C.T. 2014c. Mineral and non-carbon nutrient utilization and recovery during sequential phototrophic-heterotrophic growth of lipid-rich algae. *Appl. Microbiol. Biotechnol.*, 98 (2014), 5261-73.
- [9] Bougrier, C., Delgenès, J.P., Carrère, H. 2007. Impacts of thermal pre-treatments on the semi-continuous anaerobic digestion of waste activated sludge. *Biochem. Eng. J.*, 34 (2007), 20-27.
- [10] Burczyk, J., Smietana, B., Terminska-Pabis, K., Zych, M., Kowalowski, P. 1999. Comparison of nitrogen content amino acid composition and glucosamine content of cell walls of various *Chlorococcalean* algae. *Phytochem.*, 51 (1999), 491-497.
- [11] Buswell, A.M., Mueller, H.F. 1952. Mechanism of Methane Fermentation. *Ind. Eng. Chem.*, 44 (1952), 550-552.
- [12] Carliell, C., Wheatley, A. 1997. Metal and phosphate speciation during anaerobic digestion of phosphorus rich sludge. *Water Sci. Technol.*, 36 (1997), 191-200.
- [13] Carliell-Marquet, C.M., Wheatley, A.D. 2002. Measuring metal and phosphorus speciation in P-rich anaerobic digesters. *Water Sci. Technol.*, 45 (2002), 305-12.
- [14] Chen, Y., Cheng, J.J., Creamer, K.S. 2008. Inhibition of anaerobic digestion process: A review. *Bioresour. Technol.*, 99 (2008), 4044-4064.
- [15] Conte, M.V., Pore, R.S. 1973. Taxonomic implications of *Prototheca* and *Chlorella* cell wall polysaccharide characterization. *Arch. Mikrobiol.*, 92 (1973), 227-233.
- [16] Dabrowska, L. 2011. Speciation of heavy metals in sewage sludge after mesophilic and thermophilic anaerobic digestion. *Chem. Pap.*, 66 (2011), 598-606.

- [17] Eaton, A.D., Franson, M.A.H. 2005. *Standard Methods for the Examination of Water & Wastewater*. 21 ed. APHA, AWWA, and WEF, New York.
- [18] Eder, B., Heinz, S. 2006. *Biogas Praxis: Grundlagen, Planung, Anlagenbau, Beispiele, Wirtschaftlichkeit*. Ökobuch Verlag u. Versand, Staufen, Deutschland
- [19] Fernandez, E., Cardenas, J. 1981. Occurrence of xanthine dehydrogenase in *Chlamydomonas reinhardtii*: A common cofactor shared by xanthine dehydrogenase and nitrate reductase. *Planta*, 153 (1981), 254-257.
- [20] Gao, Y., Yang, M., Wang, C. 2013. Nutrient deprivation enhances lipid content in marine microalgae. *Bioresour. Technol.*, 147 (2013), 484-491.
- [21] Haehnel, W. 1984. Photosynthetic Electron Transport in Higher Plants. *Annual Review of Plant Physiol.*, 35 (1984), 659-693.
- [22] Hayward, G., Lau, I. 1989. Toxicity of organic solvents to fatty acid forming bacteria. *Can. J. Chem. Eng.*, 67 (1984), 157-161.
- [23] Hernandez, D., Solana, M., Riano, B., Garcia-Gonzalez, M.C., Bertucco, A. 2014. Biofuels from microalgae: Lipid extraction and methane production from the residual biomass in a biorefinery approach. *Bioresour. Technol.*, 170 (2014), 370-378.
- [24] Nelson, N.O., Mikkelsen, R.L., Hesterberg, D.L. 2003. Struvite precipitation in anaerobic swine lagoon liquid: effect of pH and Mg:P ratio and determination of rate constant. *Bioresour. Technol.*, 89 (2003), 229-236.
- [25] Pate, R., Klise, G., Wu, B. 2011. Resource demand implications for U.S. algae biofuels production scale-up. *Appl. Energy*, 88 (2011), 3377-3388.
- [26] Playne, M.J., Smith, B.R. 1983. Toxicity of organic extraction reagents to anaerobic bacteria. *Biotechnol. Bioeng.*, 25 (1983), 1251-1265.
- [27] Raven, J.A., Evans, M.C.W., Korb, R.E. 1999. The role of trace metals in photosynthetic electron transport in O₂-evolving organisms. *Photosynth. Res.*, 60 (1999), 111-150.
- [28] Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U.C., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B. 2008. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. *Bioenergy Res.*, 1 (2008), 20-43.
- [29] Siles Lopez, J.A., Martin Santos, M.d.l.A., Chica Perez, A.F., Martín Martín, A. 2009. Anaerobic digestion of glycerol derived from biodiesel manufacturing. *Bioresour. Technol.*, 100 (2009), 5609-5615.
- [30] U.S. EIA. 2013. Monthly Energy Review, September 2013, DOE/EIA-0035(2013/09). Projections: AEO2014 National Energy Modeling System, run REF2014.D102413A. . U.S. EIA.
- [31] U.S. GS. 2014. *Mineral commodity summaries 2014*. U.S. GS.
- [32] Wilkinson, S., Welch, R., Mayland, H., Grunes, D. 1990. Magnesium in plants: uptake, distribution, function, and utilization by man and animals. in: *Met. Ions Biol. Syst.*, (Eds.) H. Siegel, A. Siegel, Vol. 26, Marcel Dekker, Inc. New York, pp. 33-56.
- [33] Yu, W.-L., Ansari, W., Schoepp, N.G., Hannon, M.J., Mayfield, S.P., Burkart, M.D. 2011. Modifications of the metabolic pathways of lipid and triacylglycerol production in microalgae. *Microb. Cell Fact.*, 10 (2011), 91.

- 1
2
3
4 [34] Zhang, J., Wei, Y., Xiao, W., Zhou, Z., Yan, X. 2011. Performance and spatial
5 community succession of an anaerobic baffled reactor treating acetone–butanol–
6 ethanol fermentation wastewater. *Bioresour. Technol.*, 102 (2011), 7407-7414.
7
8 [35] Zhao, B., Ma, J., Zhao, Q., Laurens, L., Jarvis, E., Chen, S., Frear, C. 2014. Efficient
9 anaerobic digestion of whole microalgae and lipid-extracted microalgae residues
10 for methane energy production. *Bioresour. Technol.*, 161 (2014), 423-430.
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

List of Tables

Table 1. Bioreactor operational parameters

Table 2. Physicochemical composition of *Auxenochlorella protothecoides* whole algae (WA) and lipid-extracted residues (LEA) as percent of dry weight

Table 3. Theoretically calculated maximum yields of biogas, methane, ammonia and hydrogen sulfide per gram of substrate volatile solids (VS)

Table 4. Observed to theoretical methane production ratio and volatile solids reduction (VSR) depending from the operational parameters

List of Figures

Figure 1. Average volumetric (A) and specific (B) biogas and methane production rates at four different operational regimes.

Figure 2. Average volumetric (A) and specific (B) biogas and methane production rates at different operational parameters. Error bars represent the standard deviation among daily gas productions during the stable period.

Figure 3. Theoretical maximum, current and projected optimistic scenarios of energy production per 1 kgVS of algal biomass (total lipids 57%, TAGs 45% of dry weight).

Figure 4. Concentration of elements in the soluble fraction of the digestate at different operational parameters.

Figure 5. Fraction of elements concentrated in the soluble fraction of the digestate at different operational parameters.

Figure 6. (A) Cost of nutrients required for production one gallon of algal biodiesel. (B) Nutrient amount required for generation of algal biodiesel equal to 10% of the current liquid fuel consumption in the U.S., as part of their current use in agriculture.

Table 1. Bioreactor operational parameters

Run	Feed, gVS/L	HRT, days	OLR, gVS/L-day
1	19.4±1.2	20	0.97±0.06
2	40.9±1.5	20	2.05±0.08
3	38.0±2.0	40	0.95±0.05
4	61.6±2.2	40	1.54±0.06

Table 2. Physicochemical composition of *Auxenochlorella protothecoides* whole algae (WA) and lipid-extracted residues (LEA) as percent of dry weight

Parameter	WA ^a	LEA ^b	Parameter	WA	LEA
Ash	4±1	6±2	Aluminum	0.6 x 10 ⁻³	2.4 x 10 ⁻³
VS ^c	96±1	94±2	Tellurium	0.12 x 10 ⁻³	0.34 x 10 ⁻³
Total lipid	57	n/a ^d	Molybdenum	0.1x10 ⁻³	0.28x10 ⁻³
TAGs ^e	45	n/a	Cobalt	0.027x10 ⁻³	0.13x10 ⁻³
Carbohydrates	33	75	Boron	0.03 x 10 ⁻³	0.55 x 10 ⁻³
Crude protein	6	14	Chromium	0.07 x 10 ⁻³	0.22 x 10 ⁻³
Carbon	59	44.4	Rubidium	0.06 x 10 ⁻³	2.9 x 10 ⁻³
Oxygen	26	39	Strontium	0.04 x 10 ⁻³	2.4 x 10 ⁻³
Hydrogen	9.6	7.1	Barium	0.02 x 10 ⁻³	0.07 x 10 ⁻³
Nitrogen	1.5	2.3	Nickel	0.02 x 10 ⁻³	0.68 x 10 ⁻³
Phosphorus	0.4	1.2	Vanadium	0.02 x 10 ⁻³	0.11 x 10 ⁻³
Calcium	0.15	0.42	Silver	0.016 x 10 ⁻³	0.08 x 10 ⁻³
Sulfur	0.07	0.185	Arsenic	0.01 x 10 ⁻³	0.05 x 10 ⁻³
Magnesium	0.04	0.11	Indium	0.004 x 10 ⁻³	0.024 x 10 ⁻³
Iron	0.012	0.043	Lead	0.005 x 10 ⁻³	0.024 x 10 ⁻³
Zinc	0.78 x 10 ⁻³	22 x 10 ⁻³	Cadmium	0.008 x 10 ⁻³	0.03 x 10 ⁻³
Copper	0.65 x 10 ⁻³	2 x 10 ⁻³	Beryllium	0.007 x 10 ⁻³	0.01 x 10 ⁻³
Manganese	0.8 x 10 ⁻³	4.5 x 10 ⁻³	Cesium	0.002 x 10 ⁻³	0.006 x 10 ⁻³

^a WA – whole algae; ^b LEA – lipid extracted algae; ^c VS – volatile solids; ^d n/a – not available; ^e TAGs – triacylglycerols.

Table 3. Theoretically calculated maximum yields of biogas, methane, ammonia and hydrogen sulfide per gram of substrate volatile solids (VS)

Substrate type and chemical formula	Carbon oxidation state	C:N (wt/wt)	Theoretically Calculated Values				
			biogas L/gVS	CH ₄ L/gVS	CH ₄ %	NH ₃ mgN/gVS	H ₂ S mgS/gVS
<i>A. protothecoides</i> (WA) C ₂₂₅₈ H ₄₃₈₃ O ₇₃₃ N _{50.4} S	-1.23	38.5	1.15	0.75	65	16.1	0.7
<i>A. protothecoides</i> (LEA) C ₆₄₁ H ₁₂₂₇ O ₄₃₃ N _{28.6} S	-0.43	19.2	0.88	0.48	55	24.6	2.0
Glycerol C ₃ H ₈ O ₃	-0.67	n/a	0.73	0.43	58	n/a	n/a

Table 4. Observed to theoretical methane production ratio and volatile solids reduction (VSR) depending from the operational parameters

Run	Organic loading rate (OLR) and hydraulic retention time (HRT)	Obs./Theor., %		VSR, %
		biogas	methane	
1	OLR 1.0 gVS/L-d; HRT 20 d	48±5	45±5	64±5
2	OLR 2.0 gVS/L-d; HRT 20 d	43±4	38±4	59±4
3	OLR 1.0 gVS/L-d; HRT 40 d	55±5	53±4	73±6
4	OLR 1.5 gVS/L-d; HRT 40 d	34±3	30±4	55±6

Figure 1. Average volumetric (A) and specific (B) biogas and methane production rates at four different operational regimes.

Figure 2. Average volumetric (A) and specific (B) biogas and methane production rates at different operational parameters. Error bars represent the standard deviation among daily gas productions during the stable period.

Figure 3. Theoretical maximum, current and projected optimistic scenarios of energy production per 1 kgVS of algal biomass (total lipids 57%, TAGs 45% of dry weight).

Figure 4. Concentration of elements in the soluble fraction of the digestate at different operational parameters.

Figure 5. Fraction of elements concentrated in the soluble fraction of the digestate at different operational parameters.

Figure 6. (A) Cost of nutrients required for production one gallon of algal biodiesel. (B) Nutrient amount required for generation of algal biodiesel equal to 10% of the current liquid fuel consumption in the U.S., as part of their current use in agriculture.