EVALUATION OF THE FEASIBILITY OF THE ALGAE CULTIVATION-EXTRACTION-DIGESTION-RECULTIVATION PROCESS TO RECYCLE NUTRIENTS AND DETERMINE THE NITROGEN BALANCES AND IMPACTS IN THE PROCESS

by

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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Abstract

Nutrient supply is one of the critical obstacles limiting algal biofuel industrialization. It has been demonstrated that anaerobic digestion is an effective method to release nitrogen and phosphorus from algal biomass, but the nutrient recycle system of cultivation-extraction-digestion-recultivation has not been completely evaluated. In this dissertation, algae were cultivated on nutrients released from anaerobic digestion of lipid extracted algae. Algae recovery was calculated to demonstrate the feasibility of the nutrient recycle process. The quality of recultivated algae for algal biofuel was validated and the limiting nutrient was analyzed. Initial nitrogen concentration was shown to have significant impact on the entire process including algae and lipid productivity, as well as lipid and nitrogen content of the biomass. The nitrogen balance for each step of the entire process was closed within experimental error. Total nitrogen recoveries throughout the whole process were all approximately 65% regardless of the different initial nitrogen concentration. The biggest nitrogen losses occurred in lipid extraction and algae cultivation steps. The work described in this dissertation demonstrates the effectiveness of this nutrient recycle system, and therefore provides a promising method to significantly lower nutrient cost for algal biofuel production. Nitrogen analysis validates the experimental and analytical methods in this dissertation and provides points of future work to further optimize this recycle process.

Chapter One: Introduction

1. Algal biofuel

It is widely known that the demand for energy will continue to increase throughout the next decades, leading to a continuous rise in fossil fuel demand. The decline of the adequacy of fossil fuels has driven substantial investments in renewable alternative energy. Another more critical driving force for investment in renewable energy is the rise of greenhouse gas emissions and the resulting global warming issues [Malik et al., 2015]. Biofuel is one form of renewable alternative energy that has received significant attention throughout the past decades because of its environmental benefits. In recent years, the US Department of Energy (DOE) established programs with the goal of substituting 30% of transportation fuel with biofuel [Zhou et al., 2014]. The majority of the current biofuel is extracted from oil crops or bioethanol from sugar cane or corn. However, there always remains a dispute on whether crop biofuel could economically compete with fossil fuels [Pittman et al., 2011]. There are also concerns on the possible negative effects that biofuels may have on agriculture [Chisti, 2013]. Therefore, to avoid the negative impacts of crop biofuels, investments have been made in biofuel derived from algal biomass.

For a long time, several researchers have proposed the possibility of using algae as a renewable alternative fuel source [Benemann et al., 1977; Oswald and Golueke, 1960; Leite et al., 2013]. Algal biofuel is a third-generation biofuel that has certain advantages over the first- and second-generation biofuels [Voloshin et al., 2016]. First-generation biofuels are typically derived from food crops, e.g., corn. These biofuels can compete with agriculture and can negatively impact food supplies. Lignocellulose and forest residues produce second-generation biofuels. These biofuels can avoid the competition with food; however, an efficient conversion process has not yet been established. Additionally, both the first- and second-generation biofuels require large amounts of land and hence land use change must be evaluated [Vassilev and Vassileva, 2016]. Algal biofuel eliminates the disadvantages of the first- and second-generation biofuels and adds a number of other advantages as follows:

1. Algae grow in both fresh and saline water and do not compete for land use compared with terrestrial crops. A variety of algal classes have been identified. These algae have been grown in different environments. Moreover, algae are less environmentally sensitive than terrestrial crops. Therefore, algae can meet the land requirements for global cultivation [Ullah et al., 2015; Demirbas, 2010].

2. Algae adapt more easily to different climate and environmental conditions. More importantly, algae can grow in wastewater. Investment in the use of algae to remove nutrients has attracted significant attention in recent years. A number of technical advancements in the upstream/downstream processes as well as algal biofuel production have been made. Although energy consumption limits the cost effectiveness of combining cultivation to produce biofuels with wastewater treatment, future advancements and progress are expected to overcome these limitations [Bharathiraja et al., 2015].

3. Algae grow much faster than terrestrial crops and therefore have much higher productivity. Algae grow 20–30 times faster than agricultural crops and have the potential to produce 30 times more fuel than that produced using other biofuel resources (e.g., soybean, canola, or palm oil) [Ullah et al., 2015]. This is particularly true for microalgae, which grows faster than macroalgae and has a doubling time as short as 6–12 h, with a harvesting cycle of only 1–10 days [Sambusiti et al., 2015; Ziolkowska and Simon, 2014].

4. Algae have higher solar energy conversion efficiency compared with other terrestrial crops (3%–8% for algae compared with 0.2%–2% for crops) [Stephenson et al., 2011].

5. Algae have high oil content, especially some special species, such as *Chlorella sorokiniana* (oil yield from algae is 20,000–80,000 liter per acre, i.e., 7–31 times greater than that from terrestrial crops) [Demirbas and Demirbas, 2011].

6. Algae are highly efficient consumers of greenhouse gases, converting CO_2 into O_2 and liberating extra oxygen into the atmosphere. This is beneficial considering the current global warming issues [Demirbas, 2011].

However, there still are several constraints that limit the industrialization of algal biofuel. The major concern is related to the production cost and cost efficiency. For example, the production cost of microalgae is approximately 5–7 times higher than that of lignocellulosic biomass [Vassilev, 2016; Huber, 2006]. Dutta et al. (2016) estimated that the minimum fuel prices for algal biofuel could vary from $4.35/GGE to $10.55/GGE. Nevertheless, progress continues in the field of algal biofuel. Comparative studies on different technologies and modeling approaches have continued to lower the estimated fuel price [Chiaramonti et al., 2015].

Other disadvantages include the cost of CO_2, nitrogen, and phosphorus supplies, sources of water, land footprint, and lack of long-term productivity studies [Chisti, 2013]. Nutrient supply is a critical obstacle to efficient production of algal biofuel. For example, the amount of nitrogen and phosphorus required for any significant scale of algal biomass production may not currently be available. Producing an

estimated amount of algal biomass, i.e., 82 million tons per year requires ~5.4 million tons of N per year, which accounts for nearly 44% of the total yearly N consumption in the US in 2010. Similarly, algal biofuel production will consume 27.5% of the available phosphorus [Chisti, 2013]. Investing such amounts of nitrogen and phosphorous into fuel production would negatively affect agriculture industries. Thus, establishing an effective nutrient supply is necessary for producing algae prior to large-scale commercialization.

2. Nutrient recycling via anaerobic digestion

There are two potential solutions to problems associated with the high amounts of nutrients consumed to produce algal biofuel. Wastewater provides an available source of nutrients for producing algae. The application of wastewater reduces the dependence on chemicals by providing a natural source of nutrients, rendering algal biofuel more cost effective [Mallick, 2002; Chen et al., 2015]. However, the limited nitrogen in wastewater can only make a limited contribution to supplying nutrients. In an ideal scenario, algal biofuel, which uses nutrients from wastewater, can only support 1% of the petroleum demand of a large US city [Chisti, 2013].

Another method is to recycle nutrients from lipid extracted algal biomass via anaerobic digestion. Anaerobic digestion produces biogas for electrical applications and therefore has the benefit of low energy costs [Sialve et al., 2009]. The anaerobic

digestion of algal biomass, particularly for *Chlorella* sp., has received attention for a long period of time. Golueke et al. (1957) conducted the earliest study on anaerobic digestion to investigate the feasibility of using algae in wastewater treatment processes. Many studies have reported on producing biogas from algal biomass, with methane yields ranging from 189 to 403 mL g^{-1} VSS for *Chlorella* sp. [Ward et al., 2014]. Biogas produced via anaerobic digestion typically contains 55%–75% methane and 25%–45% carbon dioxide [Harun et al., 2010], where captured methane can be burned in CHP (combined heat and power) units, used in natural gas power plants, or used as vehicle fuel [Jankowska et al., 2017].

Anaerobic digestion has been used for many years for wastewater treatment. The following important factors regulate the anaerobic digestion process:

1. Environmental conditions, including loading rate, temperature, and pH. High organic loading rates enhance biogas production with typical retention times of 15–30 days under laboratory conditions. In most cases, the temperatures used for anaerobic digestion range from 30 ℃ to 38 ℃ for mesophilic conditions and from 50 ℃ to 55 ℃ for thermophilic conditions. Temperatures must be maintained constant because rapid changes in temperature can cause VFAs (Volatile fatty acids) accumulation and impede methane production. The pH during anaerobic digestion is usually adjusted to between 7 and 8, which is optimal for methanogens. Since methanogens are highly

sensitive to pH, pH valuesbetween 7 and 8 enhance methanogen growth [Bohutskyi and Bouwer, 2012; Jankowska et al., 2017].

2. Cell wall degradability. The cell walls of algae, including *Chlorella* sp., can be rigid and cannot be easily biodegraded. The longest duration time for intact algae cell walls in anaerobic digestion is 6 months for *Scenedesmus sp*. [Mussgnug et al., 2010] The rigid cell wall resulted in incomplete biogas production and nitrogen release. Various pre-treatment methods, including the mechanical, physical, thermal, and chemical pre-treatments, have been investigated to enhance the cell wall breakup [Ward et al., 2014]. Microwave pre-treatment is also an effective pre-treatment method [Passos et al., 2015].

3. C/N ratio and ammonium toxicity. The C/N ratio during anaerobic digestion should be >20 to maintain the balance between C and N, but C/N ratios for algae species range from 4.16 to 7.82, which is far below this requirement. The elevated nitrogen content results in elevated ammonium release, which can inhibit methanogen production. Higher NH_4^+ concentrations result in higher NH_3 concentrations because of a shift of the balance between them and can therefore result in an increase in pH and temperature. Increased pH and temperature are fatal to these sensitive methanogens [Ward, 2014; Jankowska et al., 2017; Bohutskyi and Bouwer, 2012]. A two-stage separation process for methanogens wherein the methanogens are separated

in the second stage effectively solves this problem of sensitivity as well as enhances biogas production and nutrient release [Vergara-Fernandez et al., 2008; Yang et al., 2011].

To further enhance the effectiveness of anaerobic digestion, algae, particularly *Chlorella* sp., are often co-digested with wastewater or other co-substrates, e.g., waste-activated sludge, cooking oil, mill residue, or paper waste [Ras, 2010; Wang, 2015; Retfalvi, 2016]. Co-digestion with additional substrates effectively adjusts the C/N ratio and enhances methane production.

In addition to biogas production, nutrients are released during the anaerobic digestion process, which provides a source of nutrients for algae regrowth. Theoretical calculations show that the digestate (i.e., the effluent from anaerobic digesters) is rich in nutrients and is a nitrogen source for biomass production [Sialve et al., 2009]. Experimental research has also demonstrated that the digestate from whole cell *Chlorella* algae is rich in ammonia. *C. sorokiniana* contains ~40 mg of ammonium for every gram of algae [Mahdy et al., 2015; Frigon et al., 2013; Ayala-Parra et al., 2017]. More recently, a photobioreactor-microbial fuel cell (MFC) anaerobic digestion system was used to demonstrate the effectiveness of nutrient recycling and energy recovery via anaerobic digestion. [Schamphelaire and Verstraete, 2009] During the 100-day continuous digestion period, there were significant

increases in ammonium and phosphorus concentrations in the digester effluent compared with the influent.

Furthermore, there has been limited research on algae regrowth using nutrients from the digestate [Prajapati et al., 2014; Erkelens et al., 2014; E et al., 2016]. Furthermore, there is limited research on anaerobic digestion of lipid extracted *Chlorella* sp. This dissertation focuses on the cultivation–extraction–digestion–recultivation process for *C. sorokiniana*.

3. Nitrogen pathway

Nitrogen is a critical limiting nutrient for algal biofuel. In this thesis, the nitrogen impacts and balances are investigated for a cultivation-extraction-digestion-recultivation system, as is shown in figure 1.

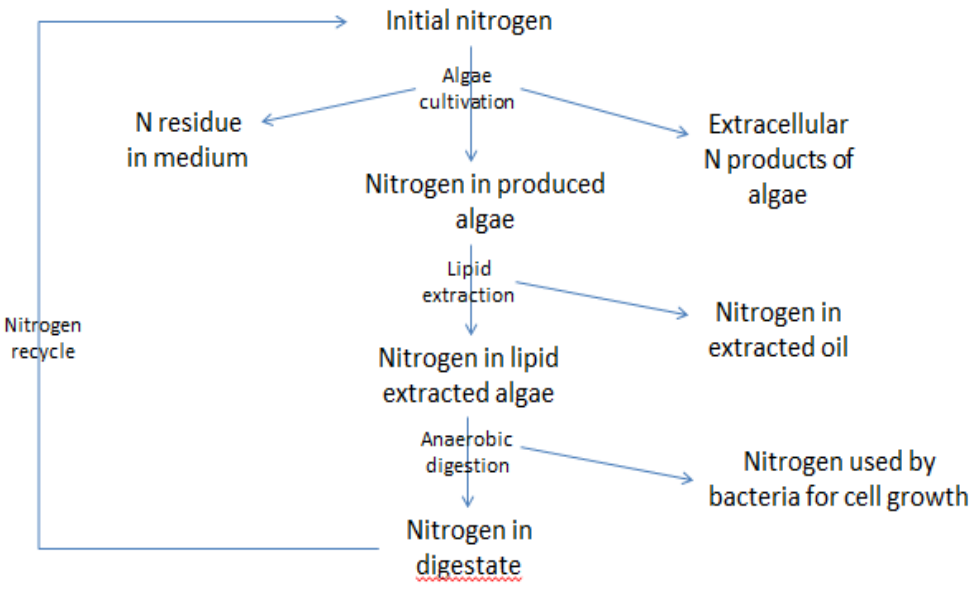

Figure 1. Scheme of process for nitrogen balance evaluation.

3.1 Summary of biological nitrogen cycle

3.1.1 Introduction to nitrogen cycle

In biology, nitrogen undergoes a variety of complex redox reactions. In the reactions, NO_3^-, NO_2^-, NO, N_2O, N_2, NH_4^+ and some other nitrogen containing compounds with oxidation states ranging from -3 to +5 can be involved and produced. These redox reactions in total form the nitrogen cycle in figure 2. As is shown in figure 2, the nitrogen cycle includes denitrification, nitrification, nitrogen fixation, assimilatory nitrate reduction, and anammox (Anaerobic ammonium oxidizing). Ammonia can be further absorbed and utilized by cells by the process of ammonia assimilation. [Bothe et al., 2007]

All reactions in the nitrogen cycle occurred in bacteria, archaea and some specialized fungi. Assimilatory NO_3^- reduction is the only exception in the nitrogen cycle that is also performed in plants. As is introduced in Section 3.4.1, plants do not absorb NO_3^- directly, but reduce it via nitrite into NH_4^+ before absorption. This process is called assimilatory nitrate reduction. In addition, NO_3^- can also serve as electron acceptor in denitrification reactions. In this pathway, NO_3^- is reduced to NO_2^- catalyzed by several different reductases, and then subsequently reduced to NO, N_2O and finally to N_2. A final nitrogen reduction process is nitrogen fixation. Several species of bacteria and archaea reduce N_2 to NH_4^+ to serve their N-requirements. N_2

fixation takes place not only in free-living prokaryotes but also in bacteria in symbiosis with plants. [Bothe et al., 2007]

In contrast to reduction reactions, some organisms can oxidize NH_4^+ or NO_2^- to meet their demand of energy. This pathway is termed "nitrification". The end product of this process is NO_3^-. Moreover, recently a pathway named anammox has been found to oxidize NH_4^+, reduce NO_2^- and produce end product of N_2. Because the end product is in the gaseous state, anammox is a promising pathway to remove nitrogen in wastewater treatment. [Bothe et al., 2007]

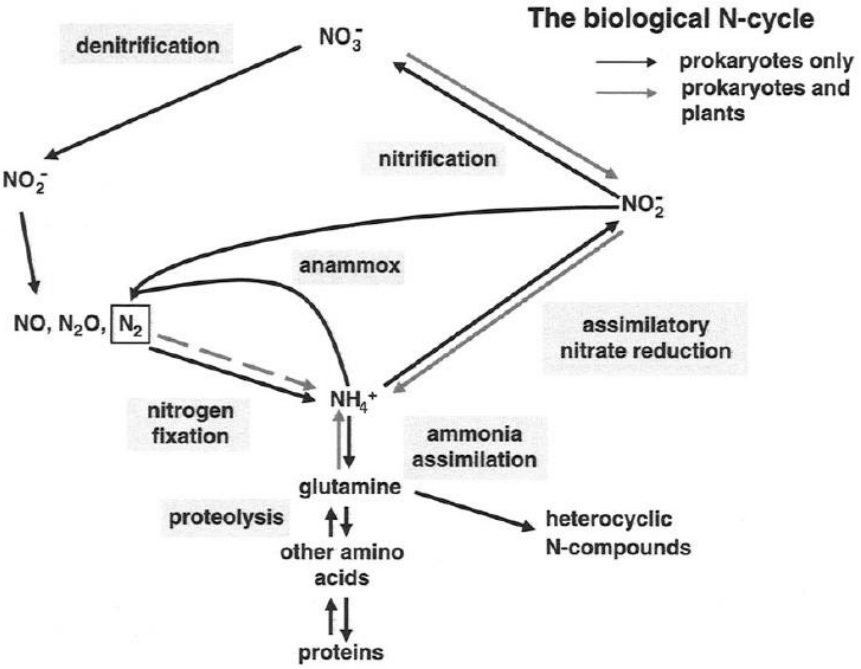

*Figure 2. Pathways of nitrogen cycle [*Bothe et al., 2007*]*

3.1.2 Denitrification

Denitrification is a reductive process that transforms NO_3^- to N_2 under an anaerobic environment. An example reaction using nitrate to degrade BOD, such as a five-carbon sugar, is shown in equation 3.1.2.1.

$$C_5H_{10}O_5 + 4NO_3^- + 4H^+ \rightarrow 5CO_2 + 7H_2O + 2N_2 \qquad (3.1)$$

[Gerardi MH, 2002]

The process of denitrification consists of four steps, NO_3^-- to NO_2^-, NO_2^- to NO, NO to N_2O and N_2O to N_2. In each step, more than one kind of reductase is involved. Figure 3 shows the detailed denitrification process in *Paracoccus denitrificans* (*Paracoccus denitrificans* is a coccoid bacterium known for its nitrate reducing properties). Different denitrifiers can have different enzymes in the process, but central reactions are similar. [Gerardi MH, 2002; Spanning et al., 2007]

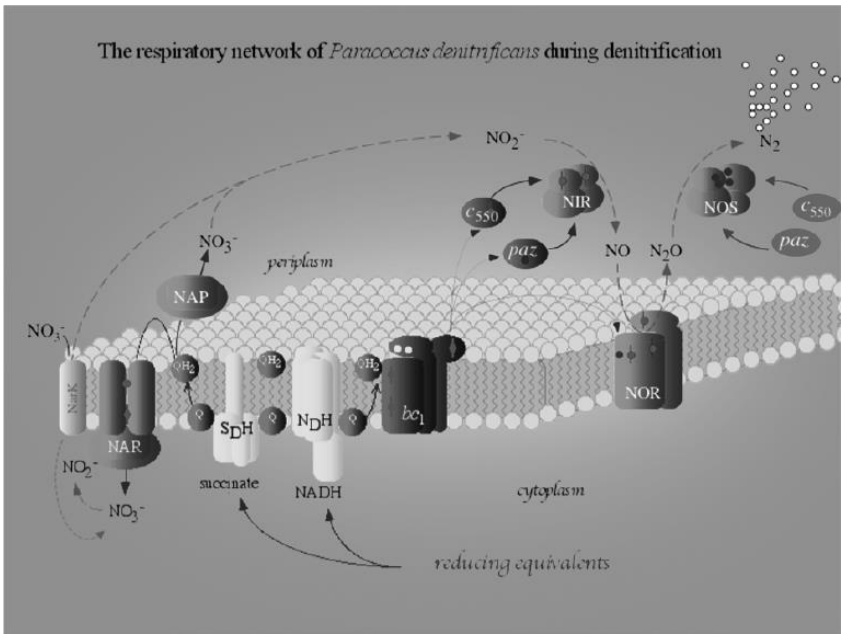

Figure 3 Scheme of a full denitrification process in Paracoccus denitrificans. Dashed arrows, N-oxide transport; straight arrows, e^--transport. SDH, succinate dehydrogenase; NDH, NADH dehydrogenase; Q, quinone; bc1, cytochrome bc1 complex; c550, cytochrome c; paz, pseudoazurin; NAR, membrane-bound NO_3^--reductase; NAP, periplasmic NO_3^--reductase (not normally involved in denitrification in this organism); NIR, cd1-type NO_2^--reductase; NOR, bc-type NO-reductase; NOS, N_2O *-reductase; NarK, NO_3^-/NO_2^- antiporter. [Spanning et al., 2007]*

3.1.3 Nitrification

Nitrification consists of two aerobic oxidations: NH_4^+ to NO_2^- and NO_2^- to NO_3^-. The catalysts of the two reactions have been proven to be either chemolithoautotrophs

or heterotrophs. Figure 4 is a typical process of biological nitrification. Oxygen is added to ammonium ions by the nitrifying bacterium *Nitrosomonas*, while oxygen is also added to nitrite ions by the nitrifying bacterium *Nitrobacter*. *Nitrosomonas* and *Nitrobacter* are the most typical examples in nitrification. They use the released energy from the oxidation reaction for cell growth. [Gerardi, 2002]

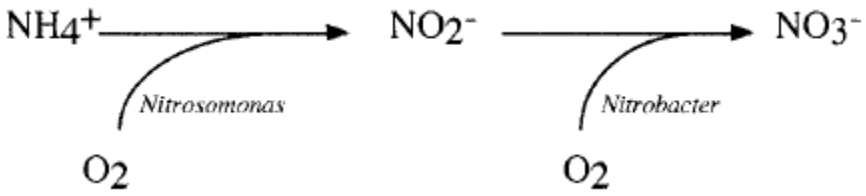

Figure 4. Biological nitrification. [Gerardi, 2002]

3.1.4 Nitrogen fixation

N_2 is the major constituent of the atmosphere, but it can't be used by organisms before nitrification. There're two pathways of nitrification: chemical nitrification and biological nitrification. Chemical nitrification produces NH_4^+ from N_2 by applying high temperature and pressure in the presence of catalyst and adding electrons and protons to N_2. The other more major pathway of nitrification is biological nitrification, which reduce N_2 by microorganisms (called diazotrophs) that carry out a process called biological nitrogen fixation. [Yang et al., 2011]

Nitrogenases are critical in biological nitrification. Most enzymes are composed of two component proteins: a large component having at least an α2β2 subunit composition and a smaller component having a γ2 subunit composition. The two proteins work together to catalyze the reduction of N_2 as is shown in equation 3.2: [Yang et al., 2011]

$$N_2 + 8e^- + 16MgATP + 8H^+ \rightarrow 2NH_3 + H_2 + 16MgADP + 16Pi \qquad (3.2)$$

3.1.5 Other reactions of nitrogen cycle

As is discussed above, nitrification, denitrification and nitrogen fixation makes a nitrogen cycle. However, there are some other major parts of the nitrogen cycle. Anammox and nitrate assimilation are both important nitrogen cycle processes.

Anammox is the process to utilize ammonium and nitrite as electron donor and acceptor, respectively, to produce nitrogen gas as the main final product. The reaction of this process is shown in equation 3.3. [Reimann et al., 2015]

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad (3.3)$$

A current understanding is that anammox catabolism essentially is comprised of three consecutive reactions with two intermediates: (1) the one-electron reduction of nitrite to NO catalyzed by a nitrite reductase (Nir), (2) the condensation of ammonium

and NO together with the input of three electrons leading to hydrazine, and (3) the oxidation of hydrazine to N_2, which generates four electrons that drive steps (1) and (2) in a cyclic way (Figure 5). [Reimann et al., 2015]

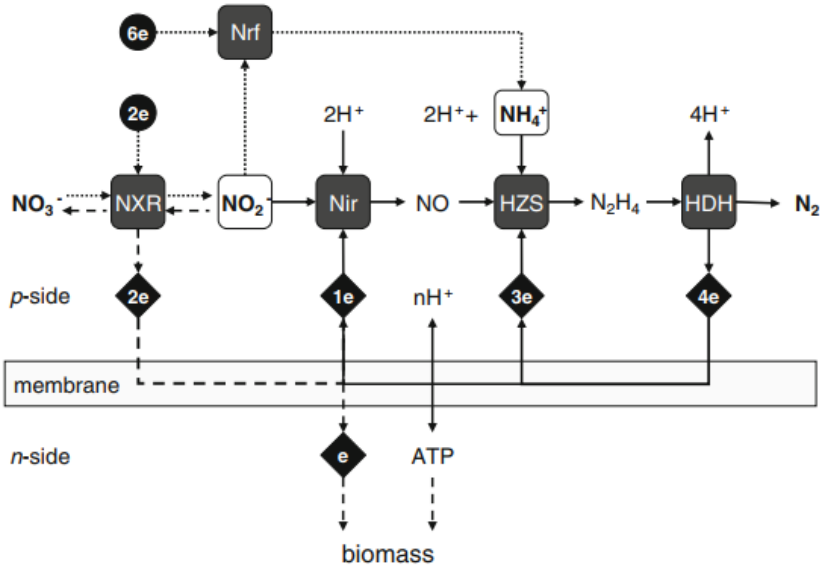

Figure 5. Scheme of the anaerobic ammonium oxidation (anammox) process with nitric oxide (NO) and hydrazine (N_2H_4) as intermediates and dinitrogen (N_2) as the end product.

Nitrogen assimilation is a major process that occurs in higher plants, fungi, algae and some bacteria. The NO_3^- is absorbed into cells and then reduced to NH_4^+, via NO_2^-, by two sequential reactions which are respectively catalyzed by nitrate reductase and nitrite reductase. The resulting NH_4^+ then undergoes ammonia assimilation and thereby be utilized by the cells. [Newton, 2007]

3.2 Nitrogen composition in algae

3.2.1 Total nitrogen in algae

The average total nitrogen in algae is about 4-9% depending on algae species, growth stages and environmental conditions. [Fogg et al., 1919-2005] The morphological form of the algae can partly influence the nitrogen content. For example, algae with thin sheaths generally contain higher nitrogen than those with thick sheaths. Also at the start of exponential growth stage, the nitrogen content is typically higher and gradually decreases as lipid and polysaccharide accumulate. For the environmental factors, high light intensity tends to lower nitrogen content and nutrient concentrations also impact the nitrogen. [Fogg et al., 1919-2005; Fay, 1969]

3.2.2 Nitrogen compounds in algae organics

Nitrogen in algae basically exists in two kinds of biochemical components, protein and nucleic acids; lipids on average contain little nitrogen. Table 1 gives mean elemental composition of overall microalgae: [Williams and Laurens, 2010]

Table 1. Mean elemental composition of different algae [Williams and Laurens, 2010]

Biochemical component	Characteristic elemental composition
Algal lipids	$C_1H_{1.83}O_{0.17}N_{0.0031}P_{0.006}S_{0.0014}$
Acylglycerides	$C_1H_{1.83}O_{0.096}$
Glycolipids	$C_1H_{1.79}O_{0.24}S_{0.0035}$
Phospholipids	$C_1H_{1.88}O_{0.173}N_{0.012}P_{0.024}$
Algal fatty acid	$C_1H_{1.91}O_{0.12}$
Methyl esters	$C_1H_{1.92}O_{0.05}$
Protein	$C_1H_{1.56}O_{0.3}N_{0.26}S_{0.006}$
Nucleic acid	$C_1H_{1.23}O_{0.74}N_{0.40}P_{0.11}$
Polysaccharide	$C_1H_{1.67}O_{0.83}$

Protein contains one or more long chains of amino acids, and nucleic acid is composed of nucleotides. The structure of amino acids and nucleotides result in high nitrogen content in protein and nucleic acids. The low nitrogen content in lipid in algae varies among different species. In this study, we focus on *Chlorella sorokiniana*. Figure 6 shows how the composition of lipid, carbohydrate and protein can vary for this species depending on the cultivation media. We typically use two different media

(Pecos 7 or BG-11) for cultivation of *C. sorokiniana*. The content of lipid is approximately 20-30%, carbohydrate 25-35%, protein 35-45% and nucleic acid 1%-3%. [Jazzar et al., 2016]

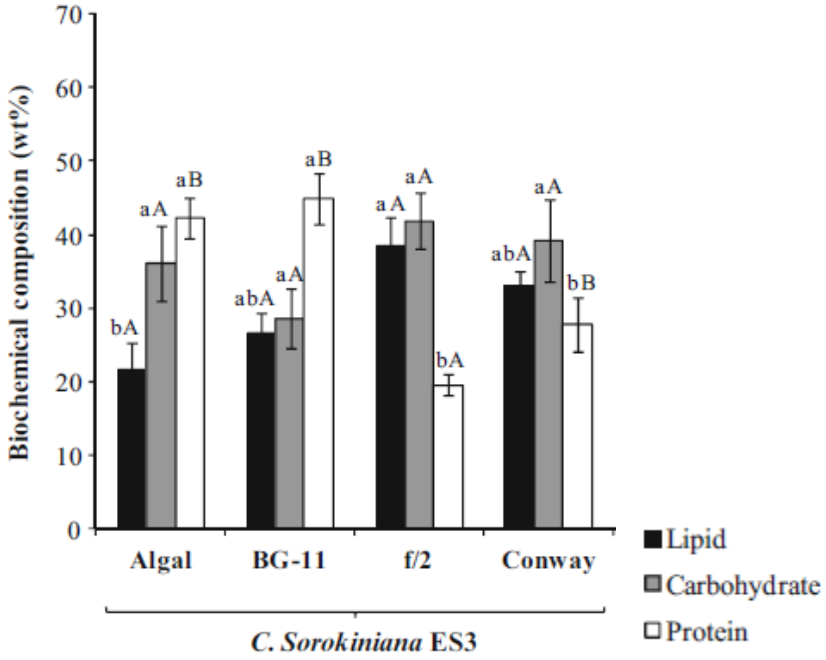

Figure 6. Proportion of lipid, carbohydrate and protein in C. sorokiniana [Jazzar et al., 2016]

3.3 Nitrogen metabolism

3.3.1 Inorganic nitrogen assimilation

The two basic inorganic nitrogen sources we use for algae cultivation are ammonia and nitrate. Ammonia is a more energetic nitrogen source that can be

directly absorbed by algae and a rapid accumulation of nitrogen inside the algae cells is observed. But ammonia is often toxic especially in high concentrations. The rapid change of ammonia concentration could lead to rapid change of pH and cause cell lysis. Nitrate-nitrogen is not directly available for algae and has to be transferred to ammonia before utilized by algal cells. The assimilation of nitrogen occurs by nitrogen reduction is shown in equation 3.3:

$$NO_3^- \rightarrow NO_2^- \rightarrow NH_2OH \rightarrow NH_3 \qquad (3.3)$$

Nitrate reductase reduces nitrate to nitrite, nitrite reductase reduces nitrite to hydroxylamine, and hydroxylamine reductase finally reduces hydroxylamine to ammonia. This process requires several organic compounds, for example NADPH2. [Fogg et al., 1919-2005; Raven and Giordano, 2016; Ullrich et al., 1990]

3.3.2 Organic nitrogen assimilation

Organic sources of nitrogen are usually cheaper and sometimes less effective nitrogen sources for algae cultivation. Urea is usually reported as an excellent nitrogen resource, although in certain circumstances it is not available due to some toxic effects. Based on studies using 14C-labelled urea, it was concluded that urea decomposed to ammonia and carbon dioxide before it was utilized. Complex

compounds and mixtures of amino acids are also excellent nitrogen sources for some blue-green algae. [Fogg et al., 1919-2005]

3.3.3 The extracellular nitrogen products of blue-green algae

Blue-green algae have been known to liberate nitrogen containing organics during metabolism. For some algae, 20-30% of the nitrogen is found as extracellular products, while for some algae less than 5% is extracellular. In most cases, algae growing in symbiotic associations release a high percentage of nitrogen. [Fogg, 1952; Magee and Burris, 1954]

The reason of the extracellular products is still not totally clear. There is evidence that the lysis of dead algae cells has little relation with the extracellular nitrogen products. Several hypotheses were established to explain it and were partly proved by experiments. One explanation is that end product repression does not occur in blue-green algae, and contributes to extra amino acid accumulation outside of the organism. When algal symbioses with higher plants are observed, nitrogen is found outside of the cells. Another probable source of extracellular products is from the decay of separation discs that are formed when hormogonia are released. The occurrence of lysis of the separation discs was observed by electron microscopy. Both

of the two explanations are conceivable, vary with species of algae, and the exact identities of extracellular products have not been established. [Fogg et al., 1919-2005]

3.4 Impact of nitrogen concentrations

3.4.1 Impact on algae cultivation

3.4.1.1 Growth and composition

Algae biomass yield is highly dependent on nitrogen source input. In most cases, higher biomass yield is accompanied with higher nitrogen concentrations before the density of algae is too high for the environment. [Li et al., 2015] If ammonia is used as a nitrogen resource, high levels of ammonia can be poisonous and not enhancing the biomass yields more. [Fogg et al., 1919-2005] Cell yield efficiency (ratio of produced biomass to nitrogen input) is generally decreasing as nitrogen concentration increases. [Li et al., 2015]

The composition of organic compounds changes dramatically on nitrogen starvation. The percentage on a weight basis of organelles containing high nitrogen levels, such as proteins, is lower when nitrogen is limited. Figure 7 is a typical result of changes in algae composition when grown on limited versus unlimited nitrogen. It

is obvious that the contents of nitrogen-adequate compounds (protein, chlorophyll; Figure 7a and b) are increasing when nitrogen is replete, while the contents of nitrogen-scarce compounds (carbohydrates, lipids; Figure 7c and d) are decreasing when nitrogen is replete. [Ikaran et al., 2015]

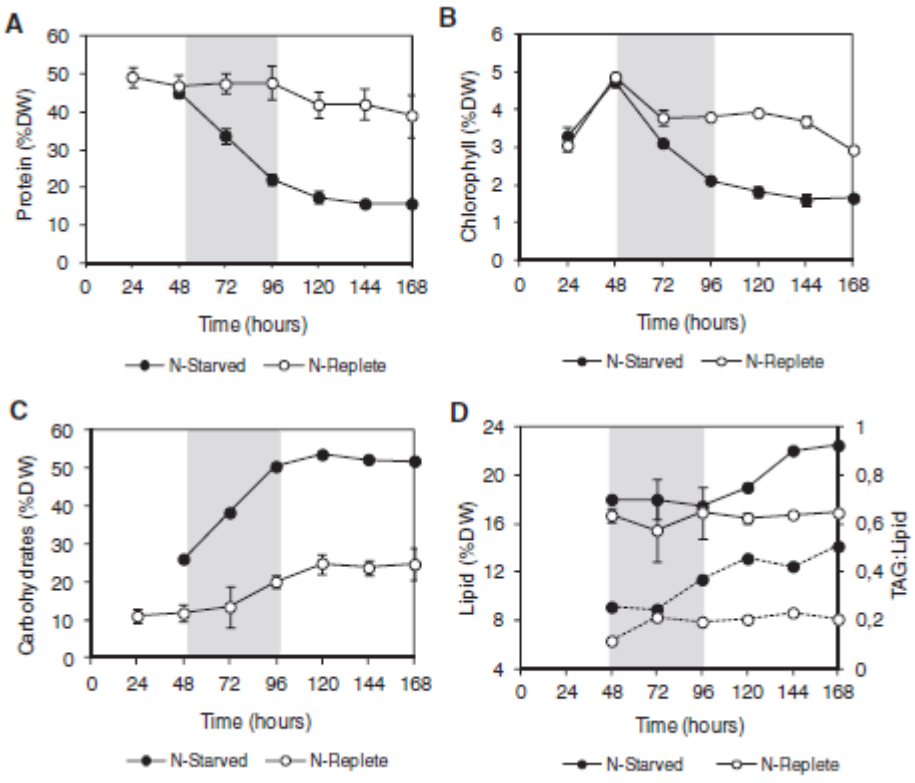

Figure 7. Changes in chemical composition of C. vulgaris cells subjected to progressive nitrogen limitation (N-Starved), and cells grown with continue availability of KNO_3 pulses (N-Replete). [Ikaran et al., 2015]

3.4.1.2 Nitrogen partitioning

As nitrogen is a key component in algae, the demand for nitrogen is high compared to other nutrients. Therefore, algae developed special mechanisms to uptake nitrogen to ensure the availability of nitrogen for critical functions. One mechanism is called “luxury uptake”, in which algae absorb more nitrogen than it needs at the start of cultivation. High levels of nitrogen accumulate in algae cells in inorganic forms or rapidly form simple N-containing compounds. This is usually more obvious when nitrogen is supplied in excess. Another is called “autophagy”, in which algae automatically biodegrade some organelles or proteins to release some nitrogen for more critical functions. This usually happens when nitrogen is not sufficient for algae growth. Both mechanisms help algae utilize nitrogen more efficiently, sequester nitrogen from competitors and guarantee that the basic metabolic functions are working. [Adams and Bugbee, 2014]

A lot of experiments have demonstrated that algae accumulate high amounts of nitrogen in the first one or two days and the nitrogen content gradually decreases in the following days. [Samori et al., 2013; Zhang and Ogden, 2017] The nitrogen is distributed amongst organic compounds, including DNA, RNA, free amino acids and chlorophyll and the percentage of these compounds decreases as the cells grow. An

initial low nitrogen concentration usually induced more rapid decrease in nitrogen content. [Adams and Bugbee, 2014]

Nitrogen concentration has an important impact on algae organic composition, especially under deplete nitrogen conditions. When limited nitrogen is used for algae cultivation, algae will not be able to have enough nitrogen for all organic compounds, therefore the content and structure of the compounds may change. For example in *C. sorokiniana*, when nitrogen input is decreased, the percentage of RNA in the pool is dramatically decreased while the amount of nitrogen in other compounds remains constant or increases slightly, as shown in Figure 8. [Adams and Bugbee, 2014]

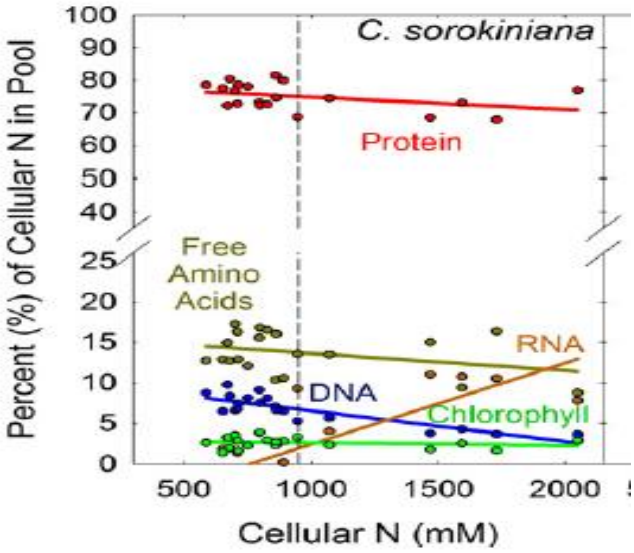

Figure 8. The percentage of C. sorokiniana cell N held in five bulk pools as a function of total cell N. [Adams and Bugbee, 2014]

3.4.2 Impact on lipid accumulation and lipid composition

Several researchers have demonstrated that nitrogen starvation results in significant increases in lipid accumulation. [Rai et al., 2017; Griffiths et al., 2014a] A typical lipid content change as a function of initial nitrogen concentration is shown in figure 9. [Griffiths et al., 2014b]

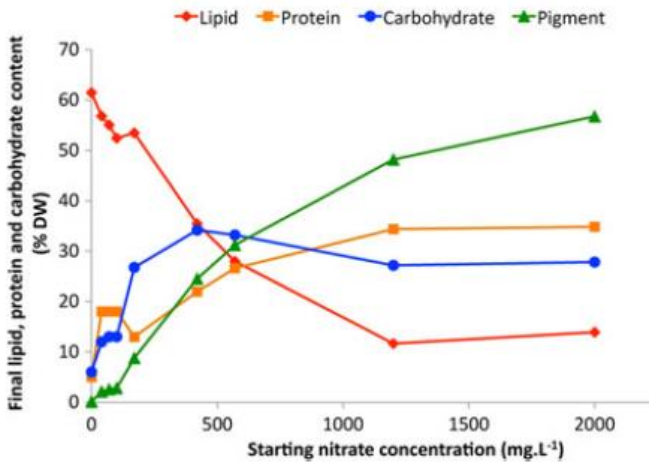

Figure 9. Organic compositions for C. sorokiniana versus increasing starting nitrate concentration. [Griffiths et al., 2014b]

There are two suggested mechanisms for lipid accumulation in the literature. Luxury uptake is one reason. Another explanation is nitrogen limitation can increase the fatty acids and active diacylglycerol acyltransferase, which converts fatty acids to triglyceride. [Li et al., 2015]

Fatty acid composition for the lipid profile also changes depending on nitrogen concentration. Ordog et al. analyzed three *Chlorella* sp., and found that although some contents could change, overall content trends for the FAMEs (Fatty acid methyl ester) are similar: C16:0 > C18:1n9c >unidentified FAMEs > C18:2n6c > C18:3n3 > C18:0. These compounds comprised 90% of the total FAME. [Ordog et al., 2016]

3.5. Nitrogen in lipid extraction

3.5.1 Nitrogen existence in algal lipid

Although there is not too much research focusing on nitrogen content in lipid, there is evidence that nitrogen is found in extracted lipid. Figure 10 is a typical polar lipid class analysis by conventional lipid extraction for *Nannochloropsis salina* and *Scenedesmus obliquus* , identified by direct-infusion FT-ICR MS. [Holguin and Schaub, 2013]

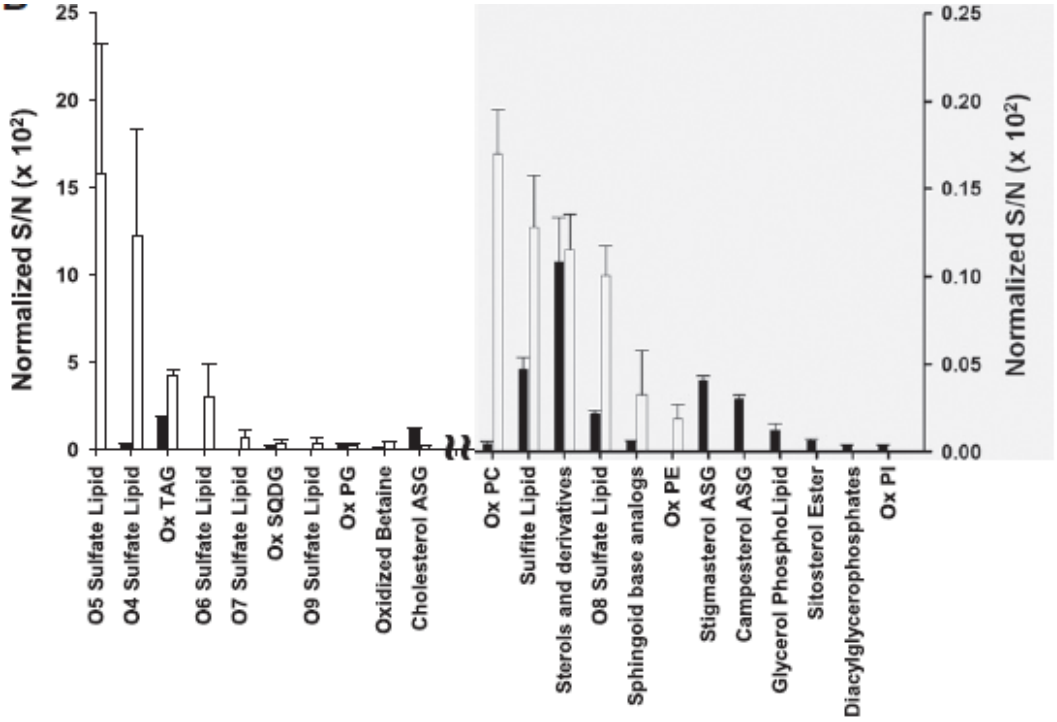

Figure 10. Biochemical composition of lipid of N. salina and S. oliquus.

[Holguin and Schaub, 2013]

Several nitrogen-containing compounds are listed in the Figure 10, including oxidized betaine and sphingoid base analogs. Lipid analysis showed that algal lipid can be composed of 300-600 different components. A nitrogen content of 4-10% was reported in several hydrothermal liquefaction oil extracts. [Vardon et al., 2012; Faeth et al., 2013] Xie et al. used fast microwave-assisted catalytic co-pyrolysis for oil extraction and measured the nitrogen content of approximately 2% at 450 ℃. [Xie et al., 2015] NAABB conversion report provided nitrogen content information by different extraction methods for different species shown in Table 2 [National Alliance

for Advanced Biofuels and Bioproducts full final report section 2] Based on this research, it is shown that nitrogen exists in extracted lipid and that the content differs depending on strain and extraction method.

Table 2 Part of characterization of algal lipid extracts from NAABB consortium producers.

Algal Biomass Supplier and Species	Oil Extractor	Oil Type	Acid Number	Oxygen	Nitrogen	Sulfur	Phospho-rous	Chlorine
			mg KOH/g	mass-%	mass-ppm	mass-ppm	mass-ppm	mass-ppm
Eldorado	Eldorado	Refined oil	0.93	10.7	5	6	<0.09	<0.1
Inventure	Inventure	FAME	2.6	11	453	158	11.9	62
Cellana N. oceanica (high lipid)	Inventure	Distilled FAME	3.12	11	6000	473	0.18	
TAMU Pecos Chlorella sp.	Valicor	Crude lipid extract	114	9.3	3600	2392	436	
Cellana N. oceanica (low lipid)	PNNL	HTL Bio-oil	55.4	19	32,000	2400	3	NR

3.5.2 Nitrogen in microwave extracted oil

Different from the conventional extraction method involving transport of heat from the heating medium to the interior of the sample, the microwave heating uses a

non-contact heat source, which can penetrate into the biomaterials, interact with polar molecules like water in the biomass, and heat the whole sample uniformly. [Mubarak et al., 2015] The mechanism of microwave extraction may explain the high nitrogen content in algal oil in our experiments.

It is suggested that in the thermochemical conversion process of raw algal biomass into bio-oil, the nitrogen-containing compounds are likely derived from proteins, carbohydrates, and lignin in the feedstocks, which undergo a complex series of depolymerization, decomposition, and reformation reactions, including Malliard reactions between amino acids and sugars. [Vardon et al., 2012] Although in microwave extraction the temperature was far lower than the hydrothermal liquefaction or pyrolysis process, in closed vessels the temperature may reach far above the boiling point of the solvent. Also in microwave extraction, the power provides localized heating in the sample, which acts as a driving force for microwave extraction to destroy the plant matrix so that the solute can diffuse out and dissolve in the solvent. [Chemat et al., 2013] The mechanism of microwave extraction results in partial lysis of algal cells. Research has demonstrated the ability of microwave treatment to help break up the rigid cell wall. [Ward et al., 2014] Therefore, it is possible that a series of depolymerization, decomposition, and reformation reactions also occur during microwave extraction of algal lipid, and thus there are two possibilities for having nitrogen containing compounds in extracted bio-oil: one could

be impurity nitrogen compounds not oil, for example amine and imidazole, and the other could be nitrogen containing oils produced from complicated reactions between original lipids and original nitrogen compounds. The classification of the nitrogen containing oils can be diverse and impacted by the power and time of microwave extraction.

3.6. Nitrogen in anaerobic digestion

3.6.1 Mechanism of anaerobic digestion

The basic pathway of anaerobic biodegradation is shown in Figure 11. The insoluble polymeric molecules are hydrolyzed into small soluble organic matter by extracellular bacterial enzymes, then fermented into acetate or hydrogen, and finally transformed into methane and CO_2 by methanogens. [Stronach et al., 1986]

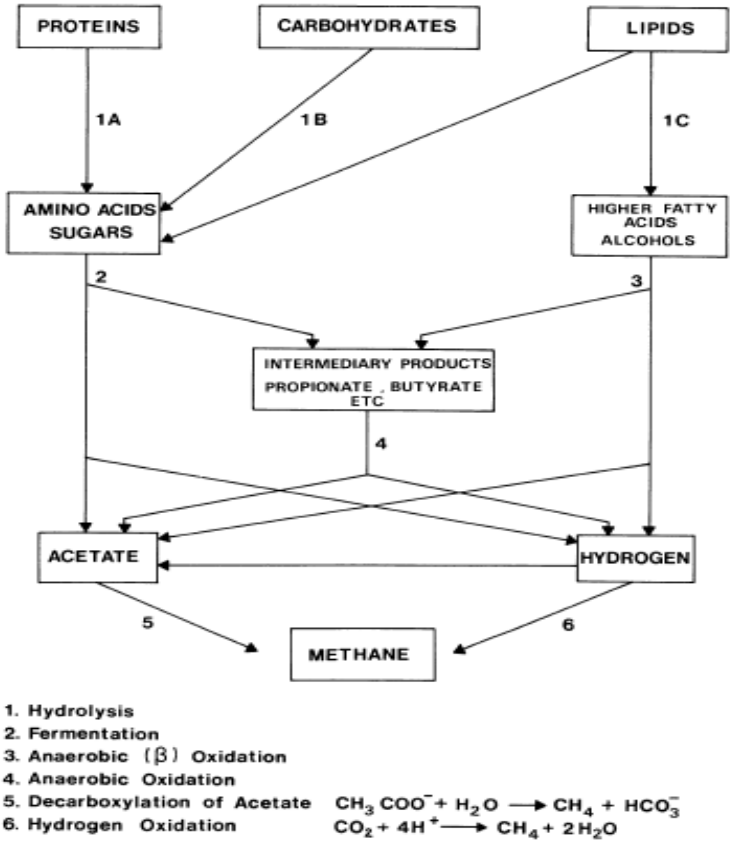

Figure 11. Pathway of anaerobic biodegradation. [Stronach et al., 1986]

Nitrogen is an essential element for the biosynthesis of new bacterial cells. The major source of nitrogen is ammonia from the hydrolysis of protein or from the hydrolysis of non-protein nitrogenous compounds like nucleic acids. Acidic end-products and ammonia are formed from the amino or amide groups of amino acids. The catabolism of these organic compounds is mediated by a large number of both obligatory and facultatively anaerobic microorganisms and the process utilizes

single amino acids, pairs of amino acids or a single amino acid in conjunction with a non-nitrogenous compound. [Stronach et al., 1986]

Ammonia is produced in fermentation, acetogenesis or methanogenesis stages. Take protein, the most abundant source of nitrogen in algae cells, as an example. The long chain proteins with peptide bonds are hydrolyzed by proteases from bacteria and released as individual amino acids, which are transported into different strains of bacterial cells. Additional degradation of amino acids occurs inside bacterial cells and produces organic acids. Equation 3.4 is a typical example of amino acid fermented into acetate:

$$4H_2NCH_2COOH + 2H_2O \rightarrow 4NH_3 + 2CO_2 + 3CH_3COOH \qquad (3.4)$$

There are three principal groups of methane-forming microorganisms including the hydrogenotrophic methanogens, the acetotrophic methanogens, and the methylotrophic methanogens. Hydrogenotrophic methanogens and acetotrophic methanogens typically produce methane and CO_2 through a methanogenic pathway shown in equations 3.5: [Neuberger and Deenen, 1981]

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

$$4CH_3OH \rightarrow 3CH_4 + 2H_2O + CO_2$$

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$$

$$4HCOO^- + 4H^+ \rightarrow CH_4 + 3CO_2 + 2H_2O \qquad (3.5)$$

Differently, methylotrophic methanogens produce methane directly from methyl groups and not from CO_2 or H_2. The decomposition of a typical substrate, methylamines [$(CH_3)_3$–N] is shown in equation 3.6 to produce ammonia:

$$4(CH_3)_3\text{-}N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3 \qquad (3.6)$$

The degradation of amino acids results in the production of a variety of organic acids including acetate and butyrate. Ammonia is released during the degradation of amino acids. [Gerardi, 2003]

3.6.2 Nitrogen distribution

For completely biodegraded algal biomass, the nitrogen should be distributed into two parts: one remains in solution in the form of ammonia, and one is utilized for bacterial growth. An important constant relationship between the mass of bacteria produced and the amount of substrate utilized is shown in equation 3.7:

$$Y = \frac{\text{mass of bacteria formed}}{\text{mass of substrate removed}}, \qquad (3.7)$$

Y is the biomass yield coefficient. The proportions of nitrogen in the VSS (volatile suspended solid) formed as a result of the degradation cycle have been estimated as approximately 10.5%. [Stronach et al., 1986] The nitrogen requirement for anaerobic digestion varies depending on loading COD shown in Figure 12. With

increasing COD loading there is a corresponding increase in nutrient needs for nitrogen. [Gerardi, 2003]

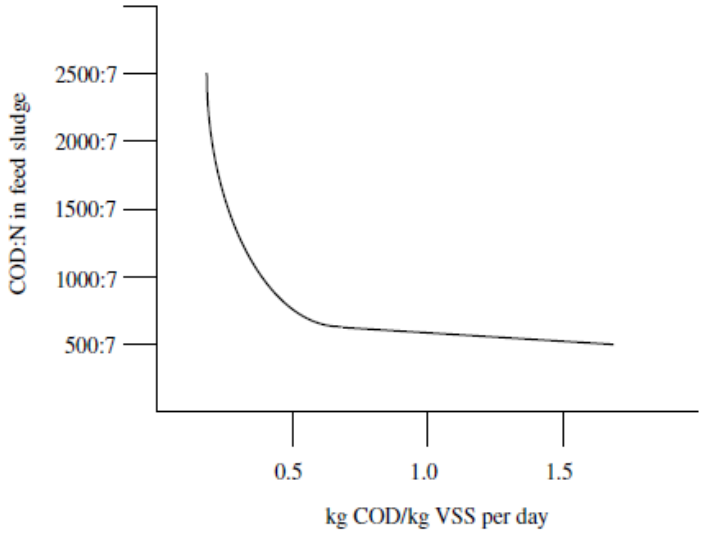

Figure 12. Nutrient needs of an anaerobic digester determined by the loading in the feed sludge. [Gerardi, 2003]

In our experiments, the nitrogen mass content in algal biomass was measured. By comparing algae and sludge inoculation, we can calculate nitrogen requirement for sludge production to decide if nitrogen is limiting. And then, if we know the biomass yield coefficient, we can calculate the nitrogen level in produced bacteria. Unfortunately, although there was some yield data for different substrates, [Stronach et al., 1986; Gerardi, 2003] there is not much information of biomass yield coefficient for algae, not to mention lipid extracted algae. Therefore, this parameter needs to be monitored to better understand nitrogen distribution.

3.6.3 Potential limiting factors on nitrogen release in this study

There are several inhibitions of algae anaerobic digestion, for example ammonia-N toxicity, salinity effect, sulfur impacts, etc. [Ward et al., 2014] In our study, three possible obstacles include rigid cell walls, ammonia concentration and C/N ratio.

As algal biomass has rigid cell walls, biodegradation of algal cells can be incomplete in some cases. Microwave extraction can partially solve the problem and results in higher ammonia concentration in anaerobic digestion. The extraction process destroys the algal cell wall and therefore facilitates the biodegradation of algae cells by anaerobic digestion. [Zhang and Ogden, 2017]

High ammonia concentration can be toxic to bacteria, while low ammonia concentration can be nutrient-limiting for the growth of bacteria. If the released ammonia concentration is too high, the inhibition of the process by free ammonia may occur. The unionized hydrophobic form of nitrogen diffuses passively across the cell membranes where it expresses its toxicity. [Sialve et al., 2009]

Proper C/N ratio is essential to achieve the highest efficiency of anaerobic digestion, and the levels of essential nutrients in the influent can be evaluated if the biomass yield coefficient is known; the COD: N ratio is frequently utilized to describe nutrient requirement. C: N ratio can be adjusted by the addition of a co-substrate to

achieve the optimized ratio, for example the elevated ammonia content of some animal wastes. [Stronach et al., 1986]

4. Dissertation objectives and format

A major objective of this dissertation is to successfully use anaerobic digestion to recycle nutrients from lipid-extracted algae for algae recultivation. *C. sorokiniana* DOE1412 was used in the following experiments because of its high productivity and lipid content.

The first study presented in this dissertation (Appendix A) mainly focuses on evaluating the effectiveness of the cultivation–extraction–digestion–recultivation system. Lipid was extracted from the harvested algae, after which the nutrients were recycled from the lipid-extracted algae via anaerobic digestion. The digestate was filtered and used for algae regrowth, and algae production was measured and were analyzed and compared with regular algae. Nutrient concentrations in both cultivation medium and anaerobic digestion medium were monitored throughout these processes. The lipid content, ash-free dry weight and lipid profiles were monitored throughout the experiments. Finally, the system feasibility and possible factors that could impact algal biofuel production were evaluated.

The second study presented in this dissertation (Appendix B) mainly focuses on evaluating the impact of nitrogen and the balance of nitrogen during the cultivation–extraction–digestion–recultivation process. Various experiments employed different initial nitrogen concentrations. Furthermore, the impacts of varying nitrogen concentrations on algae production, lipid content, nitrogen content of the algal biomass, anaerobic digestion, and algae reproduction were monitored. At each experimental stage, we observed and calculated the nitrogen balances. The distribution of nitrogen was analyzed, and possible solutions to reduce the loss of nitrogen during the production of algal biofuel were suggested.

5. Summary of appendixes

The main focus of this dissertation was to reduce nutrient costs, especially of nitrogen, by recycling nutrients from the lipid-extracted algae via anaerobic digestion. The methods, results, discussion, and conclusions are presented in Appendices A and B. A brief conclusion of the two papers is provided below:

Appendix A: Recycled Wastewater from Anaerobic Digestion of Lipid Extracted Algae as a Source of Nutrients

(Published, Fuel, 2017; 201: 705-712)

To enhance the efficiency of nutrient utilization, this study focuses on nutrient recycling from the lipid-extracted algae biomass via anaerobic digestion. Soxhlet lipid extraction and microwave lipid extraction were used. The cultivation–extraction–digestion–recultivation process released nutrients during the anaerobic digestion of the lipid extracted algae biomass. Furthermore, the recycled nutrients were collected for algae cultivation and the cycle was repeated. Results show that anaerobic digestion released nitrogen and phosphorus as well as methane as a by-product. Recycled nutrients promoted efficient algae growth. During the growth experiments, N-ammonium is the limiting macro-element and extra trace elements enhanced algae production. Ash-free dry weight, lipid content, and lipid components were monitored throughout the experiments; they did not vary when the cycle was repeated. This

nutrient-recycling system, with the addition of supplementary nutrients, has significant potential for producing algal biofuel.

Appendix B: Nitrogen Balances and Impacts on the Cultivation–Extraction–Digestion–Recultivation Process for Algal Biofuel

(Submitted to Environmental Science and Technology)

Herein, the impact of initial nitrogen concentrations on the entire cultivation–extraction–digestion–recultivation process and the observation and calculation of the nitrogen balances throughout the experiments were monitored. Results show that elevated initial nitrogen concentrations resulted in higher production, elevated nitrogen content in the biomass and lipids, and higher nitrogen concentrations in the digestate. However, elevated initial nitrogen concentrations resulted in lower lipid contents and yield coefficients. An initial amount of 75 mg/L of N-nitrate produced the highest amount of algal biomass and lipids, whereas 21 mg/L of initial nitrogen resulted in the highest yield coefficient for algal biomass and lipids. Furthermore, the nitrogen balances for algae cultivation, lipid extraction, anaerobic digestion, and algae recultivation were calculated, the results of which demonstrate that nitrogen balances are closed within experimental errors. Nitrogen recovery was ~65%, regardless of the initial nitrogen input. The lipid extraction and algae cultivation steps exhibit the

highest loss of nitrogen during the cultivation–extraction–digestion–recultivation process.

Chapter 2 Achievements and Future Work

As is introduced in Chapter 1, this dissertation focuses on nutrient recycle, especially N, for algal biofuel production by the cultivation-extraction-digestion-cultivation process. There are two major achievements:

1. The feasibility of the cultivation-extraction-digestion-cultivation process was validated. The recovered nutrients and recultivated algae demonstrated the potential to save 65% nitrogen consumption with methane biogas as a byproduct. Different from previous studies, this dissertation established a complete nutrient recycle system for algal biofuel and quantitatively analyzed the nutrient recovery at the laboratory level which was not done in previous research.

2. The nitrogen distributions were thoroughly monitored in the recycle process, and closed nitrogen balances were established in each step. Especially, the impact of initial nitrogen concentration on the distributions was evaluated. As nitrogen loss was unavoidable in the recycle process, distribution analyses clearly showed us where the nitrogen was lost and the proportion of nitrogen loss in each stage.

The results in this dissertation demonstrated a promising method to help solve the nitrogen supply shortage for algal biofuel. However, in this study there was still approximately 35% nitrogen loss in the recycle process. Moreover, if this recycle

system was to be applied in industry, there would be more obstacles. Therefore, on the basis of this research and the results presented herein, future research on the cultivation-extraction-digestion-cultivation process can be conducted considering the following points:

1) Perform futher research focusing on the impacts, distributions and recoveries of phosphorus and trace elements. Apart from nitrogen, other nutrients may also become limited especially in the recycle process and significantly impact the algae production and lipid accumulation. Determination of the nutrient requirements would avoid negative impacts and minimum the nutrients input.

2) Understand how nitrogen was lost in algae cultivation and lipid extraction steps. Analyze the nitrogen containing products in the medium at the end of cultivation and work on solutions to inhibit them or recycle them. In lipid extraction, analyze the nitrogen containing products mixed in the extracted lipid and find out method to separate them to purify the oil.

3) Evaluate the continuous cultivation-extraction-digestion-cultivation system at a larger scale. As the recycle process was not repeated for more than two generations in this study, future work should continuously monitor the nutrient recovery when the cycle was repeated more and a larger scale. This is because in large scale, there can be

some inhibition of the compounds in the recycle systems that are not apparent at the laboratory level, for example ammonium toxicity.

4) Explore effective methods to compensate for the nitrogen loss. Even if nitrogen loss can be further lowered, there are some unavoidable losses, for example nitrogen utilized for sludge growth. Therefore, in order to make the cycle continuous, extra nitrogen sources are necessary to compensate for the nitrogen loss. Investments into the combination with wastewater, waste papers or manure may be interesting.

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Appendix A: Recycled Wastewater from Anaerobic Digestion of Lipid Extracted Algae as a Source of Nutrients

(Published in Fuel, Volume 210, 15 December 2017, Pages 705-712)

Abstract

Nutrient supply, especially nitrogen and phosphorus, is one of the key obstacles limiting industrialization of algal biofuel. To help enhance nutrient utilization efficiency, our research focused on recycling of nutrients from lipid extracted algae biomass by the method of anaerobic digestion. Two methods of lipid extraction were performed, nutrients were released during anaerobic digestion of the lipid extracted biomass, the recycled nutrients were collected for algae cultivation, and the cycle was repeated: cultivation-extraction-digestion-cultivation. The results show that anaerobic digestion released nitrogen and phosphorus, and released methane as a by-product. The algae grew well on the recycled nutrients. Ammonia is the limiting macro element, and extra trace elements enhanced algae production as well. Ash free dry weight, lipid content and lipid components were monitored and did not vary when the cycle was repeated. This system for recycling nutrients with supplementary nutrients holds potential for producing biofuel from algae.

Keywords: Algal biofuel, lipid extraction, anaerobic digestion, nutrients recycle1

1 LEA- lipid extracted algae
Digestate- the nutritious effluent from anaerobic digestion.

A.1. Introduction

Micro algae have the potential to serve as a food source for fish and mammals and as a fuel source. Compared to biofuels from crop residues [1], algal biofuel has some advantages including a higher efficiency for converting sunlight into stored energy (3% for algae compared to 0.2-2% for crops) [2], a high oil content (the yield of oil from algae is expected to be 20,000 to 80,000 per acre, that is 7-31 times better than terrestrial crops) [3], and the ability to grow in saline and impaired waters. However, there are still some challenges associated with commercialization of algal biofuel [4]. The estimated cost per GGE varies greatly depending on the methods used for conversion and extraction, for example Dutta et al. [5] found that the minimum fuel selling price varied from $4.35/GGE to $10.55/GGE. Yet progress continues to be made and comparative studies on different technologies and modelling approaches have been done that continue to lower the estimated fuel selling price [5, 6]. Nutrient supply is one critical obstacle for efficient production of algal biofuel. To produce an estimated 82 million tons of algal biomass per year, approximately 5.4 million tons of N are needed, which accounts for nearly 44% of the total yearly N consumption in the US in 2010. Similarly, 27.5% of the US phosphorus will be consumed [7]. Directing so much of the nitrogen and phosphorous supply to fuel production would negatively

affect the agriculture sector. Thus, nutrient recycle is necessary for algae production prior to large scale commercialization.

One method that has been studied for nutrient recycle is anaerobic digestion of whole algae cells. This method has the added benefit of producing energy in the form of methane [8, 9, 10, 11]. Some of the previous research related to nutrient release (typically ammonia) by anaerobic digestion of algae is summarized in table A1. Frigon et al. [12] demonstrated experimentally that digestate (referred as the nutrient effluent from anaerobic digestion) from whole cell algae was rich in ammonia, and Verstraete demonstrated the availability of N and P for nutrient recycle in an open pond. [13] Although these researchers mainly focused on the application of anaerobic digestion of the algae without simultaneous production of biodiesel by oil extraction, the potential of recycling nutrients by anaerobic digestion to decrease fertilizer consumption was proven to be feasible.

If the ultimate goal is to produce biodiesel or biofuel from algal lipids, then N and P should be recycled from lipid extracted algae (LEA) or the residual after lipid extraction [14]. Neves et al. showed that anaerobically digested LEA usually produced more methane compared to whole cell algae. [15] Hemandez et al. demonstrated that digestate from LEA was rich in ammonia and the concentration could be higher than the amount obtained from digestate from whole cell algae [16].

The extraction processes destroys the algal cell wall and therefore facilitates the biodegradation of algae cells by anaerobic digestion. Morken calculated the efficiency of energy production and nutrient recycle by anaerobic digestion, [17] but stated that many inhibitory factors could lower the effectiveness of such a system, such as ammonia toxicity, algal cell resistance, and nutrient consumption by bacteria [7, 9]. Therefore, the practicality of the system needs to be further verified experimentally.

Table A1. Literature reviews of anaerobic digestion of algae

Reference	Algae strain	Oil extracted	Descriptions of experiments and conclusions
B. Sialve et al. [9]	C. vulgaris	No	Based on the gross composition the theoretical calculation of N-NH3 yield as 47.5-54.0 mg g VS-1
J. Frigon et al. [12]	C. sorokiniana	No	Different strains were tested and the ammonia yield of C. sorokiniana was calculated as 39.4 mg g VS-1
Schamphelaire, Verstraete [13]	C.reinhardtii/ P.subcapitata	No	The use of algae for energy generation by anaerobic digestion in a closed-loop system was tested and nitrogen was proven to accumulate as a result of recycle from anaerobic digestion
D.Hernandez et al.[16]	Four algae species	Yes*/No	N-NH4+ concentrations after anaerobic digestion were measure for 4 kinds of algae, both with and without lipid extraction. Higher nitrogen release from LEA than whole cell algae was shown
J. Morken et al. [17]	Model calculation	Yes	A model was established calculating the theoretical nutrient recycle efficiency and composition needed to make a closed-loop

* Supercritical fluid extraction was used in this research

In summary, previous work demonstrated the feasibility of anaerobic digestion of whole cell algae and of LEA to produced methane and recycle nutrients. However, the algal process of cultivation-extraction-digestion-cultivation has not been experimentally tested and is the focus of this study. The amount of methane produced, nutrients recycled, lipids extracted and the rate of algal growth were monitored to assess the feasibility of incorporating anaerobic digestion for nutrient recycle into an algal cultivation scheme. As trace nutrients also improve algae production [18], the impact of trace nutrients was also determined.

A.2. Methods

A.2.1. Algae Strain and Medium

The algae species used was *Chlorella sorokiniana* (DOE 1412), a species of algae that has high productivity in indoor and outdoor cultivation systems [19, 20]. The cultivation medium is shown in table AS1-AS2 and was developed by colleagues from Texas A&M Agrilife in Pecos, Texas [21] and is referred to as Pecos medium. Anaerobic sludge and secondary wastewater were received from the Pima County Ina

Road Wastewater Treatment Plant in Tucson Arizona. All solvents used in this study were analytical grade reagents.

A.2.2. Initial Algae Cultivation

C. sorokiniana was first cultivated in Pecos medium (Table AS1, AS2) in a 4L flask, and then transferred into a 40 L flat plate photobioreactor indoors with Pecos medium. Small bubbles of air and CO_2 were uniformly generated, and pH was maintained at 8 through CO_2 addition. A 12 hours on/12 hours off light/dark cycle was used. The light intensity was 80 μmol photos m^{-2} s^{-1} on the flask surface and was measured by a quantum meter (MQ-200, Apogee Instruments, Logan, UT). When the density reached a maximum of 0.85 g/L, the algae were harvested by centrifugation and stored in the refrigerator prior to lipid extraction.

A.2.3. Lipid Extraction

To experimentally test the effectiveness and impact of lipid extraction, two methods were used: soxhlet extraction and microwave extraction. Soxhlet extraction

is a typical chemical method for oil extraction which is applied in some industries, [22, 23] and microwave extraction is a typical mechanical assisted extraction which is often identified as the most easy and effective method of lipid extraction from microalgae. [24]

Algae were dried in a horizontal air flow oven (VWR International West Chester, PA) at 70℃ for 24 h and an aliquot of 8 g was weighed for extraction. Hexane was used as the extracting agent in the Soxhlet apparatus. A total volume of 300 mL of hexane were heated to 55℃ and recycled to extract lipid from the algae [25]. The extraction lasted 8 hours. For the microwave extraction, methanol-chloroform (1:2) was used as extracting agent. Algae were dissolved in methanol-chloroform overnight, and then heated in the microwave (CEM MDS 2100 Microwave oven with solvent sensor) at 70℃ for 75 min. After lipid extraction, the lipids were dried and weighted to determine the lipid content [26, 27]. The biomass was washed with hexane twice after microwave extraction to remove any residual chloroform and stored for anaerobic digestion.

A.2.4 Anaerobic Digestion

Four sets of algae were anaerobically digested: lipid extracted algae using either the Soxhlet or microwave methods, and two controls - whole algal cells and endogenous digestion with no algae. Twelve liters of anaerobic digestion medium (Table AS3, AS4) were prepared and divided uniformly into four 4L flasks. Then 35 g of anaerobic sludge were added and mixed into the medium. The 8 g LEA or whole cell algae were also added. Basal medium and operating conditions were the same as those used by Ayala-Parra et al [28].

The anaerobic digestion system is shown in Fig. A1. The 4 L flasks were sealed and the air inside was cleared by a mixture of 80% N_2 and 20% CO_2. A stir bar was used to keep the sludge and algae mixing. A safe bottle was connected to the outlet of the 4 L flask, followed by a sample-collection flask filled with 5% NaOH. The whole system was totally sealed, forming an anaerobic environment. All systems were placed in the same thermostat chamber (Southwest Instruments, Jam Scientific Company, USA). The temperature was set at 33 ℃, which is a favorable temperature for the anaerobic bacteria [9]. As methane/CO_2 gas was generated, it is collected in flask 5 that contains NaOH. The CO_2 dissolved in the NaOH, and the methane floated to the top of the flask causing the NaOH to be displaced. The volume of displaced NaOH was recorded every day to determine the volume of CH_4 generated. Samples in flask 2 were taken every two days to measure the nutrient concentration changes. Anaerobic digestion experiments were repeated twice.

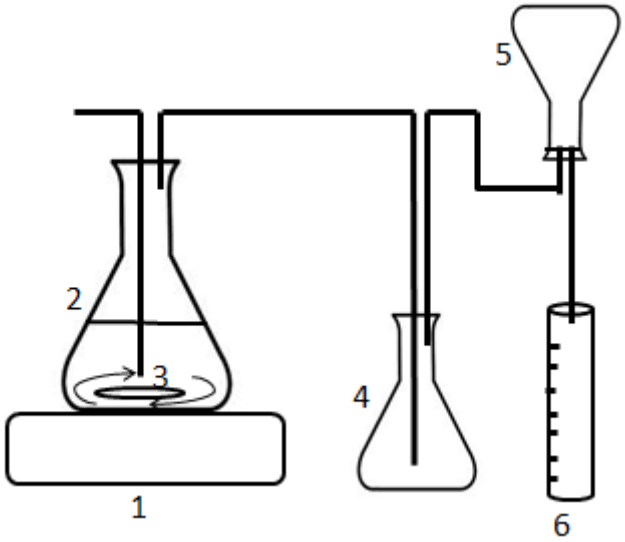

Figure A1. Scheme for the anaerobic digestion system. In the system, 1 is the magnetic stirrer; 2 is the 4L flask containing sludge, algae and medium; 3 is the stir bar; 4 is the safe bottle; 5 is the collection flask; 6 is the graduated cylinder. The entire system was sealed and controlled with switches.

A.2.5 Algae Cultivation on Digestate

After approximately 30 days, CH_4 accumulation slowed down and finally stopped, which indicates that anaerobic digestion was complete. The digestates were centrifuged and subsequently filtered through a 0.2 μm filter to remove bacteria. The purified digestates were then used for algae cultivation.

The four different types of purified digestates (500 mL) were inoculated with 15 mL of *C. sorokiniana*. Algae were grown on standard Pecos medium as a control. All cultures were grown in 1L flasks, with a continuous flow of 950 mL/min air and 50 mL/min CO_2 to provide mixing and a source of carbon. A 12/12 light/dark cycle was used; light intensity was 80 μmol/m^2.

The growth of algae was monitored by measuring the optical density (OD) on a spectrophotometer. Samples were taken every two days, diluted to assure a linear correlation between OD and cell mass, and measured at a wavelength of 750 nm. Also samples were taken every two days for nutrients analysis. Ammonia was measured with an ammonia probe (Ion Selective Electrodes Model WD-35802-00, Oakton), while phosphate concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Model 2500DV, Perkin Elmer, Shelton, CT, USA). The cultivation of algae was repeated three times and all the measurements were done in triplicate.

A.2.6. Ash Free Dry Weight (AFDW) and Lipid Analysis

After the algae stopped growing, the algae were harvested, centrifuged and dried. The ash free dry weight or AFDW of cultivated algae was determined using the method introduced by Zhu and Lee [29].

The lipid content was determined using the microwave method described in section 2.3. Furthermore, the lipid was reacted via trans-esterification with methanolic-HCl for 1 hour at 65 ℃, and was analyzed using gas chromatograph- mass spectrometry (7890 A GC, 5975C inert XL MSD, Agilent Technologies, USA) [26, 30]. Helium was used as the carrier gas with a 1 ml/min injection volume. The temperature started at 90 ℃ for 2 min and ramped 3 ℃/min to 240 ℃ and was held for 15 min for a total run time of 67 min. The instrument was tuned with a standard spectra auto tune method, and FAME was analyzed by mixing GLC10 dissolved in CH_2Cl_2.

A.2.7. Trace Elements Impact

The impact of trace elements was tested by adding trace elements into the purified digestate. The sources of trace elements included the trace elements in Pecos medium (Table AS1, AS2) and secondary wastewater. Total metals/metalloids were measured on Perkin Elmer ELAN DRC-II and Agilent 7700x (Santa Clara, California). Quality control/assurance procedures are described in detail in the US EPA Method 6020 in SW-846. Typical operating parameters are as follows: RF

power: 1400(ELAN) – 1550(7700x) watts; Plasma gas flow: 15 L/min; Carrier gas flow: 1.0 L/min ; Auxiliary(ELAN)/Makeup(7700x) gas flow: 0.15 L/min.

A.3. Results and Discussion

A.3.1 Feasibility Test of the System

As the goal is to determine the feasibility of the culture-extraction-digestion-culture approach, every part of the system needs to be monitored and evaluated. A schematic of the system is shown as Fig. A2. Starting with a large amount of biomass grown in a PBR, algae were extracted using two different techniques and solvents. The extracted biomass was anaerobically digested. Control cultures containing whole algal cells algae and no algae (endogenous) were also done for comparison. The digestate containing recycled nutrients were utilized to produce more algae, thereby forming a culture-extraction-digestion-culture system. To make the system work, every link in the cycle is important: extraction of lipids; production of methane and release of nutrients in the anaerobic digester; and cultivation of algae on digestate with similar amounts of lipids produced. A summary of the parameters monitored in each stage is shown in table A2.

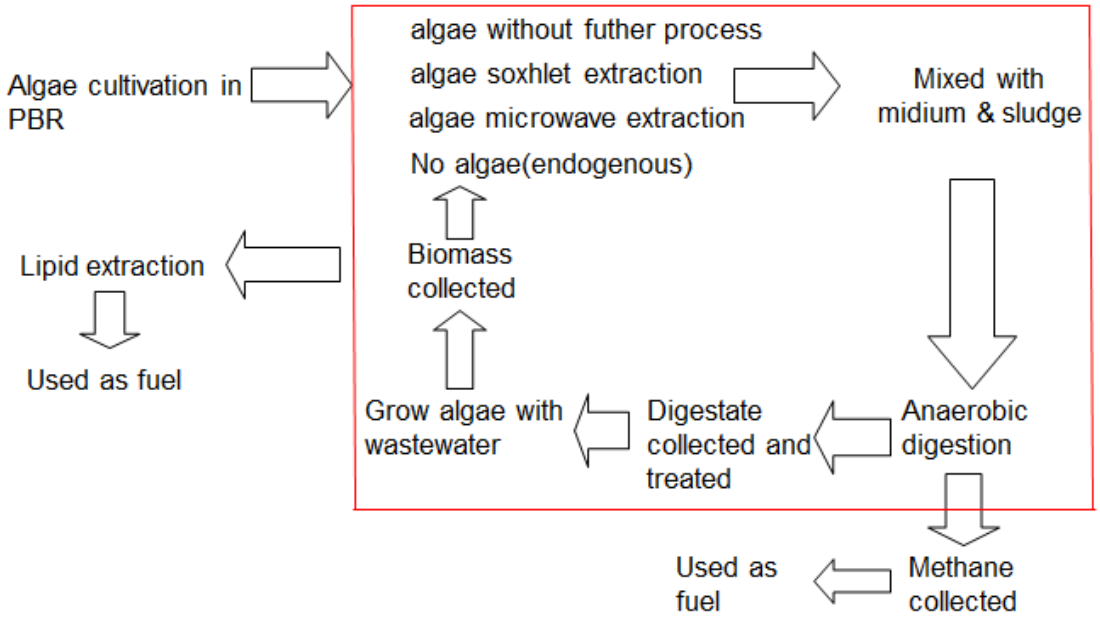

Figure A2. Scheme of culture-extraction-digester-culture system

Table A2. Summary of parameters measured in each stage

Lipid extraction	Anaerobic digestion	Algae cultivation on digestate
Lipid content	Methane production; N, P yields	Algae production; N, P concentration changes; Trace elements concentrations; Lipid content; Lipid profile

A.3.1.1 Lipid Extraction

Lipid extraction efficiency varies depending on the methods and solvents that are used. Therefore, two different methods were compared for this work. The percentage of lipid extracted using hexane in a soxhlet was 4.8%; while microwave extraction using chloroform/methanol resulted in 25.4% lipid. The extraction percentage using the microwave is comparable to the work done by Miao et al. They obtained approximately 30% lipid [31]. They used a higher extraction temperature but lower extraction time for *C. sorokiniana*. Lipid content can vary with growth condition as well. For soxhlet extraction of *C. vulgaris,* Converti et al. [32] obtained 7% lipid.

Farid and Giancarlo [33] compared microwave and soxhlet extraction methods. They stated that soxhlet extraction of lipids requires a long time and is not energy efficient. Comparing solvents, methanol-chloroform is more efficient than lipid extraction using hexane [34-36]. Therefore, if the aim is to extract more lipids at a faster rate, microwave extraction with methanol-chloroform is better choice. However, we compared the two methods because chloroform is toxic to anaerobic bacteria, and we were uncertain if the anaerobic bacteria could digest algae that had been extracted with chloroform.

A.3.1.2 Anaerobic Digestion

Anaerobic digestion has two functions in this system: production of methane gas, and degradation of additional organics and cellular material to recycle the inorganic nutrients. Fig. A3 shows the methane production during the anaerobic digestion period. The control or endogenous culture produced almost no methane, while the other three cultures containing either lipid extracted algae (soxhlet and microwave) and whole cells produced a similar methane volume of approximately 200-250 mL methane/g biomass. Previously researchers reported a methane yield for whole cell *C. sorokiniana* (without lipid extraction) as 212 mL g^{-1} VS (volatile solids) [11], a result that is similar to ours. Methane yield from other typical substrates like crops or manures are also comparable (120-220 mL g^{-1}). [37] The methane production continued for approximately 30 days. Methane production rate was low at the start and reached the highest rate of approximately 2 mL/g/day after 10 days, and gradually slowed down after 20 days. Although the rate and effectiveness are lower than some accelerated substrate digestions, [38, 39] they are comparable to the other anaerobic digestion studies [11, 28]. High methane-production rates using LEA as the organic carbon source demonstrated that lipid extracted biomass is available for methane production for biofuel. Although part of the carbon was removed during lipid extraction which tends to lower methane production, the extraction procedure makes

biomass more accessible to the digesting bacteria. This may explain the high methane production for the LEA anaerobic digestion culture. The result agreed with Hernández et al. [16]. They digested lipid extracted *Tetraselmis* sp. and obtained a methane production of 236 mL g^{-1} VS (lipid extracted biomass).

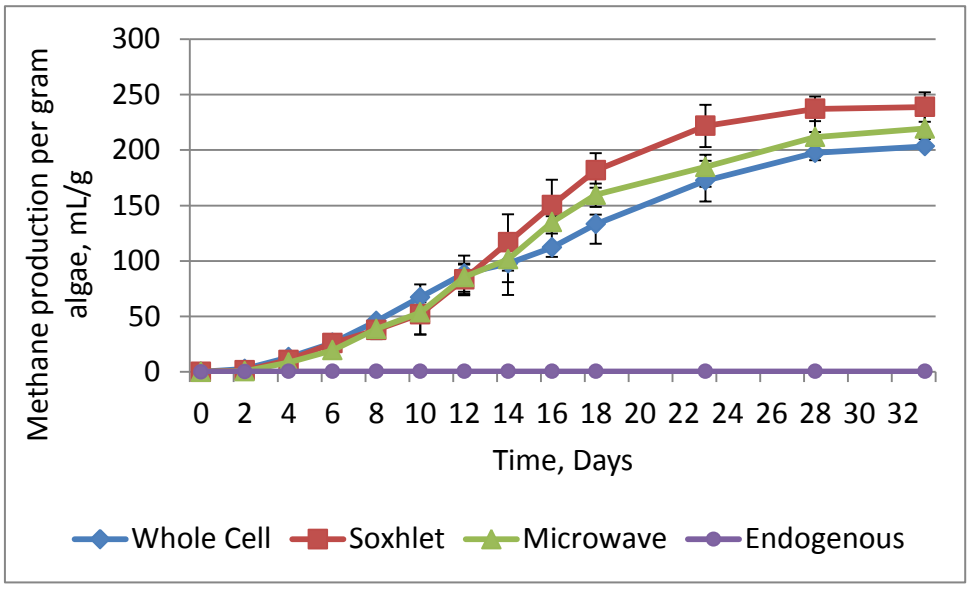

Figure A3. Methane production during anaerobic digestion of whole cell C. sorokiniana, lipid extracted algae and an endogenous control.

In the anaerobic digestion process, the bacteria degrade the algal cells and release nutrients, including nitrogen and phosphorus, into the water. Then bacteria absorb part of the nutrients for their growth and release methane and carbon dioxide produced by their metabolism. Nitrogen is in the form of ammonia due to the anaerobic environment; nitrate and nitrite concentrations in the digestate were

measured and their concentrations were almost zero. Fig. A4 shows the final concentrations of ammonia and phosphorus released. The digestate from the endogenous culture contained little ammonia and phosphorus, while the microwave one had approximately 10%-20% higher ammonia and phosphorus concentrations than the whole cell and soxhlet digestates. This agrees with the findings of Hernandez et al. [16] and may result from a more complete lipid extraction using chloroform/methanol. Since *Chlorella* sp. has rigid cell walls, the higher release of nutrients from the extracted cells may be due to a more complete lysis of algal cells, since the hemi cellulose in the cell wall tends to limit anaerobic digestion [11]. The ability of microalgae cell walls to remain intact during anaerobic digester after more than one month was demonstrated [11], and a correlation between cell wall degradation and ammonia release was also introduced [40]. Therefore use of a microwave as the pretreatment of the cell wall may result in a higher ammonia release.

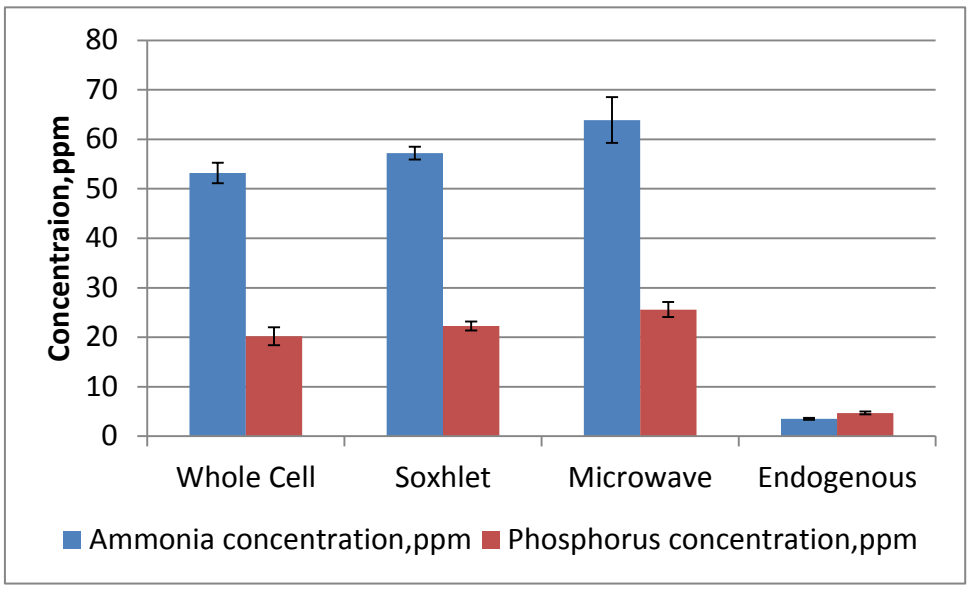

Figure A4. Ammonia and phosphorus concentration in digestates at the end of anaerobic digestion for whole cell algae, soxhlet LEA, microwave LEA and endogenous control digestates

A.3.1.3 Algae Cultivation on Digestate

The main goal of this part of study is to determine if algae can be cultivated on the digestate containing recycled nutrients (primarily N and P). Fig. A5 shows algal growth on the digestate. The digestate from the algae that were extracted using the microwave produced the highest level of algal biomass, approximately 2.29 g/L, which was higher than the control of using defined media alone (about 1.89 g/L). The other two digestates from the soxhlet extraction and the whole cell biomass reached

similar biomass levels as the media control. The endogenous control showed a small amount of algal growth reaching a final cell density of about 0.85 g/L. Although a significant amount of biomass growth was not expected for the endogenous, there is some because the anaerobic digestion media does contain some N and P (Table AS3 and Fig A4). Furthermore, since the algae are not "washed" with water prior to growth, they do have a carrying capacity for nutrients and hence some growth is always observed [41].

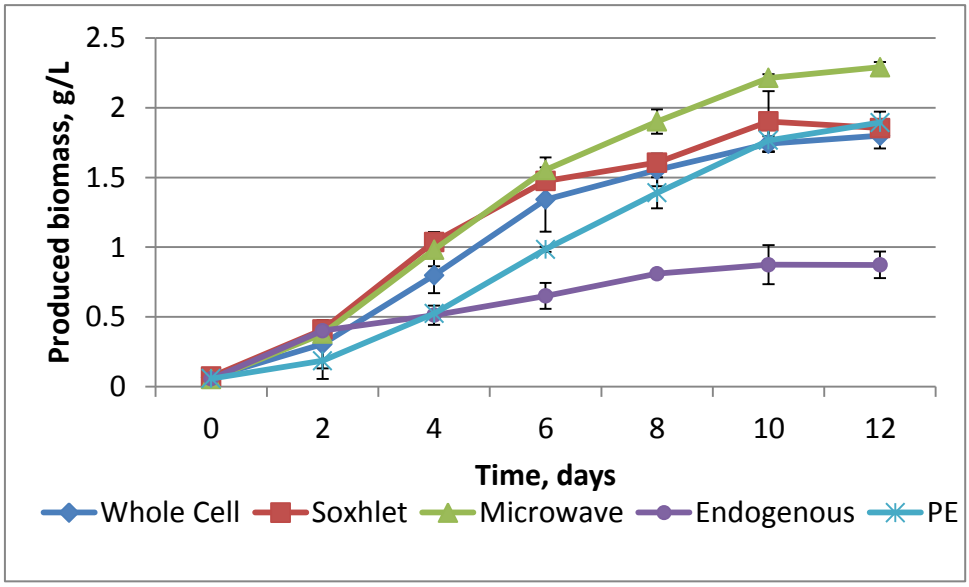

Figure A5. Algae biomass accumulation when cultivated on whole cell digestate, soxhlet digestate, microwave digestate, endogenous digestate, and on one Pecos medium control

The production of algal biomass can be better evaluated if compared with the initial biomass input, so the gross and net recoveries of algae throughout the process are defined as below:

Gross Recovery = (Produced algal biomass) / (Initial weight)

*Net Recovery = (Produced algal biomass - algal biomass produced from endogenous media) / (Initial weight)*100%*

Table A3 shows the recovery rates for the 4 cases. The gross recovery can be as high as 86%, and the net recovery is 53% by cultivation of algae that was extracted in the microwave. Being able to regrow algae and recover some of the nutrients is a first step in demonstrating the feasibility of combining lipid extraction, anaerobic digestion and regrowth. Previous anaerobic digestion work with *Chlorella* sp. only demonstrated that this species can be digested [9, 12]. Other researchers did not use *Chlorella* sp. and only demonstrated that whole cells can be digested and then grown on the resulting digestate [13]. The next step is to investigate how much of the nutrients have been recycled in this system and hypothesize why the net recovery is 35 to 53 %.

Table A3. Gross and net recovery of algal biomass after anaerobic digestion and regrowth on digestate.

	Initial weight, g	Produced algal concentration, g/L	Volume of digestate, L	Produced algal biomass, g	Gross Recovery	Net Recovery
Microwave	8	2.29	3	6.87	86%	53%
Soxhlet	8	1.85	3	5.55	69%	37%
Whole Cell	8	1.79	3	5.37	67%	35%
Endogenous	0	0.87	3	2.61	0%	0%

A.3.2 Nutrients Analysis

When algae are cultivated on digestate, the amount of nutrients present is an important factor influencing algae yield. The impact of nitrogen and phosphorus on algae cultivation rates and yields has been studied for *Chlorella sorokiniana* [42-44]. To a lesser extent, micronutrients have been studied [45-47]. In this work, macro and micro nutrients are recycled primarily as a result of the extraction/digestion process. The digestion media only includes some nutrients (Table AS3, AS4), however most of the nutrients needed for algae cultivation came from the degradation of whole cells or

LEA. This section of the paper focuses on understanding nutrient mass balances to optimize system performance.

A.3.2.1 Ammonia and Phosphorus Impact

Comparing the growth curve with the initial ammonia and phosphorus concentration (fig.A4 and fig.A5), a correspondence between the nutrient concentrations and maximum accumulated biomass is observed. The digestate from microwaved LEA had the highest initial nutrient concentrations, and the algal production from the digestate was highest. The other two digestates from soxhlet LEA and whole cells contained fewer nutrients and produced less biomass. The endogenous control contained little nutrients and produced much less. The impact of initial nutrients concentration correspond with Kim et al. [42] which showed that *C. sorokiniana* production reached 2.2 g/L on 80 ppm NO_3-N, 1.9 g/L on 40 ppm NO_3-N and 1.1 g/L on 10 ppm NO_3-N. Other researchers [43, 44] also demonstrated that *Chlorella sp.* production was enhanced by increasing the concentration of ammonia sulfate or sodium nitrate; N: P ratios as high 45:1 have been suggested.

Fig. A6 shows the ammonia concentration as a function of time for the algal cultivation experiments performed using digestate. The nitrogen cell yields for growth

on digestate were approximately 35.8 g cells / g N for the microwaved LEA, 33.8 g cells / g N for the whole cell LEA, 32.4 g cells / g N for soxhlet cell LEA. These yield values are similar to those found in the literature - approximately 37.5 g cells/ g N at 60 ppm ammonia [44]. Since the ammonia was not totally consumed, the cultures may have been light limited. The yield for the endogenous group was 249.4 g cells/ g N, which is similar to 223 g cells/ g N at 3.5 ppm ammonia from Zhang et al. [44] Fig. A7 shows the phosphorus concentration changes. For the three digestates from microwaved and soxhlet extracted biomass and whole cells, the amount of phosphate consumed was 16 ppm. Since the phosphate was not totally consumed, and all of the cell yields were approximately 120 g cells/ g P which was much lower than Kim et al. [42], phosphate was not a limiting nutrient and sufficient amounts of P are recycled in this process. The ratio of N to P was approximately 3:1 which was much lower than the optimized ratio of 16:1 [44], further demonstrating that phosphorus was not the limiting nutrient.

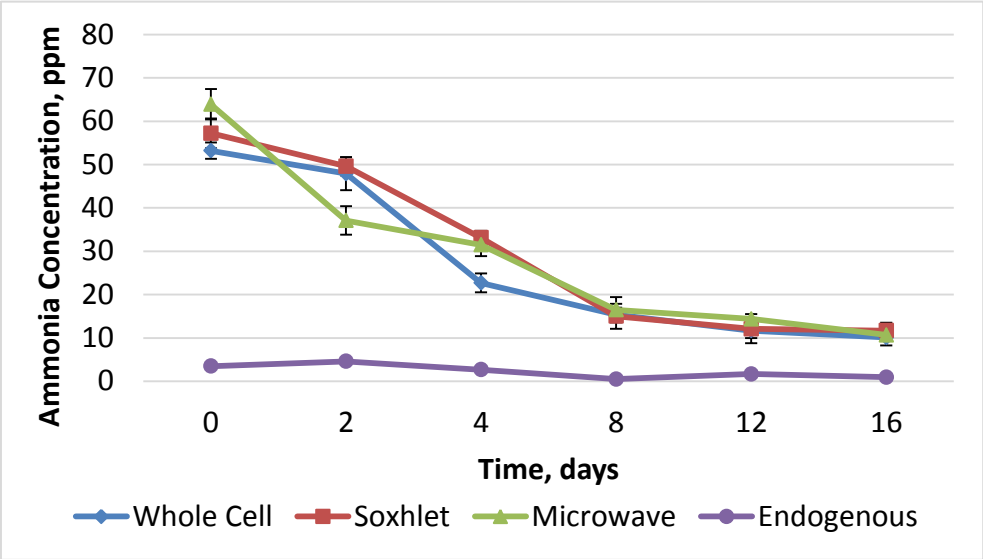

Figure A6. Ammonia concentration variations during algae cultivation on whole cell digestate, lipid extracted algae digestate and endogenous digestate control

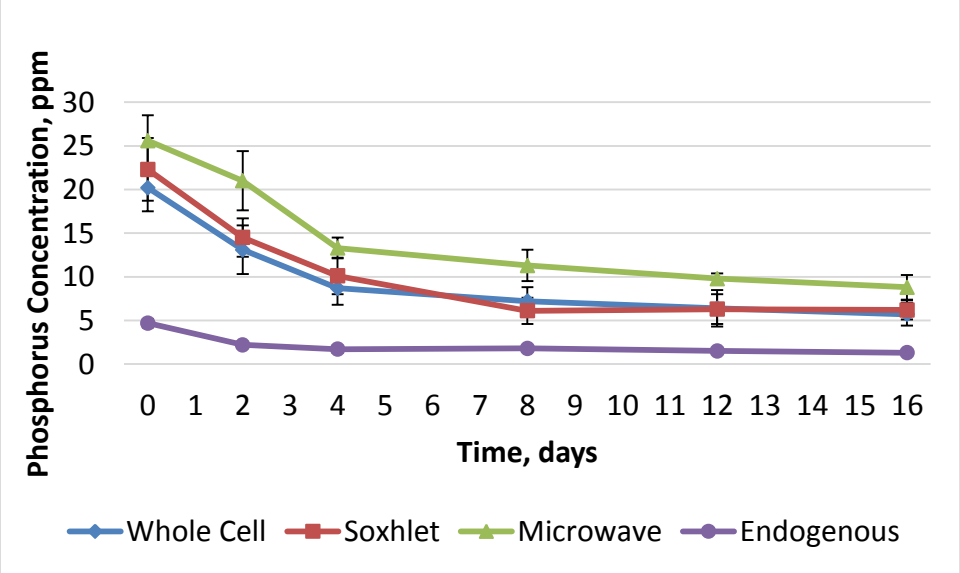

Figure A7. Phosphorus concentration variations during algae cultivation on whole cell digestate, lipid extracted algae digestate and endogenous digestate control

A.3.2.2 Trace Element Impacts

Some trace elements may also be limiting factors for algal growth, such as Fe, Mg or Zn [45-47]. The anaerobic digestion processes results in the release of trace elements in addition to N and P, however the efficiency at which these were recycled was not clear, so the concentrations of these elements were measured (Table A4) and compared to the concentrations found in media. The concentrations of Mg and K were higher in the digestate. Fe concentrations in the digestates were 100 fold lower than the growth media concentrations, and Mn was 1000 fold lower. The lack of certain elements in digestate may impede algae growth. Hence, an experiment was done to determine if the addition of trace elements would increase cell yield on digestate (Fig. A8). Algae were grown on digestate supplemented with everything in Pecos medium (Table AS1, AS2) except nitrogen or phosphorus. An improvement in algae biomass yield was observed, which demonstrates that the digestate may lack certain kinds of trace elements. This is one factor that may account for the net cell mass efficiencies of 35 to 50 %.

Table A4. Cations concentrations in the pecos medium, digestates and secondary wastewater

Element Conc [ug/l]	Pecos Medium	Whole Cell Algae	Soxhlet LEA	Microwave LEA	Endogenous	Secondary Wastewater
9 Be	4.00E-02	0.00E+00	5.00E-02	0.00E+00	0.00E+00	0.00E+00
23 Na	1.16E+03	1.06E+06	1.17E+06	1.27E+06	1.29E+06	1.04E+05
24 Mg	1.80E+03	1.51E+04	1.40E+04	5.07E+03	8.89E+03	9.43E+03
27 Al	2.75E+01	1.83E+01	2.26E+02	4.49E+01	4.50E+02	1.15E+01
39 K	5.21E+02	2.48E+04	2.51E+04	2.81E+04	2.50E+04	1.57E+04
55 Mn	9.23E+04	1.23E+02	1.35E+02	2.25E+01	4.39E+00	1.47E+01
56 Fe	1.65E+03	9.89E+00	1.13E+01	8.90E+00	1.02E+01	1.79E+01
60 Ni	2.96E+00	6.20E+00	1.13E+01	1.43E+01	5.57E+00	4.32E-01
63 Cu	3.98E+00	1.21E+01	4.77E+01	7.47E+01	1.46E+01	4.27E+00
66 Zn	1.26E+02	1.94E+01	8.98E+01	6.90E+01	1.61E+01	5.66E+01
95 Mo	1.41E+02	7.27E+00	1.44E+01	2.26E+02	5.01E+01	4.09E+00
107 Ag	2.00E-02	7.33E-03	1.79E-01	8.94E-02	2.94E-02	0.00E+00
111 Cd	1.79E+00	3.23E-02	6.93E-02	1.89E-01	6.01E-03	0.00E+00
118 Sn	2.00E-02	2.58E-01	1.92E+00	1.23E+00	4.71E-01	3.06E-02
121 Sb	1.50E-01	4.09E-02	2.16E-01	3.17E-01	5.41E-01	2.82E-01
137 Ba	2.90E+00	2.24E+01	2.58E+01	1.27E+02	8.51E+00	1.87E+01
208 Pb	1.00E-02	4.71E-01	6.99E-01	6.63E+00	1.80E-01	1.10E-01

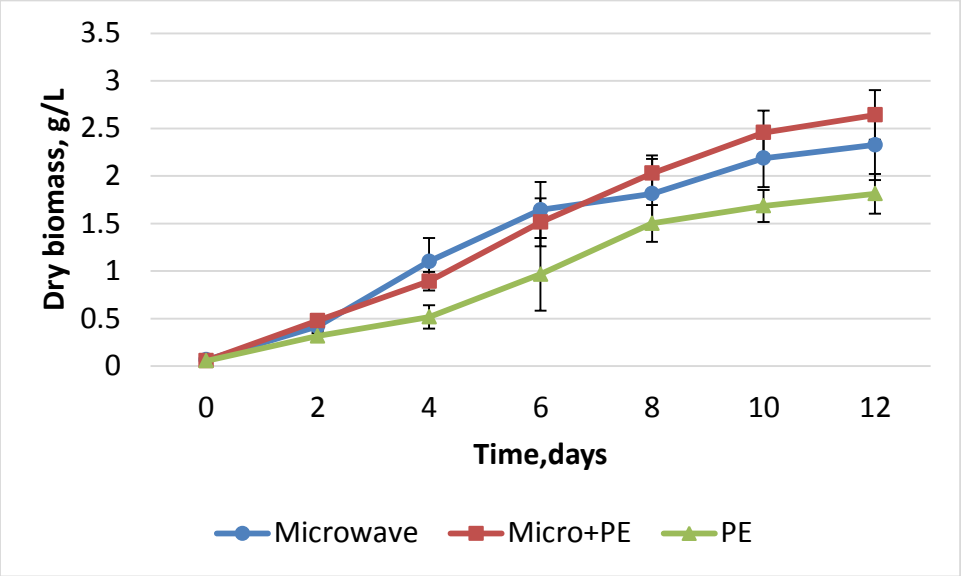

Figure A8. Algal growth on digestate added with trace elements in Pecos medium, digestate control and Pecos medium control

** The micro+PE curve shows algae growth on digestate mixed with all nutrients except N, P from Pecos medium.*

Secondary wastewater (SWW) is an additional source of trace nutrients (Table A4) and contains only 5.8 mg L^{-1} of nitrogen. In particular, the concentration of Fe in SWW was 10 fold higher than the concentration in the digestates and may limit growth. Hence we investigated using SWW as an inexpensive source of Fe and other trace elements. Fig. A9 shows the cultivation condition when digestate was mixed with secondary wastewater. A small amount of secondary wastewater mixed with a large amount of digestate produced more algae.

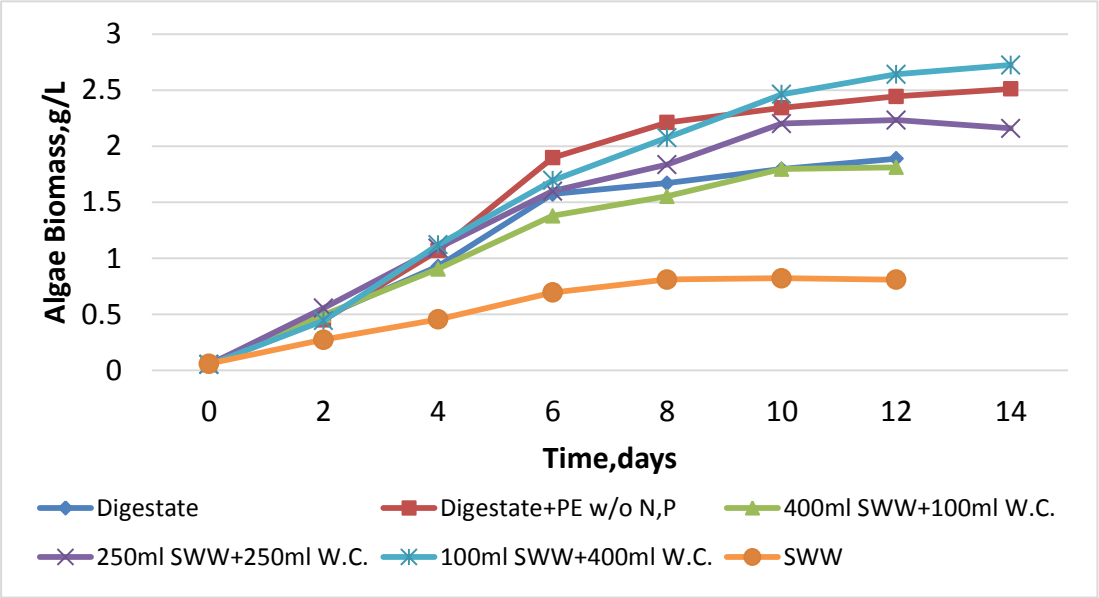

Figure A9. Algae growth on digestate mixed with Secondary Wastewater at different ratio of digestate to SWW

Comparing the two sources of trace elements, Pecos medium can be controlled more accurately, while secondary wastewater can be cheaper, and may help to compensate the evaporation of water. The conclusion is that some extra trace elements did enhance algae production.

A.3.3 Algae Examination

In addition to production of algal biomass, the ash content and the lipid content were measured. The ash free dry weight was consistently 95% of the total dry weight,

and hence the algae contained only about 5% inorganic matter. This agreed with the results by Phukan et al. who reported ash content of *Chlorella* as 5.93% [48] and demonstrates that use of digestate is not causing an increase in ash content. Fig.A10 shows the lipid content for the algae. An average of 25% lipid was observed for the algae, which is similar to what is reported in the literature. For example, Wan et al. [49] observed a lipid content of 25% for *C. sorokiniana* cultured in freshwater. Fig. A11 shows the fatty acid components from esterification of the lipid. C16 and C18 dominate in the profile, which is similar to the profiles obtained by Neofotis et al. [20]. The lipid composition of the cells cultivated on digestate was similar to the composition of the cells cultivated in media.

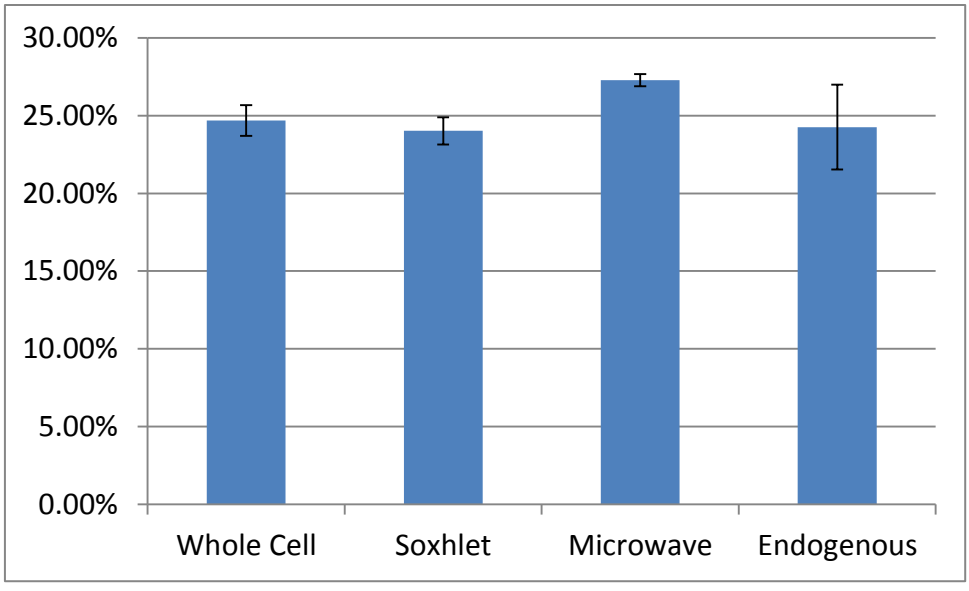

Figure A10. Lipid content of algae produced on digestates from whole cell algae, lipid extracted algae and endogenous control

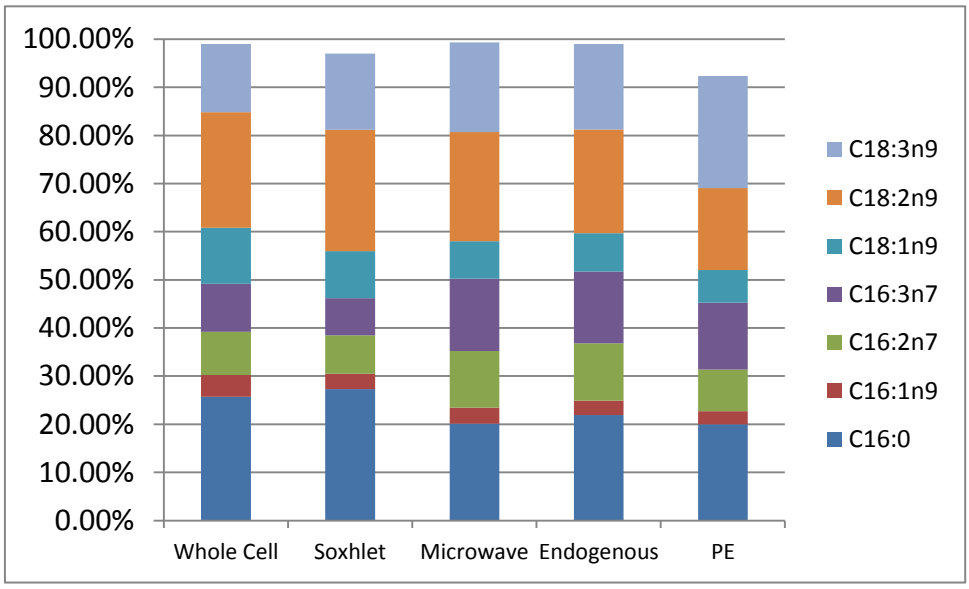

Figure A11. Lipid profile of algae produced on digestates from whole cell algae, lipid extracted algae, and the endogenous control. The profile of algae grown on PE media is shown for comparison.

Combining all the algae analysis, we may draw conclusion that the algae growing on recycled anaerobic digested wastewater were comparable to those grown on media, and are suitable for production of algal biofuel.

A.4. Conclusion

This paper demonstrated the feasibility of using recycled wastewater from anaerobic digestion of lipid extracted algae as a source of nutrients. In the closed system, methane was produced, nutrients were recycled and algae were produced. The lipid extracted algal biomass produced more than 200 mL methane per gram algae by anaerobic digestion, which can be used as a gas fuel. Ammonia concentration in the digestate from microwave LEA was higher, and this is one reason for the observed higher algae yield when grown on digestate. More than half of the algal biomass was recovered without additional nutrients, and this recovery holds potential to decrease nutrients consumption for algal biofuel. Ammonia/phosphorus tracking proves ammonia as a key growth-limiting factor in algal cultivation on digestate. Fe may be one growth-limiting trace element for digestate. Pecos's medium or SWW can be sources of trace elements and help enhance algae reproduction. Finally, AFDW and lipid analysis further ensured that the process is a feasible system. More work needs to be done in the future to further understand the process. Nutrient balances should be calculated and nutrient recycle rate should be optimized. From all results mentioned above, we may draw the conclusion that by anaerobic digestion, we can effectively recycle the nutrients from LEA for algae cultivation, and thus build a recycle system and effectively decrease the nutrients consumption.

Acknowledgements

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Supplementary Data

Table AS1. Pecos medium

Chemical	Final Concentration, g/L	Stock solution concentration	Volume, mL
(NH2)2CO	0.1	4g/200mL	5
MgSO4·7H2O	0.012	2.4g/200mL	1
NH4H2PO4	0.025	5g/200mL	1
Potash	0.075	7.5g/200mL	2
FeCl3	0.00315	0.63g/200mL	1
Na2CO3	0.02	4g/200mL	1
EDTA	0.00436	0.218g/200mL	4
*Allen's solution			1

*Table AS2. *Allen's solution*

Component	Amount	Final concentration
H3BO3	2.86 g/L	46 μM
MnCl2·4H2O	1.81 g/L	9 μM
ZnSO4·7H2O	0.22 g/L	0.77μM
Na2MoO4·2H2O	0.39 g/L	1.6μM
CuSO4·5H2O	0.079g/L	0.3μM
Co(NO3)2·6H2O	0.049g/L	0.17μM

Table AS3. Anaerobic Digestion Medium

Compound	Concentration, g/L
K2HPO4	0.009
NaH2PO4·H2O	0.004
CaCl2·2H2O	0.02
MgCl2·6H2O	0.003
NH4Cl	0.01
NaHCO3	3
Yeast extract	0.02
Trace element solution*	1mL

*Table AS4. *Trace element solution*

Compound	Concentration
Nitrilotriacetic acid	1.50 g/L
MgSO4·7H2O	3.00 g/L
MnSO4·H2O	0.50 g/L
NaCl	1.00 g/L
FeSO4·H2O	0.10 g/L
CoSO4·7H2O	0.18 g/L
CaCl2·2H2O	0.10 g/L
ZnSO4·7H2O	0.18 g/L
CuSO4·5H2O	0.01 g/L
KAl(SO4)2·12H2O	0.02 g/L
H3BO3	0.01 g/L

Na2MoO4 ·2H2O	0.01 g/L
NiCl2 ·6H2O	0.03 g/L
Na2SeO3 ·5H2O	0.30 mg/L
Na2WO4 ·2H2O	0.40 mg/L

Appendix B: Nitrogen Balances and Impacts on the Cultivation-Extraction-Digestion-Cultivation Process

(Submitted for review in Algal Research)

Abstract

Anaerobic digestion of lipid extracted algae is a method for recycling nutrients to a cultivation system. Growth of algae on recycled nitrogen from these systems has been demonstrated. This study focuses on how the initial nitrogen concentration impacts each stage of an integrated cultivation-extraction-digestion-cultivation process; and how the nitrogen is distributed in each step. The entire process was performed at five different initial nitrogen concentrations, ranging from nutrient deplete to excess. Algae production and lipid content were measured to evaluate the impact of initial nitrogen concentration. A thorough nitrogen mass balance for every step of the process was completed. The results show that initial nitrogen concentration had significant impacts on the whole process. The nitrogen recoveries through the nutrient recycle process were all approximately 65% regardless of initial nitrogen concentration. All nitrogen balances closed within experimental error. The largest losses of nitrogen occurred in the extraction and cultivation steps.

Key words: Algal biofuel, nitrogen recycle, anaerobic digestion, *Chlorella sorokiniana*

List of Abbreviations

LEA	Lipid extracted algae
Digestate	The nutrient effluent from anaerobic digestion
$\mathbf{N_0}$	Initial nitrogen input in the initial algae cultivation
$\mathbf{N_{algae}}$	N in produced algal biomass on cultivation
$\mathbf{N_{nitrate}}$	Excess nitrogen in the form of N-nitrate in cultivation
$\mathbf{N_{ex}}$	Extracellular nitrogen products in spend medium
$\mathbf{N_{lipid}}$	Nitrogen in lipid of algae
$\mathbf{N_{LEA}}$	Nitrogen in lipid extracted algae
$\mathbf{N_{SI}}$	Nitrogen in initial sludge
$\mathbf{N_{SF}}$	Nitrogen in final sludge
$\mathbf{N_{digestate}}$	Nitrogen in digestate
$\mathbf{N_{ReAlgae}}$	Nitrogen in recycled algae in re-cultivation
$\mathbf{M_{algae}}$	Weight of algae production in initial cultivation
$\mathbf{M_{ReAlgae}}$	Weight of algae production in re-cultivation

B.1. Introduction

The increasing demand for energy and potential shortages of non-renewable petroleum in the future have been reported, thus alternative sources of renewable fuel require continued evaluation (Ghasemi et al., 2012). Algae based biofuels are promising and have the advantages of high photosynthesis efficiency, conversion of CO_2, high biomass productivity per acre, and high oil content (Su et al., 2017; Bradley et al., 2015). Although currently algal biofuel is not financially competitive with fossil fuels, it has the potential to become so with improvements in cultivation practices and effective recycling of nutrients such as nitrogen and phosphorous (Quinn and Davis, 2015).

Algal fuel production on a commercial scale may have negative impacts on agriculture. In particular, sources of nitrogen and phosphorous need to be closely monitored (Zhang and Ogden, 2017; Chisti, 2013; Aida et al., 2017). Nitrogen as a primary nutrient required by algae has a significant impact on algal growth, algal oil and biochemical compositions (Li et al., 2015; Mubarak et al., 2015; Ikaran et al., 2015; Griffiths et al., 2014). In most cases, higher nitrogen concentrations result in higher algal production, unless the culture becomes light limited (Li et al., 2015). However, nitrogen deficiency usually results in higher biomass yield coefficients (algal production per unit nitrogen input) and often promotes increases in oil content in many algal species (Li et al., 2015; Mubarak et al., 2015). Nitrogen starvation

usually results in lower protein and chlorophyll content and higher carbohydrate and lipid content (Ikaran et al., 2015; Griffiths et al., 2014). The biochemical composition of the algal lipid also changes depending on concentration and source of nitrogen (Ordog et al., 2016).

Nitrogen supply requires thoughtful sourcing and reducing nitrogen cost is a necessary step to improve the operating costs of algae cultivation (Talbot et al., 2016). Nitrogen recycle via anaerobic digestion is a promising technology (Ayala-Parra et al., 2017). This process can be integrated into the algae cultivation scheme. After the algae are grown, harvested and the lipids are extracted to produce fuel, the resulting lipid extracted algae (LEA) is biodegraded in an anaerobic digester, methane is produced, and ammonium is released. The ammonium is recycled as a source of nitrogen for algal regrowth. Effective algae cultivation on recycled nitrogen and theoretical energy efficiency were demonstrated for this process (Zhang and Ogden, 2017; Morken et al., 2013). However, the efficiency of the nitrogen recycling process correlated with starting nitrogen input has not been quantitatively evaluated. Nitrogen mass balances for the process have not been completed.

This research focuses on performing nitrogen mass balances on the entire process of algae cultivation, extraction, anaerobic digestion, and re-cultivation. Previous studies do not quantify the amount of nitrogen found in the lipid fractions,

that is therefore lost from the system and not able to be recycled. In this study, the amount of nitrogen was monitored in the liquid media, the algal cells, the extracted cells, and in the digestate (digestate is referred as the nutrient effluent from anaerobic digestion) throughout the process. Furthermore, the initial nitrogen concentration for algae cultivation was varied to determine if there were any variations in the ability to recycle nitrogen if the initial culture was nitrogen rich or nitrogen replete.

B.2. Materials and Methods

The system consisted of four stages: algae cultivation, lipid extraction, anaerobic digestion and algae recultivation. A schematic is shown in Figure B1. This process started by cultivating the algae with different (0.012-0.089 g/L) initial amounts of N-nitrate (N_0). After cultivation, the nitrogen was distributed as produced algae (N_{algae}), N-nitrate residues in medium ($N_{nitrate}$) and extracellular nitrogen products in the spent media (N_{ex}). The second step was the lipid extraction step. The amount of nitrogen in the lipid (N_{lipid}) and lipid extracted algae (N_{LEA}) was determined. The LEA was biodegraded by anaerobic digestion and the initial nitrogen in this stage was determined as the sum of N_{LEA} and initial nitrogen in the sludge (N_{SI}). At the end of this process, part of nitrogen remained in the final sludge (N_{SF}), and the rest was released into the digestate ($N_{digestate}$) typically in the form of ammonium. Nitrogen in the digestate was used for algal recultivation. Again, the nitrogen was monitored and

was distributed into algal biomass ($N_{ReAlgae}$) or as extracellular nitrogen products (N_{ex}). The nitrogen values in the mainstream or center stream of Figure B1 were considered to be recycled N streams (N_0, N_{algae}, N_{LEA}, $N_{digestate}$, $N_{ReAlgae}$), while the nitrogen values in tributaries or the right-hand column were considered as nitrogen losses ($N_{nitrate}$, N_{ex}, N_{lipid}, N_{SF}) from the system since they were not able to be recycled to the algal bioreactors. Nitrogen distributions in all stages were monitored to calculate nitrogen balances. The whole process was repeated three times and the mean values are reported. Error bars represent standard deviations.

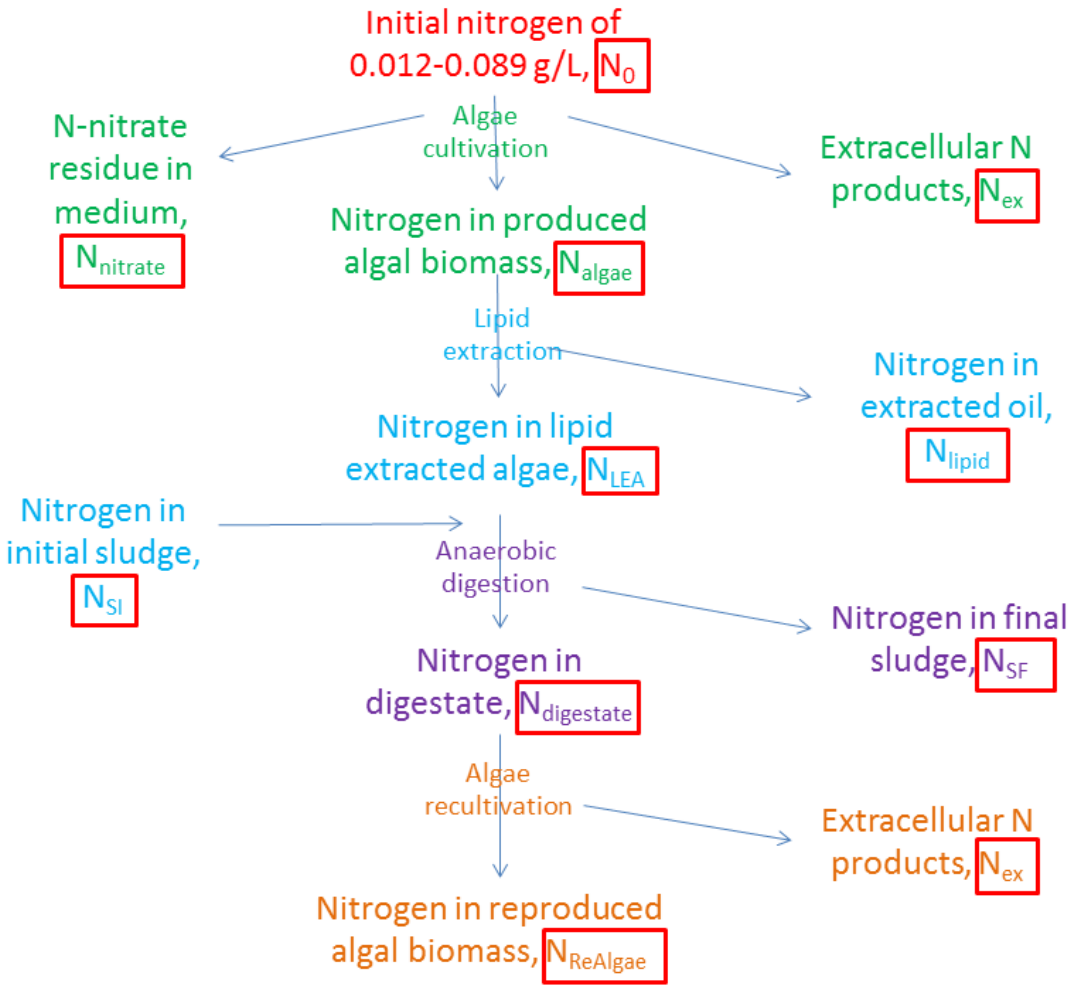


Figure B1. Schematic of the nitrogen recycle process highlighting the nitrogen concentrations that were measured during experimentation.

B.2.1 Algae Cultivation

Chlorella sorokiniana was chosen for the experiments due to its high productivity (Zhang and Ogden, 2017; Unkefer et al., 2017). Algae were cultivated in 500 mL of Pecos medium developed by colleagues from Texas A&M Agrilife in

Pecos, Texas (constitutions shown in Table BS1). The $NH_4H_2PO_4$ was replaced by the same concentrations of NaH_2PO_4, and $(NH_2)_2CO$ was replaced by five different concentrations of N-$NaNO_3$: 0.012 g/L, 0.021 g/L, 0.045 g/L, 0.075 g/L, 0.089 g/L.

The medium was added to 1 L flasks, autoclaved, and inoculated with 15 mL of an approximately 0.4 g/L starter culture of *C. sorokiniana*. The algae were cultivated with a continuous flow of 950 mL/min air and 50 mL/min CO_2 to provide mixing and a source of carbon. A 12/12 light/dark cycle was used; the light intensity was 80 μmol m^{-2} s^{-1}. Algae were harvested when they reached stationary phase due to light limitation. The culture was sampled daily to determine the biomass and nitrate concentrations. In addition, samples were taken on day 0, day 4 and day 8 to determine the total nitrogen in the media.

The growth of algae was monitored by measuring the optical density (OD) using an ultraviolet-visible spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, Inc., Waltham, MA) at a wavelength of 750 nm. Samples were taken from medium every day and diluted to ensure that the measured OD_{750} values were within the range of 0.1-0.8. A calibration curve relating biomass weight to OD was determined for algal cell growth. The conversion from OD to biomass is 0.1725 g biomass / OD_{750} (Zhang and Ogden, 2017).

At the end of the growth experiment, the culture was centrifuged; the supernatant was analyzed for nitrate and total nitrogen while the harvested algae were dried and weighted to monitor the biomass production. The nitrogen content of the solid algal biomass was also determined.

B.2.2 Lipid Extraction

Microwave extraction with methanol-chloroform (1:2) was done to extract the lipid (Veggi et al., 2013; Ryckebosch et al., 2013). Algae were dissolved in methanol-chloroform overnight, and then heated in the microwave (CEM MDS 2100 Microwave oven with solvent sensor) at 70 ℃ for 75 min. After the sample cooled to room temperature, the liquid was separated from the LEA by filtering through glass fiber, and then dried by continuous nitrogen gas flow. Methanol-chloroform was evaporated, and the extracted lipid was collected. Extracted lipid and lipid extracted algae were both weighted, and the nitrogen content of both lipid and LEA were measured.

B.2.3 Anaerobic Digestion

Lipid extracted algae were washed with hexane twice to avoid any chloroform residue. Hexane was removed by centrifugation and the LEA was dried in a horizontal

air flow oven (VWR International West Chester, PA) at 70 ℃ for 24 h. This LEA was used as substrate for anaerobic digestion. Anaerobic sludge was received from the Pima County Ina Road Wastewater Treatment Plant in Tucson, Arizona. The initial ratio of wet sludge to LEA was 4:1. The volatile suspended solid (VSS) per gram of wet sludge was determined by drying and was 0.03 g VSS per gram wet sludge. The dried sludge sample was ground, and the nitrogen content of dry sludge was measured.

The anaerobic digestion system was the same as the one used previously (Zhang and Ogden, 2017; Table BS2 shows the anaerobic digestion medium used in this study). Lipid extracted algae were mixed with wet sludge in the ratio of 1:1 in 500 mL basal anaerobic digestion medium in 1L flasks. Initial water samples were taken. The system was sealed, and inside air was removed by flushing with a mixture of 80% N_2 and 20% CO_2. Samples of water were collected every two days to determine the total nitrogen content. When the anaerobic digestion was complete, the digestate and sludge were separated by centrifugation. The wet sludge before and after anaerobic digestion was weighted, and samples from wet sludge were dried and analyzed for nitrogen content to calculate nitrogen balances.

B.2.4 Algae Recultivation

The digestates were purified by filtration through 2.0 μm filters to remove any bacteria. Algae were cultivated on the digestate, and the growth conditions were the same as initial algae cultivation described in section 2.1. Nitrogen concentration changes throughout the cultivation process were monitored and the nitrogen content in the produced algal biomass was measured.

B.2.5 Measurement of Nitrate in Liquid Media ($N_{nitrate}$)

N-nitrate concentration was measured by ion chromatography (Dionex ICS-5000; Dionex Corporation, Sunnyvale, CA, USA). A Dionex IonPac AS18 (2 ×250mm) column was used with a sample injection size of 10 μL. KOH eluent flow rate was kept constant at 0.38 mL min^{-1}. The operating temperature was set at 30 ℃. Ion peak detection was undertaken by suppressing conductivity measurements at 22 mA. Chromeleon provided by Dionex was used to analyze the spectra. N_0 (g) is initial N-nitrate equivalent weight.

B.2.6 Measurement of Total Nitrogen in Medium and Solid Samples

Total nitrogen in the medium including N_{ex} and $N_{digestate}$ was measured for the mass balance analysis. N_{ex} was determined by measuring the total nitrogen in the medium at the end of algae cultivation and re-cultivation steps. Total nitrogen

concentration was analyzed on the Model TOC-L carbon analyzer with TN-L module (Shimadzu Scientific Instruments of Columbia, MD). Samples were diluted to final concentrations between 0.05 mg/L and 3.5 mg/L, the measurement range used to create a standard curve. All samples, standards, and quality standards (QCs) were prepared in 24 mL scintillation vials that were baked for 4 h at 475 C ºand made using RO water (18.2 mΩ). QCs include a calibration blank check (CCB), continuing calibration check (CCC), and a certified reference material check (CRM). All QCs were within ±10% error and were run before and after each batch of samples.

The total nitrogen content of solid samples including dried algae, lipid, LEA and sludge were measured to calculate total nitrogen of N_{algae}, N_{lipid}, N_{LEA}, N_{SI} and N_{SF}. Solid samples were ground and dried at 60 ℃ for 1 h. Nitrogen contents of solid algae were analyzed on the ECS 4010 CNS analyzer (Costech Analytical Technologies, Valencia, CA). The system used flash combustion, GC separation, and a thermal conductivity detector (TCD) to effectively determine total nitrogen content. Calibration standards were sulfanilamide and LECO samples that include soil, corn gluten, and cysteine.

B.2.7 Nitrogen Balance Calculations

To evaluate the nitrogen throughout the entire process, nitrogen containing components in each stage were monitored and mass balances were calculated. Figure B1 highlights with red rectangles all components that were analyzed for nitrogen content and used in the mass balances.

The initial nitrogen source for cultivation was the N-nitrate in the medium, N_0. At the end of the algae cultivation period, nitrogen existed as: nitrogen in the produced algal biomass (N_{algae}), excess nitrogen in the form of N-nitrate ($N_{nitrate}$), and as extracellular nitrogen products (N_{ex}) that were in the spent media. Only the nitrogen found in the algal biomass had the potential to be recycled. The initial nitrogen balance (input is red; output is green in Figure B1) is:

$$N_0 = N_{algae} + N_{nitrate} + N_{ex} \quad (1)$$

After lipid extraction, N_{algae} was divided between the extracted lipid (N_{lipid}) and lipid extracted algae (N_{LEA}). In this step, the N_{lipid} was lost from the system and N_{LEA} continues down the recycle nutrient path. The total nitrogen balance for this step (input is green; output is blue in Figure B1) is:

$$N_{algae} = N_{LEA} + N_{lipid} \quad (2)$$

After anaerobic digestion, N_{LEA} was either found in the digestate ($N_{digestate}$) or in the sludge primarily as part of the newly produced bacterial cells. Since the sludge used as inoculum contained nitrogen (N_{SI}), this was also taken into account. The

$N_{digestate}$ continues in the recycled nutrient path while the N_{SF} in the final sludge was lost. The resulting nitrogen balance (input is blue; output is purple) is:

$$N_{SI} + N_{LEA} = N_{digestate} + N_{SF} \quad (3)$$

Finally, during algal recultivation, $N_{digestate}$ served as the nitrogen source in the form of ammonium and it was completely taken up by the cells. The nitrogen therefore was either part of the algae ($N_{ReAlgae}$) or was excreted by the cells as extracellular nitrogen products (N_{ex}). The nitrogen balance (input is purple; output is orange) is:

$$N_{digestate} = N_{ReAlgae} + Nex \quad (4)$$

To better evaluate the impact of nitrogen, yield coefficients are reported. Algal biomass and lipid yield coefficients are calculated by relating biomass to "In" nitrogen as follows:

$$Y_{Algae} = M_{algae} / N_0 \quad \text{and} \quad Y_{ReAlgae} = M_{ReAlgae} / N_{digestate} \quad (5)$$

$$Y_{Lipid} = Y_{Algae} \times Lipid\% \quad \text{and} \quad Y_{ReLipid} = Y_{ReAlgae} \times Lipid\% \quad (6)$$

B.3. Results and Discussion

The objective of the cultivation-extraction-digestion-cultivation process is to recycle as much nitrogen as possible. However, the initial nitrogen may distribute into extracellular products, extracted oil, and newly produced sludge making it challenging to be totally recycled. To better understand how nitrogen is distributed and thus help

lower nitrogen waste, nitrogen balances were analyzed. Moreover, since the nitrogen concentration has a significant influence on algal cultivation, the impact of initial nitrogen concentration was monitored (Li et al., 2015; Mubarak et al., 2015; Ikaran et al., 2015; Griffiths et al., 2014; Ordog et al., 2016).

The initial concentration of N-nitrate (N_0) in this study ranged from 0.006 to 0.045 g in 500 mL of media or 0.012 to 0.089 g/L. When the nitrogen concentration was lowered to less than 0.01 g/L, algal growth was not apparent. The algae inoculum formed clumps and turned yellow. When the nitrogen concentration was increased above 0.089 g/L, the final yield of algal biomass did not increase, and extra nitrate was not removed from the media because the culture was light limited. The significant impact of light intensity on growth of *Chlorella sorokiniana* has been demonstrated by previous research (Kumar et al., 2014).

B.3.1 Nitrogen Impacts on Algae Cultivation – Lipid Extraction – Anaerobic Digestion – Algae Recultivation Process

B.3.1.1 Algae Cultivation

The algae growth curves for different initial nitrogen concentrations are shown in Figure B2(a). As expected, the medium with 0.012 g/L of initial nitrogen produced the lowest level of algal biomass (0.41 g/L); based on algal growth and nitrogen data,

less than 0.021 g/L can be considered as deplete nitrogen. Additional nitrogen significantly enhanced algal biomass production when the initial nitrogen was lower than 0.075 g/L. The medium with sufficient or excess nitrogen (> 0.075 g/L) produced the maximum level of algal biomass (1.16 g/L) for these cultivation conditions. There was no statistically observable difference in the final algal biomass production when N_0 was between 0.075 g/L and 0.089 g/L. In accordance with previous studies, the lower nitrogen concentration resulted in less algae production (Zhang and Ogden, 2017; Can et al., 2016).

Figure B2(b) shows the corresponding N-nitrate changes during algal growth. Nitrogen was rapidly absorbed by algae in the first few days due to the high demand for nitrogen. This was most likely due to luxury uptake, in which algae absorbed more nitrogen than required at the start of cultivation for future use. The high levels of nitrogen accumulated in algae cells in inorganic forms or rapidly form simple N-containing compounds. No N-nitrate residue was found at the end of cultivation when the initial nitrogen concentration was less than 0.075 g/L. Using a range of initial nitrogen concentrations allowed us to compare and contrast the recycling and assimilation of nitrogen by the algae across a range of growth conditions from nitrogen starvation to excess nitrogen.

The percent nitrogen (N_{Algae} /M_{Algae}, or the ratio of nitrogen in algal biomass to the total mass) of the resulting produced algal biomass is provided in Figure B2(c). Higher nitrogen content corresponded with higher initial nitrogen concentration, especially when the nitrogen amount was above deplete conditions. This is supported by previous research that states that a limited nitrogen supply dramatically lowers protein content for *Chlorella* sp. The major nitrogen containing product is typically protein (Ikaran et al., 2015). The reason for the differences in protein content can be the mechanism of autophagy, in which algae automatically biodegrade some organelles or proteins to release some nitrogen for more critical functions. This generally happens when nitrogen is not sufficient for algae growth (Adams and Bugbee, 2014). Under deplete nitrogen conditions, the algae often biodegrade many of their proteins, and as the nitrogen concentration increases, more of the proteins are kept intact.

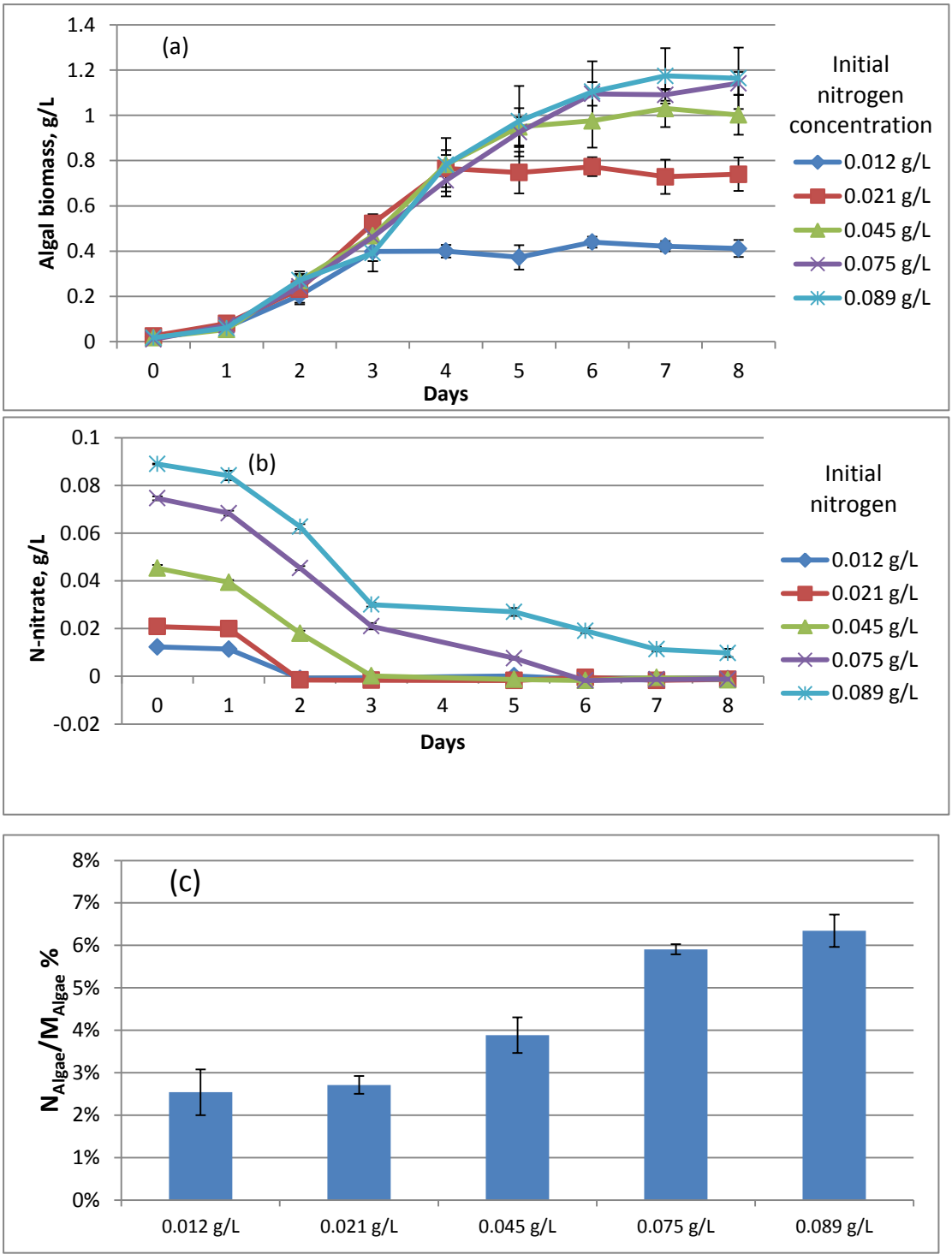


Figure B2. Impacts of different initial nitrogen concentrations: (a) algal growth curve in 500mL medium; (b) N-nitrate concentration changes in 500 mL medium; (c) nitrogen content in produced algal biomass.

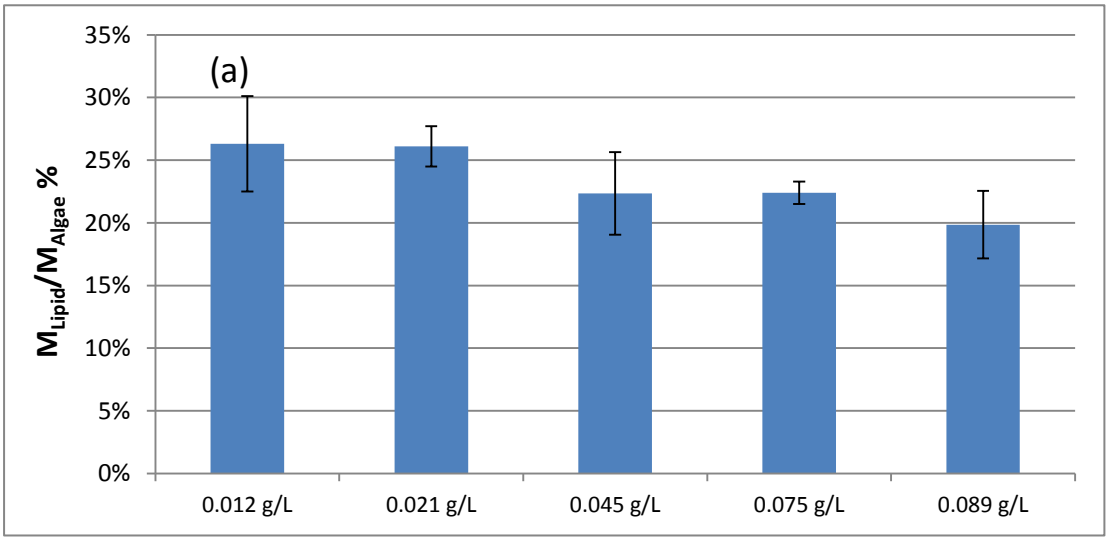
B.3.1.2 Lipid Accumulation

Lipid content of the algal biomass is shown in Figure B3(a). The lipid content in the algae was higher when the nitrogen was limited. As the initial nitrogen concentration increased, the lipid content decreased from 26.3% to 19.9%. This trend is often observed for *Chlorella sp*. (Li et al., 2015; Griffiths et al., 2014). There are two suggested mechanisms for lipid accumulation in the literature. One is that under nitrogen-deficiency, algal cells often accumulate a surplus of carbon metabolites as lipids (Yeesang and Cheirsilp, 2011). Another explanation is that nitrogen limitation leads to biodegradation of nitrogen containing macromolecules, increasing the amount of the fatty acids and active diacylglycerol acyltransferase, which converts fatty acids to triglyceride (Li et al., 2015; Yeesang and Cheirsilp, 2011).

Figure B3(b) shows the percent nitrogen of the lipid fraction (ratio of the mass of nitrogen to the total lipid mass). Nitrogen content of lipid was quite low (< 2%) when there was not excess nitrogen. Therefore, to limit nitrogen loss in the lipid fraction, the amount of nitrogen provided to the algae during cultivation should be optimized as a function of light and temperature, the two factors that influence algal growth rate and productivity the most. (Kumar et al., 2014) The amount of nitrogen in the lipid fraction was as high as 6.2%. It is reported in the literature that algal lipid can be composed of 300-600 different components including various nitrogen containing

components. Some examples found in *Chlorella* sp. include oxidized betaine and sphingoid base analogs (Holguin and Schaub, 2013; Vardon et al., 2012). As algal cells reproduce, part of the nitrogen is used for new cells but some excess nitrogen may stay in the lipid, which accounts for the higher nitrogen content (Adams and Bugbee, 2014).

Figure B3(c) shows the nitrogen content in LEA (ratio of mass of nitrogen in LEA to total mass of LEA). The nitrogen content increased with higher initial nitrogen concentration. Since the majority of the LEA is protein, and protein content increases with increasing nitrogen, the trend is expected. This trend is in accordance with Griffiths el al.'s results of whole cell algae (Griffiths et al., 2014).



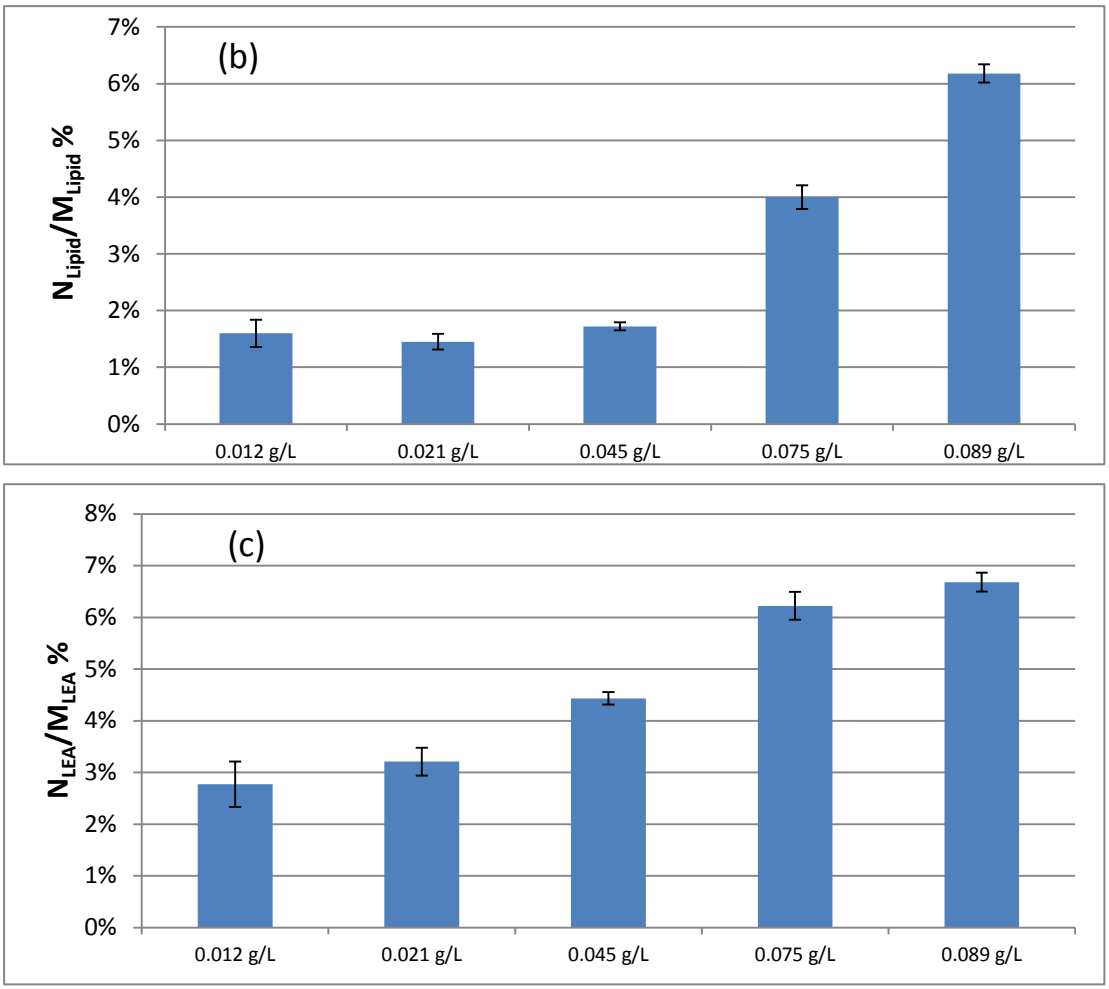


Figure B3. Impact of initial nitrogen concentration on lipid accumulation in algal biomass: (a) lipid content of whole algal biomass; (b) nitrogen content in lipid; (c) nitrogen content in LEA.

B.3.1.3 Production and Yield Coefficient of Biomass and Lipid

Based on the algae production from Figure B2(a) and lipid content from Figure B3(a), the initial nitrogen concentration of 0.075 g/L resulted in the highest production of both algae and lipid at a light intensity of 80 μmol m^{-2} s^{-1} used in this

study (Fig BS1). However, the nitrogen yield coefficients for biomass and lipid decreased as initial nitrogen increased (Figure B4). Under deplete nitrogen conditions the yields were the highest, more than double the amount when there was excess initial nitrogen. This trend has been observed by others (Yeesang and Cheirsilp, 2011; Talbot et al., 2016; Griffiths et al., 2014).

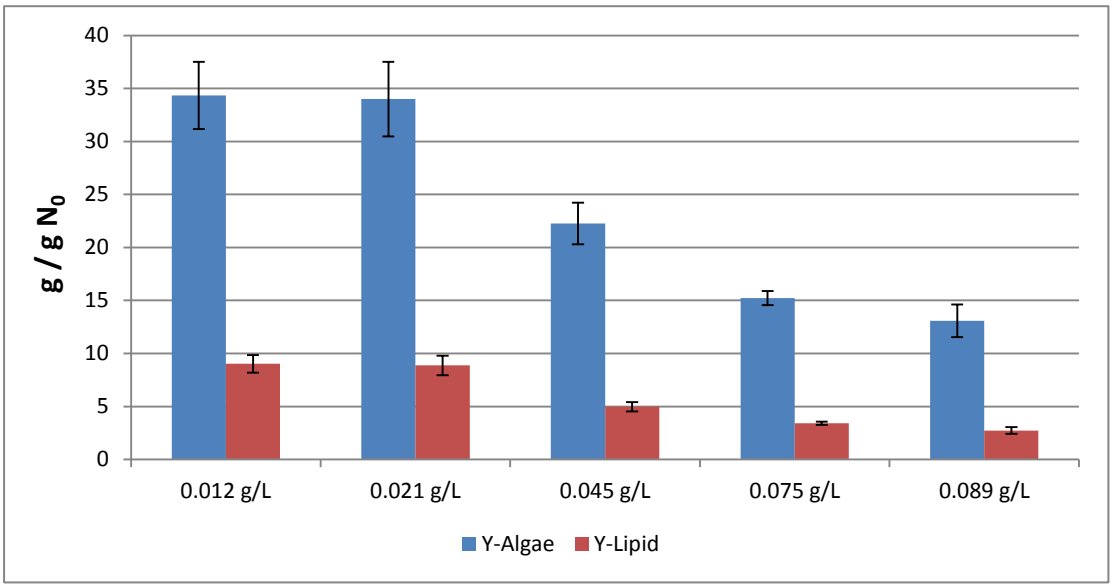


Figure B4. Impacts of initial nitrogen concentration on algal biomass yield coefficient and lipid yield coefficient versus initial nitrogen inputs.

B.3.1.4 Anaerobic Digestion

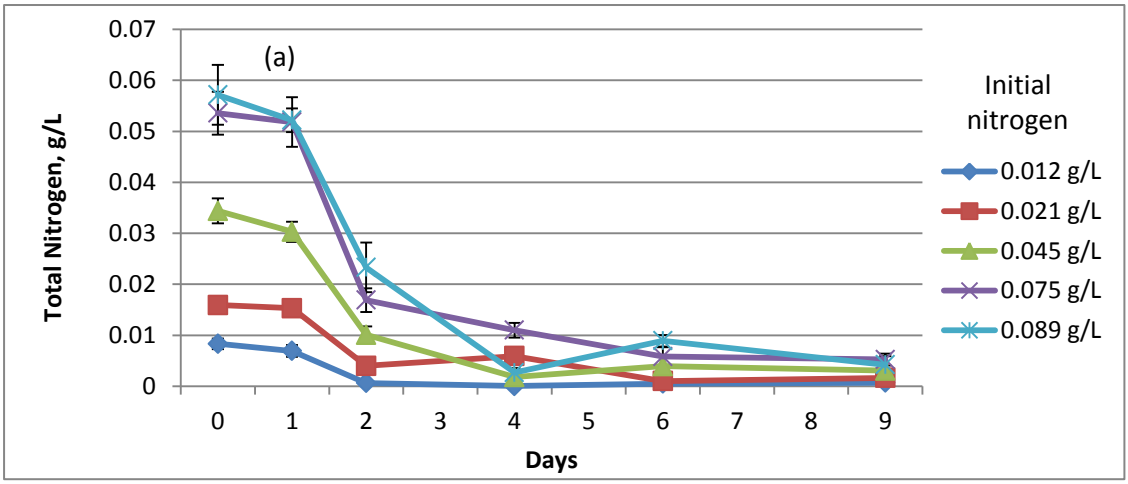
The amount of nitrogen released into the digestate during anaerobic digestion is shown in Fig BS2(a). LEA was biodegraded and nitrogen was rapidly released. A

minimum of 42% of the nitrogen was released in the first day of anaerobic digestion Fig BS2(b). After 5 days, the majority of the nitrogen originally contained in the LEA was found in the digestate regardless of the total amount contained in the LEA. This is supported by previous research on anaerobic digestion of LEA (Zhang and Ogden, 2017; Ayala-Parra et al., 2017). Apart from the nitrogen in the digestate, some nitrogen was utilized for sludge growth; although only 2.9 to 5.2 %.

B.3.1.5 Algae Recultivation

To complete the cycle, the algae were cultivated using digestate as the nitrogen source. The form of the nitrogen was primarily ammonium. Previous work demonstrated that ammonium in high concentration can be toxic and inhibit algae growth due to the rapid pH changes during cultivation. In this work, the initial N-ammonium concentrations during recultivation were lower than 0.06 g/L which was not high enough to be toxic for *C. sorokiniana* (Fogg et al., 1973; Gutierrez et al., 2016). Figure B5(a) shows the nitrogen removal as a function of time during cultivation. As the initial nitrogen concentrations in the digestate were lower than 0.075 g/L, there were no environmental limitations and the nitrogen was be totally absorbed by algae. Any nitrogen found in the liquid media was in the form of extracellular nitrogen products (N_{ex}). The production (Fig BS3) and yield coefficients

(Figure B5(b)) of algal biomass and lipid for algal recultivation were calculated. Similar to the initial cultivation that was done on nitrate, the biomass nitrogen yield was highest at low concentrations of digestate nitrogen and decreased when more nitrogen was supplied to the system.



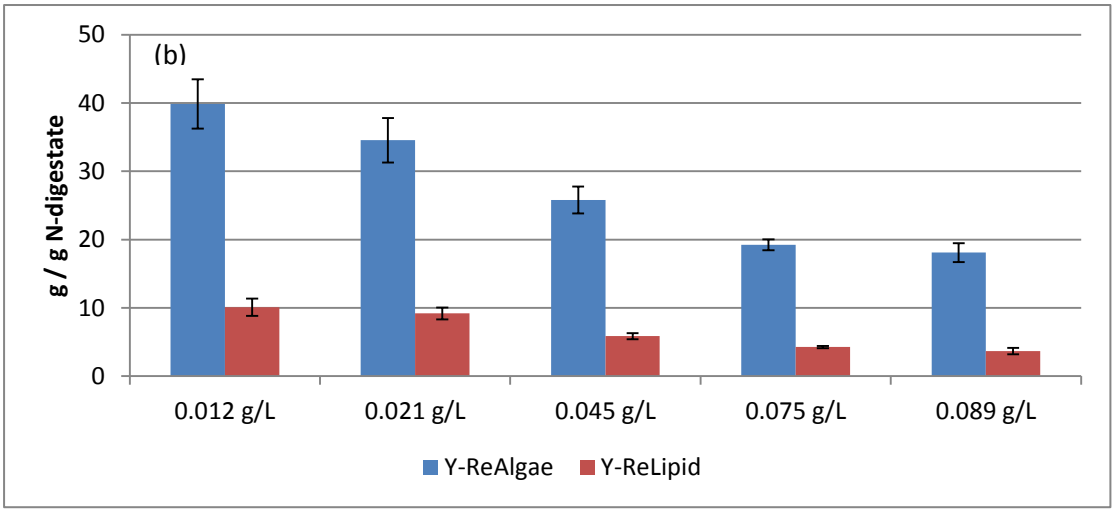


Figure B5. Impacts of initial nitrogen concentration (N_0) on algae recultivation: (a) total nitrogen changes in 500 mL digestate during algal recultivation; (b) yield coefficient of reproduced algal biomass and lipid versus initial N-digestate.

B.3.2 Nitrogen Balances

B.3.2.1 Nitrogen Balance Calculations

In this study, nitrogen balances were analyzed throughout the algae cultivation, lipid extraction, anaerobic digestion and algae recultivation process. The balances with different initial nitrogen (N_0) were monitored and compared. N_0, N_{algae}, N_{LEA}+ N_{SI} and $N_{digestate}$ are respectively considered as "In-Nitrogen" in the four stages (Figure B1). Eqns. (1) to (4) are mass balance equations used in the calculations. The difference between "In-Nitrogen" and "Out-Nitrogen" divided by the "In-Nitrogen" was determined to show how well we were able to account for nitrogen throughout the process. Table B1 shows the results of balance calculations. The data are average values based on three repeated experiments, and the +/- values represent the standard deviations. The mass balance analysis showed that the percent difference, regardless of processing step or initial nitrogen concentration, was very low (less than 4.0%). This validated the experimental and analytical methods in this research. Throughout the entire process, the nitrogen balance was statistically closed even though a variety of techniques were used to monitor nitrogen concentration including ion chromatography for N-nitrate, CNS for nitrogen in solids, and TN-L for N-ammonium and mixed total nitrogen. This work demonstrates that nitrogen can be tracked throughout the process and hence nitrogen loss can be minimized using this

information. Different initial nitrogen concentrations did not impact the ability to monitor nitrogen even though there were variations in the amount of nitrogen found in the protein and lipid fractions.

Nitrogen weight, g	0.012 g N_0/L	0.021 g N_0/L	0.045 g N_0/L	0.075 g N_0/L	0.089 g N_0/L
Algae cultivation step					
In (N_0)	0.006 +/-0.0001	0.0105 +/-0.0001	0.0225 +/-0.0003	0.0375 +/-0.0005	0.0445 +/-0.0004
Out 1 (N_{Algae})	0.0052 +/-0.0009	0.0097 +/-0.0004	0.0195 +/-0.0008	0.0337 +/-0.0009	0.0369 +/-0.003
Out 2 ($N_{nitrate}$)	0	0	0	0	0.005 +/-0.0008
Out 3 (N_{ex})	0.0006 +/-0.0001	0.0010 +/-0.0001	0.0022 +/-0.0002	0.0034 +/-0.0004	0.0036 +/-0.0006
Out sum	0.0058 +/-0.001	0.0107 +/-0.0005	0.0217 +/-0.001	0.0371 +/-0.001	0.0455 +/-0.005
Difference%	3.13%	-1.62%	3.65%	1.14%	-2.32%
Lipid extraction Step					
In (N_{algae})	0.0052 +/-0.0009	0.0097 +/-0.0004	0.0195 +/-0.0008	0.0337 +/-0.0009	0.0369 +/-0.003
Out 1 (N_{lipid})	0.0009 +/-0.0002	0.0014 +/-8 E-05	0.0019 +/-0.0003	0.0051 +/-0.0002	0.0075 +/-0.0008
Out 2 (N_{LEA})	0.0042 +/-0.0009	0.0082 +/-0.0004	0.0172 +/-0.001	0.0279 +/-0.001	0.0309 +/-0.004
Out sum	0.0051 +/-0.001	0.0095 +/-0.0005	0.0191 +/-0.001	0.0331 +/-0.001	0.0384 +/-0.005
Difference%	2.94%	1.61%	2.01%	1.99%	-3.93%
Anaerobic digestion Step					
In 1 (N_{SI})	0.00091	0.00190	0.00290	0.00337	0.00346
In 2 (N_{LEA})	0.0042 +/-0.0009	0.0082 +/-0.0004	0.0172 +/-0.001	0.0279 +/-0.001	0.0309 +/-0.004

In Sum	0.00512 +/-0.0009	0.0101 +/-0.0004	0.0201 +/-0.001	0.0313 +/-0.001	0.0343 +/-0.004
Out 1 (N_{SF})	0.00108 +/-0.0001	0.00228 +/-0.0004	0.00365 +/-0.0008	0.00498 +/-0.0006	0.00469 +/-0.0008
Out 2 ($N_{digestate}$)	0.00417 +/-0.002	0.00796 +/-0.001	0.0169 +/-0.003	0.0268 +/-0.002	0.0286 +/-0.002
Out sum	0.00525 +/-0.002	0.0102 +/-0.002	0.0206 +/-0.004	0.0318 +/-0.002	0.0333 +/-0.003
Difference%	-2.50%	0.99%	-2.48%	-1.51%	3.11%
Algal recultivation step					
In ($N_{digestate}$)	0.00417 +/-0.002	0.00796 +/-0.001	0.0169 +/-0.003	0.0268 +/-0.002	0.0286 +/-0.002
Out 1 ($N_{ReAlgae}$)	0.00377 +/- 0.0005	0.00708 +/- 0.0006	0.0155 +/-0.001	0.0236 +/-0.001	0.0247 +/-0.005
Out 2 (N_{ex})	0.00036 +/-4 E-05	0.00082 +/- 3 E-05	0.00152 +/- 0.0002	0.00262 +/- 0.0006	0.00212 +/-0.0002
Out sum	0.00413 +/-0.0006	0.00791 +/-0.0007	0.0170 +/-0.001	0.0262 +/-0.002	0.0268 +/-0.005
Difference%	0.97%	0.69%	-0.61%	1.97%	6.13%

Table B1. Nitrogen balance calculations for algae cultivation, lipid extraction, anaerobic digestion and algae recultivation.

B.3.2.2 Nitrogen Distribution Analysis

To clearly understand how nitrogen was recycled or lost in each step, N_{Algae}, N_{LEA}, $N_{Digestate}$ and $N_{ReAlgae}$ are compared to N_0 in Figure B6. Nitrogen found in these forms was part of the recycle system, whereas the nitrogen found in the rest of the forms were lost from the system at each step of the process. Regardless of the cultivation conditions, deplete or sufficient initial nitrogen, approximately 65% of N_0

was recycled and used to grow additional algae. The biggest nitrogen loss of approximately 14% of N_0 occurred in the lipid extraction step. The second largest loss, 8.0%-10% of N_0 occurred in the algae cultivation step. The least amount of loss, approximately 3.0% of N_0 occurred in the anaerobic digestion step. Initial nitrogen concentration showed no obvious impacts on nitrogen recovery for this process when not in excess.

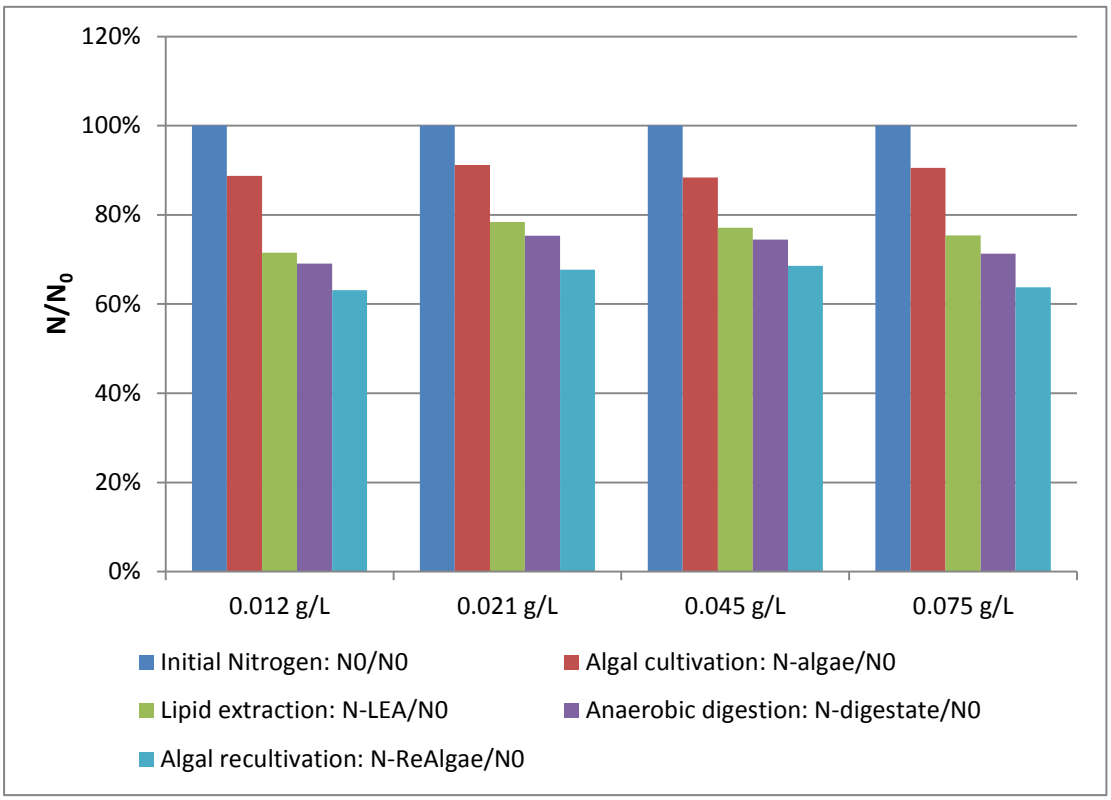


Figure B6. Percent of nitrogen compared to the initial nitrogen (N_0) at the end of algae cultivation, lipid extraction, anaerobic digestion and algal recultivation steps.

B.4. Conclusions

This work shows that nitrogen can affectively be recycled through anaerobic digestion of lipid extracted algae. The state of the algae, whether it has been grown under conditions of nitrogen starvation or with access nitrogen, has little effect on the ability to recover and recycle nitrogen. If a continuous cyclic system is developed to cultivate algae, extract algae, anaerobically digest the LEA, and then use the nutrients again, this is feasible, but around 35% of the nitrogen is lost in the cycle. Additional nitrogen would always have to be added. The majority of the loss occurs during the extraction process, and some is lost during cultivation. To increase the efficiency of this process, the extraction process should be optimized further to decrease losses.

This study has significant potential to indicate further investigation on nitrogen recycling. N impact analysis provided reference to optimize nitrogen utilization by controlling nitrogen concentration in the recycle process. Nitrogen balance analysis illustrated nitrogen distribution schematics and pointed out the potential future works to further enhance nitrogen recycle efficiency.

B.5. Acknowledgements

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Supporting Information

Table BS1. Pecos medium

Chemical	Final Concentration, g/L	Stock solution concentration	Volume, mL
$NaNO_3$	0.283	5.66 g/200 mL	10
$MgSO_4 \cdot 7H_2O$	0.012	2.4 g/200 mL	1
NaH_2PO_4	0.024	4.8 g/200 mL	1
Potash	0.075	7.5 g/200 mL	2
$FeCl_3$	0.00315	0.63 g/200 mL	1
Na_2CO_3	0.02	4 g/200 mL	1
EDTA	0.00436	0.218 g/200 mL	4
*Allen's solution			1

**Allen's solution*

Component	Amount	Final concentration
H_3BO_3	2.86 g/L	46 μM
$MnCl_2 \cdot 4H_2O$	1.81 g/L	9 μM
$ZnSO_4 \cdot 7H_2O$	0.22 g/L	0.77 μM
$Na_2MoO_4 \cdot 2H_2O$	0.39 g/L	1.6 μM
$CuSO_4 \cdot 5H_2O$	0.079 g/L	0.3 μM
$Co(NO_3)_2 \cdot 6H_2O$	0.049 g/L	0.17 μM

Table BS2. Anaerobic Digestion Medium

Compound	Concentration, g/L
K_2HPO_4	0.009
$NaH_2PO_4 \cdot H_2O$	0.004
$CaCl_2 \cdot 2H_2O$	0.02
$MgCl_2 \cdot 6H_2O$	0.003
NH_4Cl	0.004
$NaHCO_3$	3
Yeast extract	0.02
Trace element solution*	1mL

**Trace element solution*

Compound	Concentration
Nitrilotriacetic acid	1.50 g/L
$MgSO_4 \cdot 7H_2O$	3.00 g/L
$MnSO_4 \cdot H_2O$	0.50 g/L
NaCl	1.00 g/L
$FeSO_4 \cdot H_2O$	0.10 g/L
$CoSO_4 \cdot 7H_2O$	0.18 g/L
$CaCl_2 \cdot 2H_2O$	0.10 g/L
$ZnSO_4 \cdot 7H_2O$	0.18 g/L
$CuSO_4 \cdot 5H_2O$	0.01 g/L
$KAl(SO_4)_2 \cdot 12H_2O$	0.02 g/L
H_3BO_3	0.01 g/L
$Na_2MoO_4 \cdot 2H2O$	0.01 g/L
$NiCl_2 \cdot 6H_2O$	0.03 g/L
$Na_2SeO_3 \cdot 5H_2O$	0.30 mg/L
$Na_2WO_4 \cdot 2H_2O$	0.40 mg/L

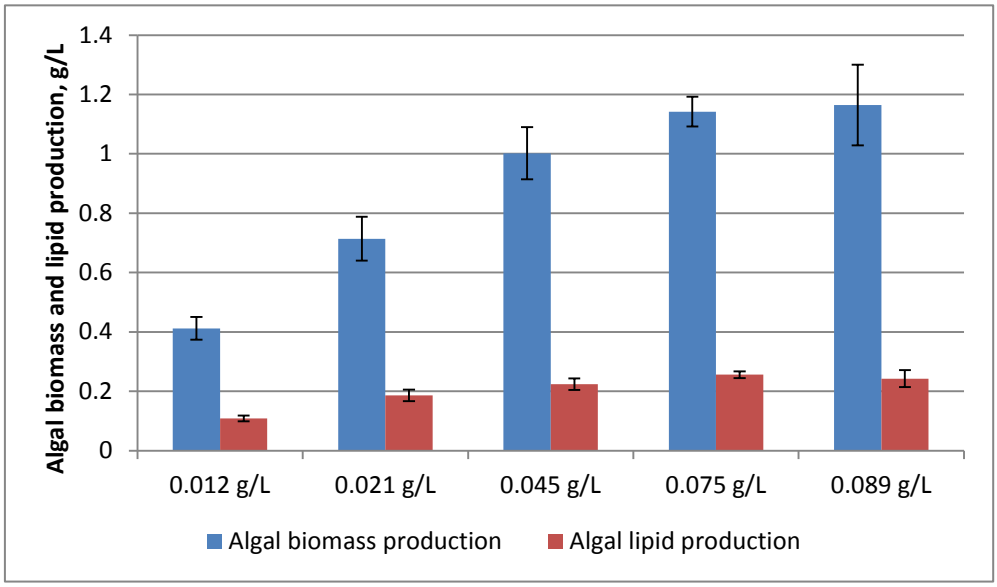

Figure BS1. Impacts of initial nitrogen concentration on algal biomass production and lipid production on 500 mL medium in initial cultivation

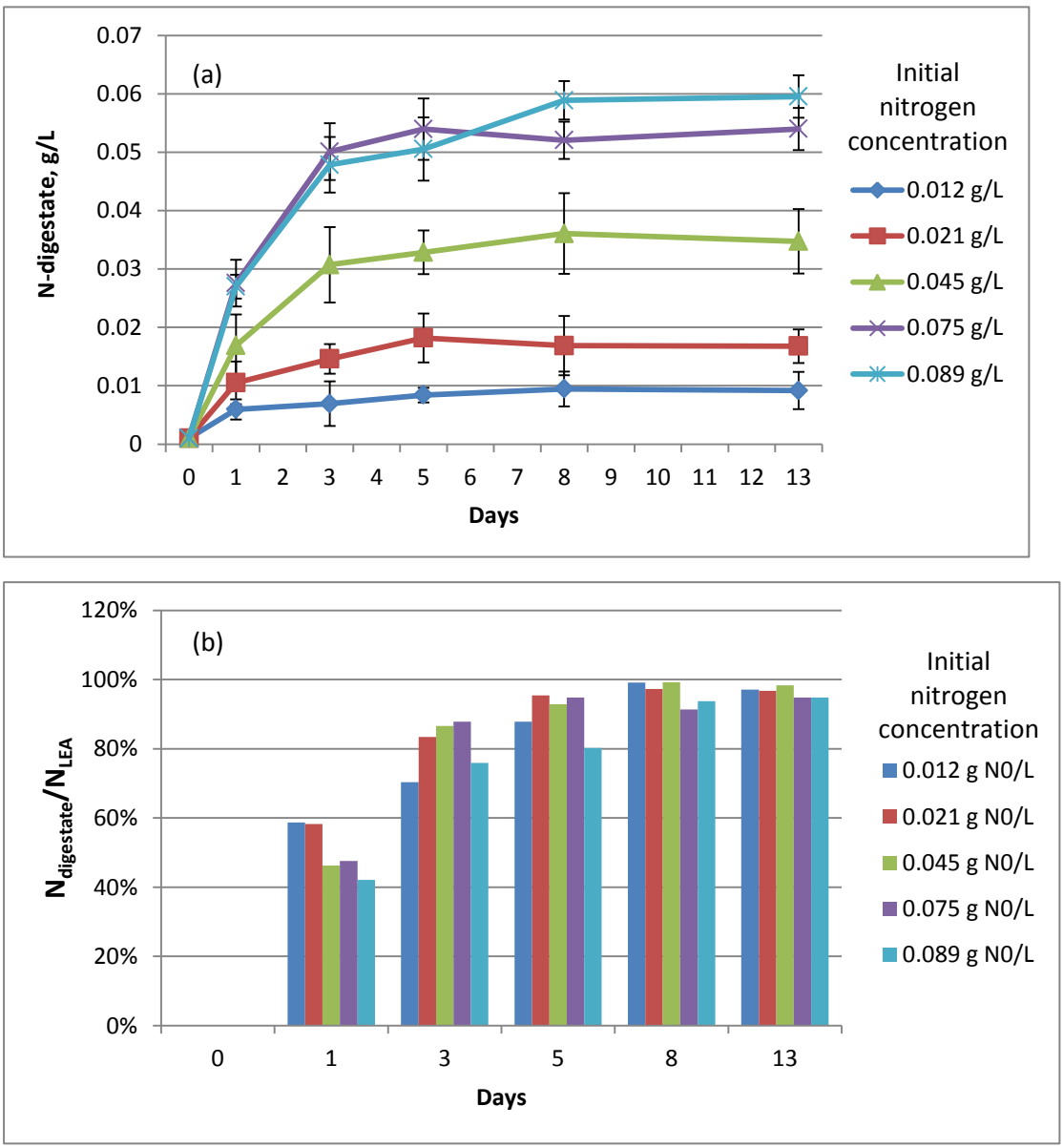

Figure BS2. Impacts of initial nitrogen concentration on nitrogen release in anaerobic digestion: (a) total nitrogen concentration in digestate in anaerobic digestion; (b) percent of nitrogen released in digestate compared to N_{LEA}.

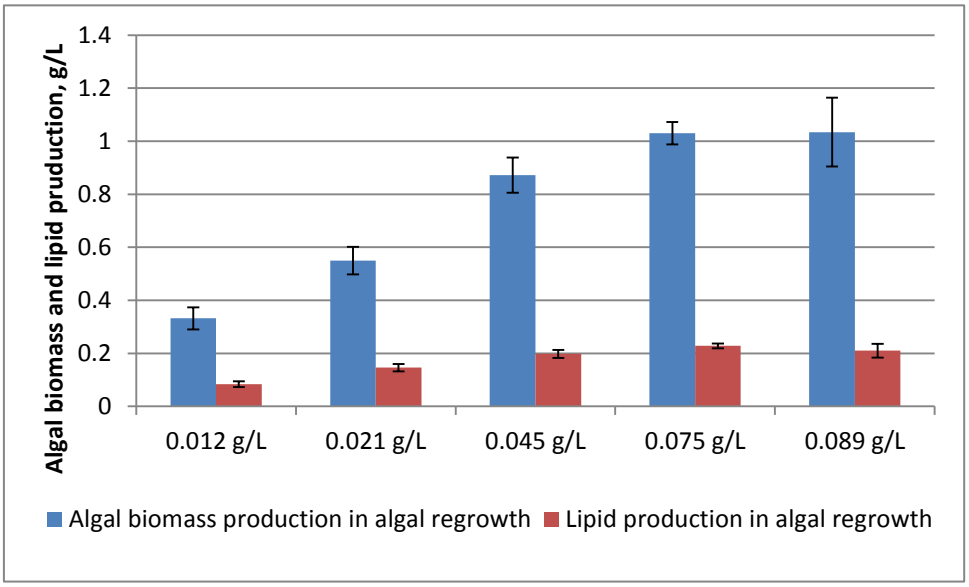

Figure BS3. Impacts of initial nitrogen concentration (N_0) on algal biomass production and lipid production on 500 mL digestate in algae recultivation