FRESHWATER ALGAL CULTIVATION WITH ANIMAL WASTE FOR NUTRIENT REMOVAL AND BIOMASS PRODUCTION

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ABSTRACT

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Excess nutrients, particularly nitrogen and phosphorus remained in the anaerobically digested (AD) manure effluent, have major impacts on the environment if disposed inappropriately. Algal cultivation, with the advantages of faster uptake of nutrients in waste streams, year-round production, and higher photosynthetic efficiency, represents one of the best processes for the removal of excessive nutrients. Meanwhile, algae have also been proved as one of the most promising non-food-crop-based feedstock for biofuels production. This study focuses on a practically and economically feasible algal cultivation system that satisfies the needs of nutrient removal, carbon sequestration, and biofuels production. Non-filamentous green algae, especially *Chlorella* sp., were able to tolerate high nutrient loadings in a five-month cultivation; a chemically pretreated AD effluent which initially contained 200 mg/L of total nitrogen and 2.4 mg/L of total dissolved phosphorus (TDP) provided an optimal nutrient concentration for the cultivation of selected algae strain. Additionally, the cultivation of selected algal strain with optimal pretreated AD effluent in a pilot-scale semi-continuously fed raceway pond revealed a stable productivity of 6.83 g volatile solids (VS)/ m^2/day.

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Dedicated to my parents and people I love.

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LITERATURE REVIEW

Algal biofuels: a rising star in renewable energy industry

The world has consumed over a trillion barrels of oil since the first oil well was drilled in 1895. With such furious consumption, world oil reserves are set to dwindle. In less than 300 years we are going to deplete what took hundreds of millions of years to form. As outlined by the federal government in the Advanced Energy Initiative (AEI), nation's dependence on imported oil can be reduced by accelerating the development of domestic, renewable alternatives to power and liquid transportation fuel. The "2007 Energy Independence and Security Act (EISA)" contains provisions designed to increase the availability of renewable energy that decreases greenhouse gas (GHG) emissions while establishing an aggressive Renewable Fuels Standard (RFS) at the same time. The new standard mandates the production of 36 billion gallons of renewable fuels by 2022, and of which at least 21 billion gallons must be advanced biofuels. In 2009, U.S. President Obama also expressly emphasized that only the leader in clean energy technologies could lead the world economy in the 21st century. Among all types of clean energies, bio-based fuels show significant promise in helping to achieve the 21-billion-gallon goal; and of these candidates, algal biofuels have the potential to help the U.S. meet the new RFS while moving the nation ever closer to energy independence (Department of Energy, 2009).

Algae (Latin for "seaweed"), are a large and diverse group of simple, typically photoautotrophic organisms, ranging from unicellular to multicellular forms. The term "microalgae" refers to the microscopic, photosynthetic, free-living organisms that flourish in diverse habitats, for example, freshwater, brackish, marine, hyper-saline, snow, and even hot springs (DOE, 2009). As the larger counterparts of microalgae, "macroalgae" or "seaweeds" have cells organized into structures resembling leaves, stems and roots of higher plants, and

dominantly exist in marine waters. Macroalgae have many commercial and industrial uses, but due to their size and the specific requirements of the environment in which they need to grow, they do not lend themselves as readily to cultivation. The majority of algae that are intentionally cultivated fall into the category of microalgae, especially as emphasis switched to production of natural oils from biodiesel, microalgae became the exclusive focus of national renewable energy laboratory because they generally produce more of the right kinds of natural oils needed for biodiesel (Sheehan et al., 1998).

Table 1: Comparison of potential oil yields from feedstock (adapted from Chisti, 2007). Theoretically, a moderate growth of algal biomass (10 g/m^2/day, containing 15% triglyceride of dry weight) may produce 1200 gallons/acre/year of advanced biodiesel, which is 67 times the oil yield of corn. A more intense cultivation of algae (50 g/m^2/day, which contains 50% triglyceride of dry weight) may even be able to produce 10000 gallons/acre/year of biodiesel, which is 556 times the oil yield of corn.

Crop	**Oil Yield (gallons/acre/year)**
Corn	18
Cotton	35
Soybean	48
Mustard seed	61
Sunflower	102
Rapeseed/Canola	127
Jatropha	202
Oil palm	635
Algae (10 g/m^2/day at 15% triglyceride)	1,200
Algae (50 g/m^2/day at 50% triglyceride)	10,000

As photosynthetic organisms, algae can convert solar energy into useable forms for all organic life in our environment; however, different from other terrestrial plants which have

been used as traditional biofuel feedstock, algae show a lot of distinguishing advantages.

Firstly, due to their simple structure, algae do not need to specify any support or reproductive organs, and with a ready supply of water and nutrients, the algal cell can contribute the majority of the energy it takes to biomass growth. According to the estimation by Field et al. (1998), microalgae, though making up only 0.2% of global photosynthetic biomass, have been found to be responsible for nearly 50% of the earth's annual carbon-dioxide consumption and more than 45% of the oxygen production. Biofuels derived from algal biomass feedstock show considerable promise as a major contributor to the replacement of petroleum-based fuels (Table 1; DOE, 2009).

Meanwhile, the majority of current biofuels are produced from crop-based stock (corn, rice, wheat, soybean, etc.), which would not only deprive us of agricultural lands (Figure 1), but also cause food deficiency and many social problems in the long run. For instance, soy is the most common vegetable oil used because of its availability and high quality of the fuel product; however, Pimentel and Patzek (2005) have implied that the energy intensity of soybean production limits the economic feasibility and energy saving. Tanlens et al. (2007) also indicated that producing ethanol from corn represents a loss in net energy due to the energy required for agriculture and the process of producing ethanol itself. On the other hand, algal biomass is a non-food-based feedstock and has the capability of producing around 40 times the oil yield per acre per year given controlled conditions compared to terrestrial crops like soybean and cotton. Since algae are able to thrive in a lot of habitats, they have the potential to be cultivated in some otherwise non-productive lands.

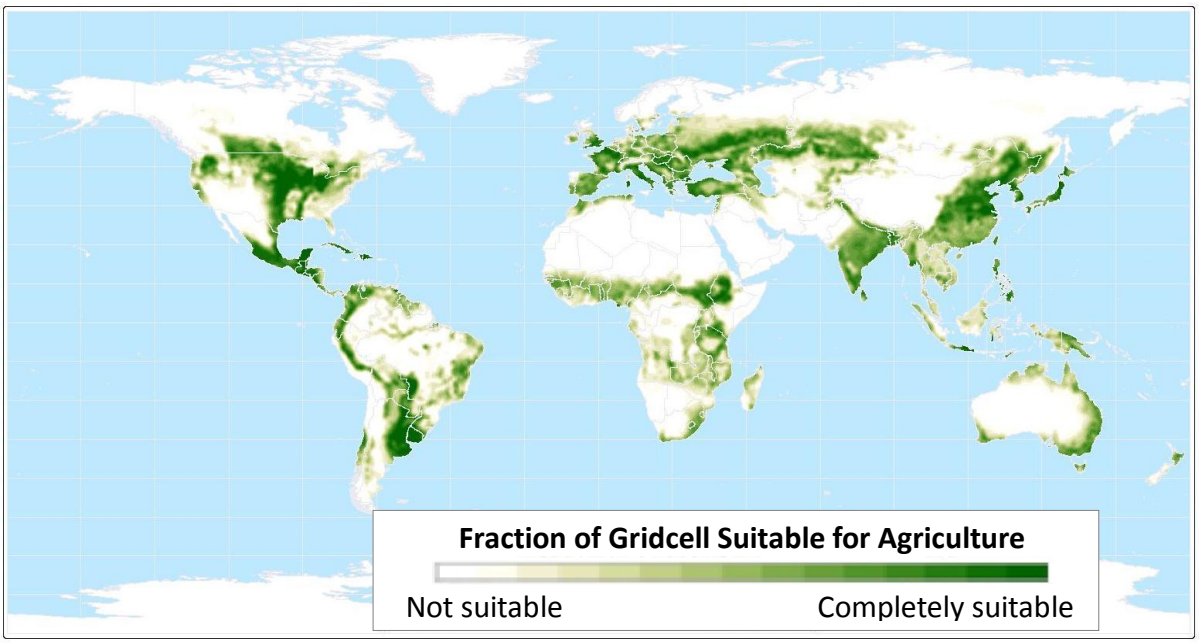


Figure 1: The global distribution of cultivable lands. Approximately 71% of the earth's surface is covered by ocean, and only less 1/3 of the land is suitable for high levels of terrestrial agriculture. However, algae grow in freshwater, oceans, and even on some non-productive lands, which may increase the potential productivity of biomass and return lands to agriculture and food production. (Center for Sustainability and the Global Environment, University of Wisconsin-Madison). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

From the environmental point of view, due to high absorption rates of CO_2 (the only inorganic carbon source for photosynthesis) and tolerance to high loading nutrients (mainly nitrogen and phosphorus), algal cultivation could also be used as post-treatment processes to utilize both carbon dioxide from fossil fuel combustion (or other industrial processes) and nutrients in waste streams to reduce pollution and generate value-added products. For example, algal cultivation on the wastewater stream from a medium-size dairy farm (1,000 heads) can produce about 720 tons of algal biomass each year (Sheehan et al., 1998). The carbohydrate from this amount of algal biomass could yield approximately 27,000 gallons of ethanol, which is equivalent to the ethanol produced from 167 acres of corn. In fact, results

from National Renewable Energy Laboratory suggested that large scale algal cultivation for biofuel production should be integrated with wastewater treatment.

Last but not least, although biodiesel and bioethanol are the most commonly discussed energy outputs from algae, they are not the only ones. Numerous studies of energy and of algae point to a wide range of energy outputs that can be theoretically derived from algae, e.g. hydrogen, methane and gasoline. Moreover, since many algal species are rich in protein, vitamins (including A, B_1, B_2, B_6, niacin and C) and minerals (such as iodine, potassium, iron, magnesium and calcium) (Kay and Barton, 1991), residuals from biofuel production can also be used as high-quality fertilizer or even as nutritious food supply for human and animals.

Making good use of the wastes

Raw manure can cause air pollution due to volatized ammonia and other odor compounds, pathogens and excessive nutrients from manure can leak to the surrounding watershed and aggravate surface water quality. Over 100 years ago, people started to use anaerobic digestion (AD) to process municipal sewage and a wide variety of industrial wastes. Anaerobic digestion is a series of processes that converts any biodegradable/organic matter from plants, animals or their wastes to biogas. This process not only removes an immense majority of the odorous compounds, it also significantly reduces pathogens (Lusk, 1995). However, the nutrient-rich AD effluent hasn't been fully put under control yet. Even though people around the world have been using it as fertilizer for decades, the rapid growth in the size and the number of dairy operations has resulted in an even larger amount of excessive but "nutritious" wastes (Burke, 2001). Therefore, nutrient removal has become one of the major objectives for livestock waste management. It is expected that new federal environmental and agricultural policies for agricultural nutrient management will be

implemented in the foreseeable future. Therefore, among the various processes for nutrient management, algal cultivation represents one of the best biological treatments with faster uptake of nutrients in waste streams, year-round production, and higher photosynthetic efficiency.

Latest research and development of manure-fed algal fuels

Although the concept of algal biofuels, combined with agricultural nutrient management, is relatively new in the field of renewable energy, it has been developing rapidly over the past 10 years. The first outdoor test of using algae to clean liquid manure from dairy farms has started at a U.S. Department of Agriculture research center in Maryland by the end of 20th century (Cosmic, 2000).

Microbiologist Walter Mulbry has evaluated and developed an algal turf scrubber (ATS) method of growing filamentous algae to remove nitrogen, phosphorus and soluble carbon from dairy manure (Mulbry and Wilkie, 2001). As a forerunner, his team conducted a series of experiments, indoors and outdoors, regarding algal cultivation with animal wastes. They measured nitrogen and phosphorus removal rates using small ATS and found that the removal of NH_4-N and PO_4-P from manure effluent was clearly influenced by both manure concentration and turf biomass density (Pizarro et al., 2002). They found higher irradiance enhanced algal growth, but it also decreased the N and P removal rate. One of the explanations was that low irradiance levels promote higher production of photosynthetic pigment and higher N content in algal cells (Kebede-Westhead et al. 2003). The researchers also compared effects of two effluent-AD dairy manure and raw swine manure-with the same ATS method, results showed that algae preferred the AD dairy manure better as an N resource, but the elemental composition (such as Al, Cu, Fe, Mn, Mo, Zn and P) showed close correspondence (Kebede-Westhead et al., 2006). In 2006, Pizarro et al. even did an economic

assessment of ATS technology for treatment of dairy manure effluent and pointed out many potentials and opportunities.

Recently, biofuels production with manure-fed algal biomass has gathered a lot of attention, which also increased investigation of downstream operations. For example, how can we extract the valuable products (sugar, lipid, protein, etc.) from the algal biomass as much as possible? Different from traditional biomass feedstock, considerable research is needed to solve problems which are unique to algae, such as cell wall chemistry, high water content, small cell size, and the lack of standardized agronomic methods for the harvest of biomass or the extraction of value-added energies and chemicals.

For products from algal carbohydrates, the main bottleneck is that biodegradation rates of algal biomass could be low depending on both the biochemical composition and the nature of the cell wall. Pretreatment of algal biomass prior to fermentation (final product is ethanol) allows significant improvement in biodegradability while acting on its physico-chemical properties, it makes the organic matter more accessible to the biomass and thus more easily degraded (Sialve et al., 2009). Chen and Oswald (1998) conducted a study to determine the influence of thermochemical pretreatment of algal biomass, and they discovered the optimal pretreatment condition (heating at 100 ℃ for 8 h, without sodium hydroxide) could improve the efficiency of methane production by 33%. Nguyen et al. (2008) used a dilute acid hydrothermal method, which was low cost and high efficiency, for algal biomass pretreatment. Their results indicated that glucose released from the biomass was greatest at 58% (w/w) after pretreatment with 3% sulfuric acid at 110 ℃ for 30 min. Furthermore, it has been proposed that other components (e.g. peptide/protein) in the algal hydrolysate may be applied as surfactants to enhance the enzymatic hydrolysis process. Because cellulase can be absorbed non-productively on lignin during the enzymatic hydrolysis of lignocelluloses (Tatsumoto et al., 1988), high enzyme loadings are required. However, an addition of

exogenous protein enhanced the lignocellulose hydrolysis (Tengborg et al., 2001). Yang and Wyman (2006) reported that adding bovine serum albumin into the system prior to cellulase increased effectiveness of the hydrolysis process, which was consistent with Tengborg's results.

Another valuable final product from algae is oil. At present, one of the biggest breakthroughs for this procedure was finding the particular biological component for extraction depends heavily on the algal species and growth status, which is highly characterized for higher plants as compared to algae (DOE, 2009). Therefore, although some extraction methods used for terrestrial oilseed plants have been applied to algae, most are ineffective and have little utility. Numerous approaches have been used to solve, or at lease to ease this problem. The first approach is selecting algal strains which have high lipid content in biomass. For instance, Rodolfi et al. (2008) selected four out of thirty microalgal strains (two marine and two freshwater) in the laboratory based on their robustness, highly productivity, and relatively high lipid content. They also found that fatty acid content increased with high irradiances and following both nitrogen and phosphorus starvation. The second approach, genetic and metabolic modification, is likely to have great impact on improving the economics of production of microalgal diesel, although it has not received much attention yet (Chisti, 2007). Thirdly, environment may also influence chemical contents of algal biomass. Mulbry et al. (2008) studied on how the fatty acid content and composition of algae responded to changes in the type of manure and manure loading rate, as well as to whether the algae was grown with supplemental carbon dioxide. They found that both indoor and outdoor culture units showed no consistent relationship to loading rate, type of manure, or supplemental carbon dioxide. Another way to increase algal oil productivity is to optimize the procedures for oil extraction. A very recent study (Mulbry et al., 2009) reported comparing a high temperature/pressure extraction method (a.k.a. accelerated solvent

extraction or ASE) and a manual extraction method (a.k.a. modified Folch extraction), and finding ASE method yielded higher values for oil extraction and extraction efficiency of the ASE method for fatty acid was dependent on the extraction solvent. In this case, chloroform/methanol was the best.

INTRODUCTION

Nutrient pollution from animal manure, particularly excess nitrogen and phosphorus, has a major impact on the environment (Jongbloed and Lenis, 1998). Nitrogen in the form of ammonia is volatized to the atmosphere to cause air pollution, and phosphorus can leak to the surrounding watershed to impact surface water quality. Treatment of these wastes is a major objective for livestock waste management (Lanyon, 1994). New federal environmental and agricultural policies are expected for agricultural nutrient management in the future (Ruhl, 2000). Among various processes for nutrient management, algal cultivation, with the advantages of faster uptake of nutrients in waste streams, year-round production, and higher photosynthetic efficiency, represents one of the best biological treatments (Kebede-Westhead et al., 2004). In addition, algal biomass is rich in starch, lipid, and proteins, which can be used as a non-food-based feedstock for value-added energy and chemical production. The United States Department of Energy's Aquatic Species Program recommended that an integrated approach, which combined wastewater treatment with algal biofuel production, should be studied (Sheehan et al., 1998).

Many studies of algal cultivation on wastewater (municipal and agricultural) and algal biofuel production have been conducted recently. Kebede-Westhead et al (2006) reported the increased loading rates of anaerobically digested dairy manure could significantly facilitate the algal productivity, but raw swine manure could barely comparable. In 2003, the team also found algae grew faster in higher irradiance, but it could also inhibit N and P removal rates. Pizarro et al. (2002) introduced algal turf scrubber (ATS) system to farm wastewater treatment. With the system, they discovered elemental (such as Al, Cu, Fe, Mn, Mo and Zn) and organic matter (carbohydrate, lipid and protein) composition response to different manure effluent sources and loadings (Mulbry et al., 2008; Kebede-Westhead et al., 2003 &

2006). However, we know little about algal cultivation with anaerobically digested (AD) manure effluent. For example, culture conditions (nutrient concentration, light intensity, flow rate, temperature, pH, etc.) have not been fully optimized (Mulbry, 2005), and analyses of algal community assemblages in manure-based culture have not been reported.

Meanwhile, the high turbidity of AD effluent attenuates the light availability and accumulates solids, and thereby limits the growth of algae (Hamdani et al., 2004). A pretreatment of AD effluent to reduce the solids content is highly recommended for large scale algal cultivations in wastewater (Barnet et al., 1994; author's unpublished data).

In wastewater treatment industry, conventional coagulation and flocculation are essential pretreatments because the processes aggregate suspended solids together into larger bodies so that physical filtration processes can more easily separate them from water (Global Health and Education Foundation, 2007). Various types of coagulants are being used to condition water before sedimentation and filtration. The most widely used coagulants are: aluminum potassium sulfate (alum), poly aluminum chloride, calcium hydroxide (lime), ferrous sulfate, silicon derivatives and synthetic organic polymers (Sivaramakrishnan, 2008). Hamdani et al. (2004) showed that compared with ferric chloride, the coagulation-decantation by calcium hydroxide considerably reduced the suspended matter and total phosphorus; however, both chemicals were insufficient for the removal of nitrogen. Atsuko et al. (1989) combined calcium hydroxide, ferrous sulfate and acrylamide sodium acrylate polymer for coagulation precipitation processes, they found that the mixture was effective in removing anionic surfactants, but not in removing nonionic surfactants. Meric et al. (2002) also reported a combination of ferrous sulfate and calcium hydroxide applied in coagulation-flocculation could increase the removal rate of COD, *E. coli*, and *Daphnia magna*.

This study focused on culturing fresh water algae on liquid effluent of anaerobically digested animal manure to remove nutrients and accumulate algal biomass for value-added

applications. The objectives of this study were to: 1) discover the impact of high nutrient concentration on the assemblages of algal community; 2) determine an optimal pretreatment method of AD effluent to eliminate the impacts of suspended solids on algal cultivation; 3) specify an optimal dilution of pretreated AD effluent for algal cultivation on a bench-scale; and 4) apply the optimal dilution of AD effluent in a semi-continuously fed raceway culture system and verify the productivity of selected algal strain in pretreated AD effluent.

MATERIAL AND METHODS

Effect of AD effluent on algal community assemblage

Raw dairy manure was collected from a private dairy farm with 3,000 cows and anaerobically digested for 30 days at 35℃ in a completely stirred tank reactor (CSTR). A screw press was used to separate liquid and solid fractions of AD effluent. The liquid effluent from the digester was used as nutrient source in this study. The effluent was stored at 4℃ prior to use.

Algae were collected from a local pond in Okemos, Michigan (*42N 42' 18.64", 84W 23' 49.8"*) in February 2009. Original AD effluent (non-pretreated) was diluted to three concentrations, and the diluted AD media were named by their total nitrogen (TN) concentration, 40, 100, 200 mg TN/L, throughout this study. 5 mL algal suspension (Total Solid = 4.52 g/L) was inoculated in 120 mL dilute AD media of three concentrations. Each nutrient level had two replicates. The flasks were cultured on the orbital shakers (150 rpm) at 18±3 ℃ under continuous illumination from fluorescent lamps (6×10^{19} quanta m^{-2} s^{-1}). AD effluent was added to each culture every 4 to 6 days to keep nutrient levels stable around 40, 100 and 200 mg TN/L in respective treatments. 1.0 mL sample of each culture was collected periodically for algal community assemblage analysis.

Chemical Pretreatment of AD effluent

15 mL of AD liquid effluent was mixed with 15 mL of tap water and 2 g of dry carbon dioxide (dry ice). Calcium hydroxide ($Ca(OH)_2$) and aluminum potassium sulfate dodecahydrate ($AlK(SO_4)_2 \cdot 12H_2O$) were applied as coagulants. Independent and interactive

effects of different concentrations of $Ca(OH)_2$ (4.5, 6, 7.5, and 9 g/L) $AlK(SO_4)_2 \cdot 12H_2O$ (1, 1.5, 2, and 2.5 g/L) were tested using a completely random design, with three replications. After 24 h settlement, the turbidity of supernatants was measured with UV spectrophotometer (UV-1800, SHIMADZU, Columbia, MD) under OD 600 nm. The volume of supernatant was measured. Total nitrogen (TN) and total phosphorus (TP) in the supernatants were tested as well. This study aimed to determine the supernatant with relatively low turbidity, high supernatant-to-sediment (v/v) ratio, and appropriate nitrogen and phosphorus concentrations (Redfield, 1958).

Bench culture of selected algal strain with chemically pretreated AD effluent

Algae survived from the first experiment (cultured in 200 mg TN/L of non-pretreated AD effluent for 5 months) were used as inocula in this study. About 98% of the community consisted of spherical unicellular green algae, which are commonly referred to as *Chlorella* sp. because of their morphological similar to this genus. However, no further genetic tests were performed to identify this taxon. Chemically pretreated AD effluent using the optimal method from previous experiment was used as nutrient source. Six dilutions of pretreated AD effluent having 10, 40, 100, 200, 300 and 400 mg TN/L and 0.7, 1.0, 1.4, 2.5, 3.5, 4.4 mg total dissolved phosphorus (TDP)/L, respectively, were used as media for algal cultivation. 5.0 mL of algae (TS = 5.01 g/L) were inoculated in 45 mL of medium for each dilution with three replicates. The flasks were cultured on the orbital shaker (150 rpm) at 18±3 ℃ under continuous illumination from daylight fluorescent lamps (about 6×10^{19} quanta m^{-2} s^{-1}). Total dissolved nitrogen (TDN), TDP, total solids (TS), volatile solids (VS), turbidity and algal cell number of the culture were monitored every 5 days.

Optical density (OD 750 nm) was determined to evaluate the quantity of algal biomass

presented in different nutrient concentrations.

The average cell biovolume (mm^3/cell) for all 6 treatments was measured using the imaging software NIS-Elements D 3.00 (Nikon Instruments Inc., Melville, NY), and the cell density (cells/mL) was measured using a microscopic counting chamber (hemocytometer) every 5 days throughout the cultivation. Standardized biovolume (volume of algae/volume of sample, mL/mL), multiplying the average cell biovolume times the cell density (cells/mL), was used to compare the growth in different nutrient concentrations.

Chlorophyll content was measured to verify the optimal nutrient concentration. Algal biomass on Day-0 and Day-20 of cultivation were analyzed.

Algal kinetics were studied based on the analyses of OD, TS, VS, and standardized biovolume. Nutrient uptake was derived indirectly by subtracting TDP and TDN left in the media after 20 days of cultivation from the initial concentrations of each culture. Both the kinetics and the nutrient uptake rate were used as reference for the pilot-scale study.

Pilot algal culture in a semi-continuously fed raceway pond

Algae from the first experiment (cultured in 200 mg TN/L of non-pretreated AD effluent for 5 months) were used as inocula. Chemically pretreated AD effluent using the optimal method from the second experiment was used as nutrient source. 200 mL of algal inocula (TS = 5.4 g/L) and 19.8 L of pretreated AD effluent with optimal concentration were mixed to the total volume of 20 L in a raceway pond driven by an aquarium pump (1200 L/h, 296 gph; LIFEGARD® Quiet1One, Pentair Aquatics™, Chino, CA) (Figure 2). The culture was kept at 18±3 ℃ under continuous illumination from daylight fluorescent lamps (about 6×10^{19} quanta m^{-2} s^{-1}). The feeding and biomass collection strategy were both based on previous kinetics. Nutrient uptake rates of TDN and TDP in 200 mg TN/L culture were 5.96 and 0.08

mg/L per day, respectively. The TDN and TDP in chemically pretreated AD effluent were 397 and 5.7 mg/L, respectively; which meant that about 400 mL of pretreated AD effluent would be needed every day for a 20 L pilot culture. After the algal growth in the pilot culture reached the exponential phase (the eighth day, estimated by daily consumed TN and TP, OD-750 nm and VS), its biomass density was 1.47±0.12 g VS/L and the growth rate was 0.0164 g/g/day. Considering the mass balance in a semi-continuously fed culture, 300 mL of broth from the raceway pond was collected daily, and TS, VS, and OD (750 nm) of collected algal biomass was measured. Water was added frequently to keep the total volume constant at 20 L.

Figure 2: Semi-continuously fed and harvested raceway pond, the culture was circulated using an aquarium pump and exposed under fluorescent lamps.

Analytical Methods

Algal biovolume and the assemblage of algal community were analyzed throughout cultural period (Academy of Natural Sciences of Philadelphia (ANSP), Protocols for Analysis of National Water-Quality Assessment (NAWQA) Algae Samples). A compound microscope

(Nikon Eclipse 50i, 100× objective, 1000× total system magnification) was used to identify (on genus-level) and enumerate algae (Sheath and Wehr, 2002; Anderson, 2005; Prescott, 1982).

Chlorophyll content of algae was tested by centrifuging 1 mL of diluted samples for 10 min. Sediments were re-suspended using 10 mL of 90% (v/v) ethanol. Samples were sonicated for 30 min to break the cells. Sonicated samples were left in a refrigerator at 4 ℃ for 24 h. Appropriate dilution was applied to the extracted samples for measurement of chlorophyll content by using a fluorometer (TD-700, Turner Designs, Inc., Sunnyvale, CA).The optical densities (OD), 750 nm for cell density and 600 nm for chemical solution, were measured using a UV spectrophotometer (Van de Veer and Bolier, 1991).

Throughout the cultivation, all samples left from community, chlorophyll and OD analysis were centrifuged at 3500 rpm for 15 min. TS and VS of algal biomass were measured (American Public Health Association, 1998). Supernatants were saved for chemical analyses of pH, temperature, TN and TP. Supernatants from kinetics study were also filtered with Millex-GS 0.22 mm membrane, TDN and TDP were measured. All nitrogen and phosphorus tests followed the persulfate digestion method using “Total Nitrogen High Range Reagent Set” and “Total Phosphorus Test ’N Tube Reagent Set” (HACH, Loveland, CO), respectively.

Total chemical oxygen demand (COD), TN and TP in AD liquid effluent were also analyzed using HACH testing reagent sets. TS and VS of AD liquid effluent were analyzed according to APHA (1998).

Statistical Analysis

A pair-wise comparison using the Statistical Analysis System program 9.0 (SAS Institute, Inc., Cary, NC) was conducted to analyze the change of algal community assemblage.

A two-way ANOVA using SAS 9.0 was conducted to analyze the significance of impacts of two chemicals ($Ca(OH)_2$ and $AlK(SO_4)_2 \cdot 12H_2O$) on AD effluent pretreatment, based on turbidity, volume, TN and TP of the supernatants.

Considering the complexity of AD effluent, a first-order equation was chosen to describe the algal growth on AD effluent. The model can be written as

$$\frac{dN}{dt} = kN \quad \text{, or } N(t) = N_o e^{kt}$$

where N is the algal biomass (described as the responses: OD, TS, VS, and standardized biovolume), k is the growth rate, and t is time; $N(t)$ is the algal biomass at time t, and N_0 is the algal biomass of inoculants.

One-way ANOVA, Hsu's multiple comparisons with the best (MCB) and z-test using Minitab 15.1.30.0 (Minitab, Inc., State College, PA) were conducted to evaluate the impact of nutrient concentration on algal growth rate for all responses.

RESULTS

Effect of AD effluent on Algal Community Assemblage

The original algal community in the freshwater pond mainly consisted of six algal species: *Scenedesmus* sp., *Synechocystis* sp., *Chlorella* sp., *Pseduanabaena* sp., *Limnothrix* sp., and *Phormidium* sp. (Figure 3-a; Figure 4). Taxonomic composition of the algal assemblage differed among AD concentration treatments through time ($P < 0.01$).

In 40 mg TN/L AD effluent (Figure 4), the relative densities of filamentous cyanobacteria (*Pseduanabaena* sp., *Limnothrix* sp., and *Phormidium* sp.) significantly decreased ($p < 0.001$) in the first 15 days of culture, and they were hardly observed afterwards. The density of *Chlorella* sp. kept increasing in 3 months of culture ($p = 0.002$, R-square = 95.2%), from 19% to 79% of the community. The density of *Synechocystis* sp. was high at day 15 (51%) and day 90 (42%), but low at day 30 (16%) and day 50 (13%), compared to the original (29%). The density of *Scenedesmus* sp. kept increasing in the first 30 days, and started to decrease afterwards, especially after day 50.

In 100 mg TN/L AD effluent, the change of algal community assemblage was similar to the one in 40 mg TN/L, except that the *Chlorella* sp. increased more dramatically in the last month of cultivation.

In 200 mg TN/L AD effluent, the density of *Chlorella* sp. also dramatically increased after day 50. Moreover, the density of *Synechocystis* sp. increased in the first 15 days, but then decreased till the end of the cultivation (5 months); *Scenedesmus* sp. did not decreased until day 50. After three months, all three concentrations of AD effluent were dominated by the three non-filamentous algal taxa. Although the density of cyanobacteria *Synechocystis* sp. was high in 40 mg TN/L effluent, while the densities of green algae, *Scenedesmus* sp. and *Chlorella* sp., were high in 100 and 200 mg TN/L effluent. After five months, 98% (density)

of the algal community in 200 mg TN/L AD effluent consisted of *Chlorella* sp.

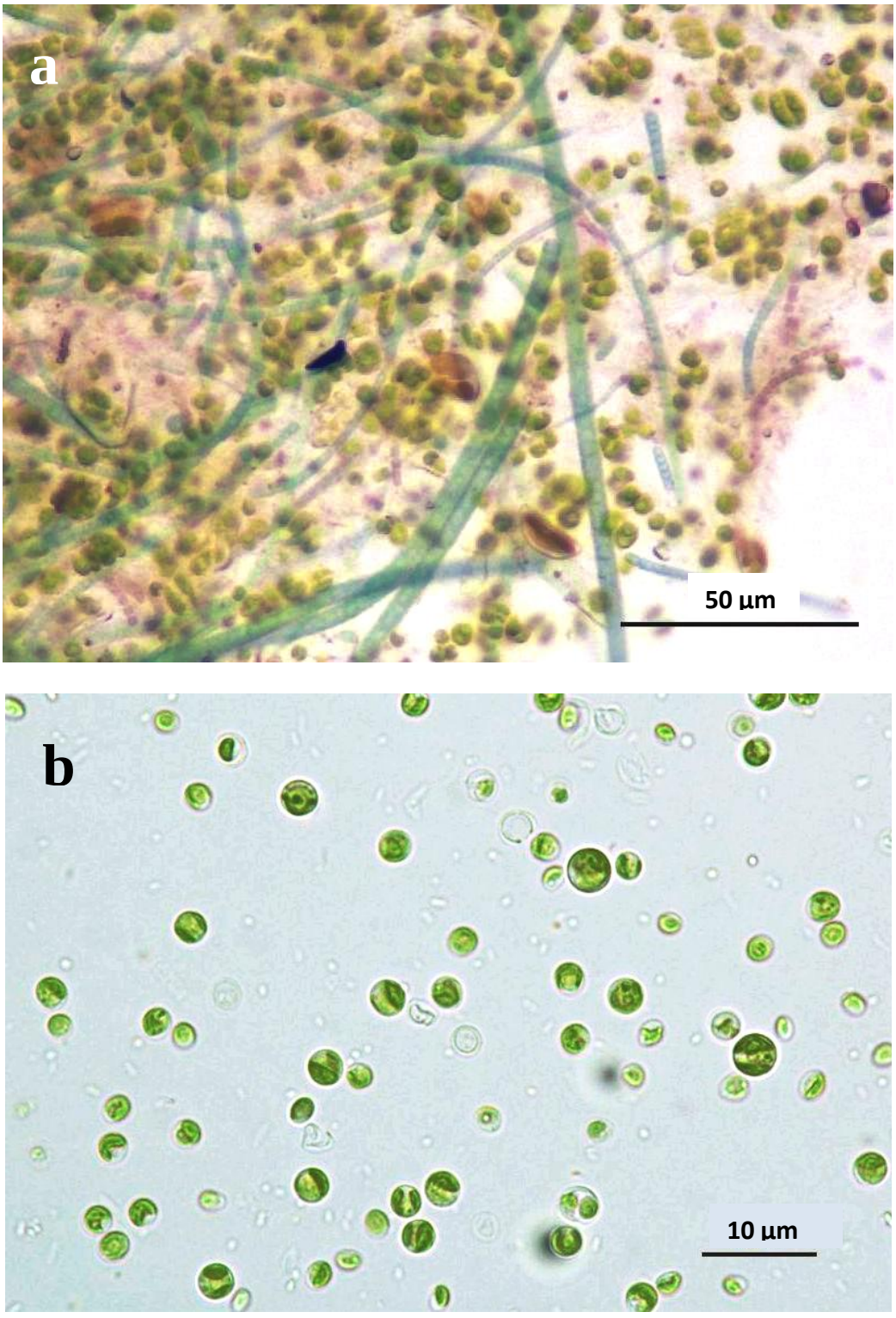


Figure 3: Algal community assemblages before (a) and after (b) 5-month cultivation with AD effluent. Most of filamentous cyanobacteria were eliminated by high concentration of AD effluent.

Figure 4: The count (in unit) of algal community in different concentrations of AD effluent (40, 100, and 200 mg TN/L) through time (3-5 months). *Pseudanabaena, Phormidium, Limnothrix, Chlorella, Synechocystis* and *Scenedesmus* were the six dominant algal genera in the original inoculum. The data were acquired from two replications. The error bars imply the 95% confidence intervals (Mean ± 1.96 SE) of each count.

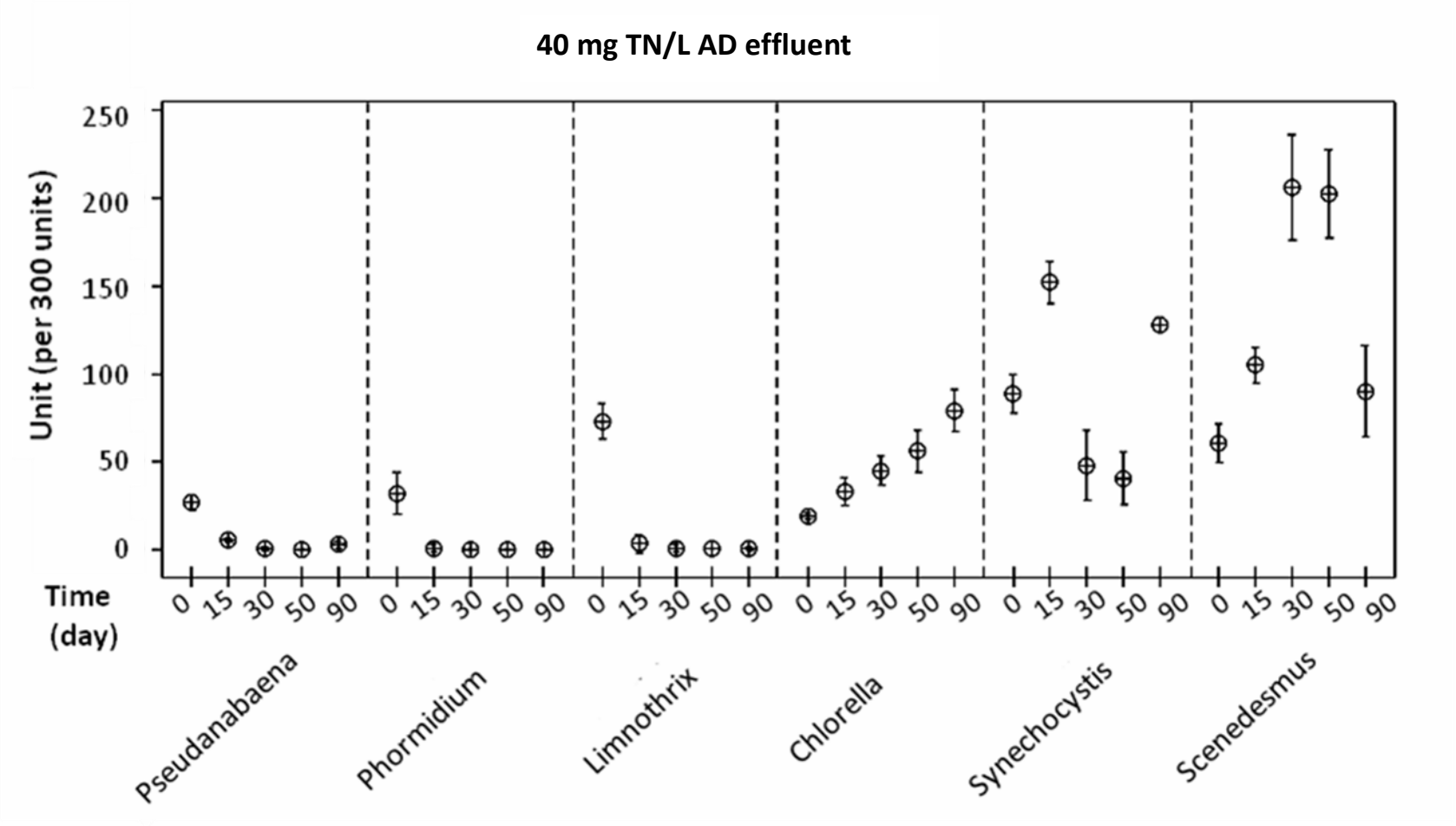
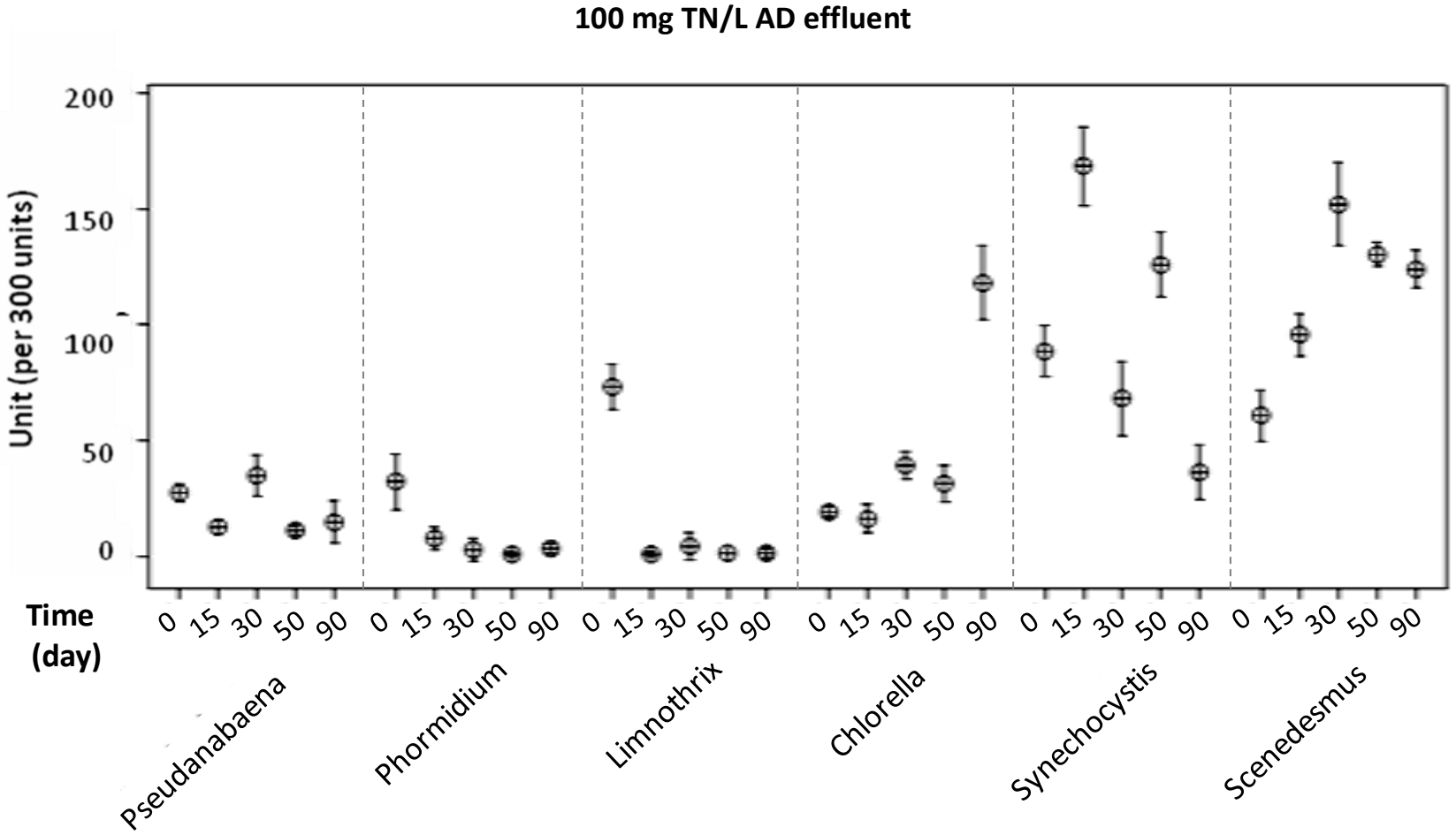


Figure 4: (cont'd)



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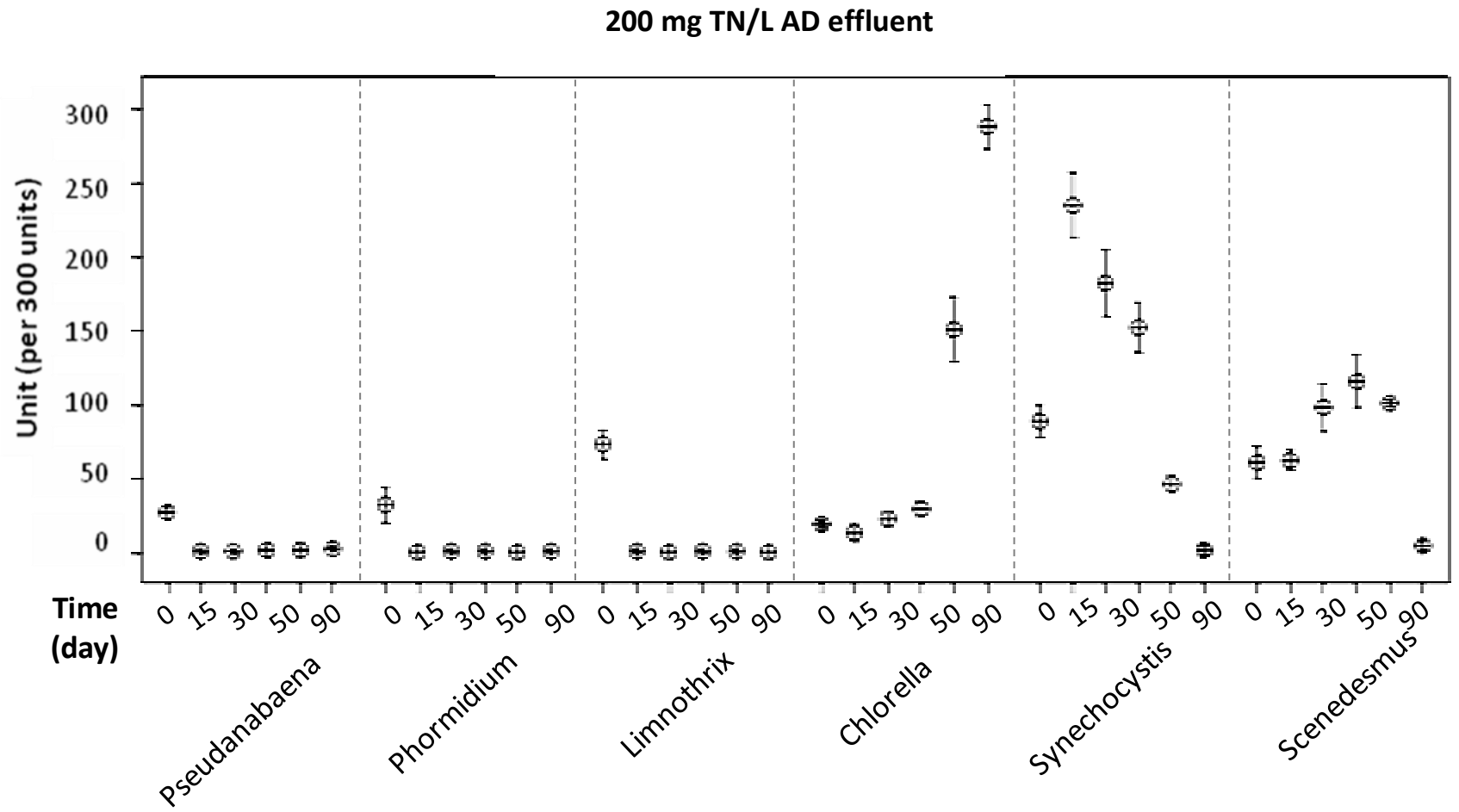


Chemical pretreatment of AD effluent

Different combinations of chemical treatments had different effects on AD effluent (Figure 5). Both chemicals, $Ca(OH)_2$ and $AlK(SO_4)_2 \cdot 12H_2O$, had significantly negative independent and interaction effects on turbidity and TP (2-way ANOVA, $p < 0.007$; Table 2). With 4.5 g/L $Ca(OH)_2$, OD readings of turbidity for the treatments with 1, 1.5, 2, 2.5 g/L $AlK(SO_4)_2 \cdot 12H_2O$ were 1.63, 1.0, 0.41, 0.15, respectively; with 6 g/L $Ca(OH)_2$, the readings were 1.65, 0.97, 0.31, 0.24, respectively; with 7.5 g/L $Ca(OH)_2$, they were 0.86, 0.44, 0.22, 0.17; and with 9 g/L $Ca(OH)_2$, they were 0.57, 0.23, 0.14, 0.17. And the TP of these treatments were: with 4.5 g/L $Ca(OH)_2$, they were 18.81. 9.68, 3.42, 0.81 mg/L for treatments with 1, 1.5, 2, 2.5 g/L $AlK(SO_4)_2 \cdot 12H_2O$; with 6 g/L $Ca(OH)_2$, they were 19.27, 7.66, 2.15, 0.97 mg/L, respectively; with 7.5 g/L $Ca(OH)_2$, they were 13.58, 6.91, 4.53, 1.08 mg/L; and with 9 g/L $Ca(OH)_2$, they were 7.13, 4.7, 2.08, 0.86 mg/L. Both chemicals had significantly negative effects on TN, but their interaction did not. With 4.5 g/L $Ca(OH)_2$, TN for the treatments with 1, 1.5, 2, 2.5 g/L $AlK(SO_4)_2 \cdot 12H_2O$ were 172.4, 156, 148.9, 158.9 mg/L, respectively; with 6 g/L $Ca(OH)_2$, the results were 183.1, 158.9, 141.8, 155.3 mg/L, respectively; with 7.5 g/L $Ca(OH)_2$, they were 158.9, 133.7, 137.1, 149.9 mg/L; and with 9 g/L $Ca(OH)_2$, they were 135.8, 146.2, 112.3, 132.8 mg/L.

Only $AlK(SO_4)_2 \cdot 12H_2O$ had significantly positive effect on the volume of supernatant. After the comparisons among pretreatments (Figure 6), the pretreatment containing 7.5 g/L of

$Ca(OH)_2$ and 1 g/L of $AlK(SO_4)_2 \cdot 12H_2O$ was selected as the optimal method.

The characteristics of pretreated AD effluent with optimal method were 475, 397, 23.4, and 5.7 mg/L of TN, TDN, TP, and TDP, respectively.

Table 2: The significance of impacts of $Ca(OH)_2$ and $AlK(SO_4)_2 \cdot 12H_2O$ on turbidity, volume, TN and TP of pretreated AD effluent (supernatant). P-value's and R-square's were derived from two-way ANOVA.

Response	P-value's			R-sq
	$Ca(OH)_2$	$AlK(SO_4)_2 \cdot 12H_2O$	Interaction	
Turbidity	<0.001	<0.001	0.007	0.942
Volume	0.097	0.001	0.911	0.725
TN	<0.001	0.002	0.36	0.812
TP	<0.001	<0.001	0.002	0.963

Figure 5: Turbidity, volume, TN and TP of supernatant under the pretreatments with different concentrations of $Ca(OH)_2$ (4.5, 6, 7.5, 9 g/L) and $AlK(SO_4)_2 \cdot 12H_2O$ (1, 1.5, 2, 2.5 g/L).

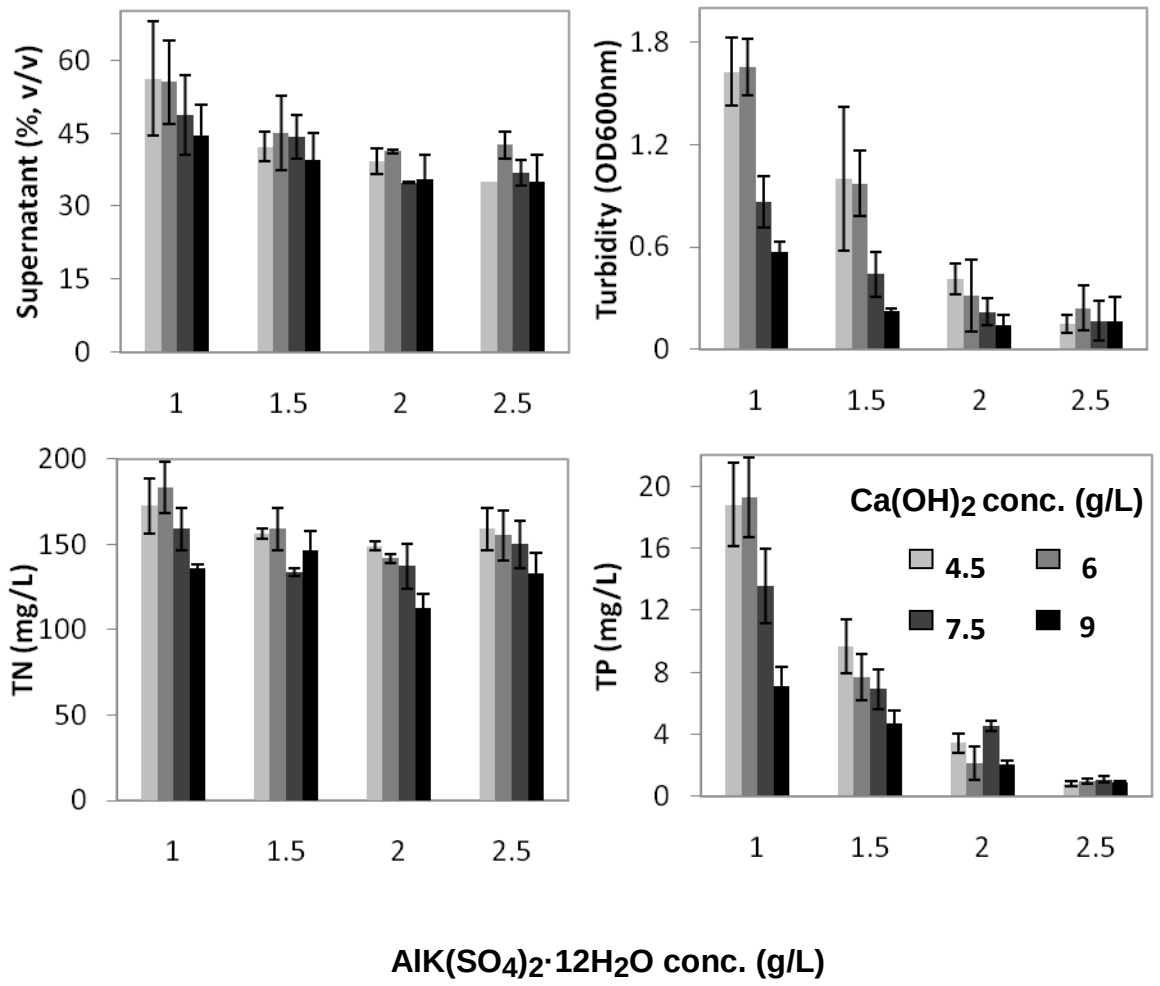
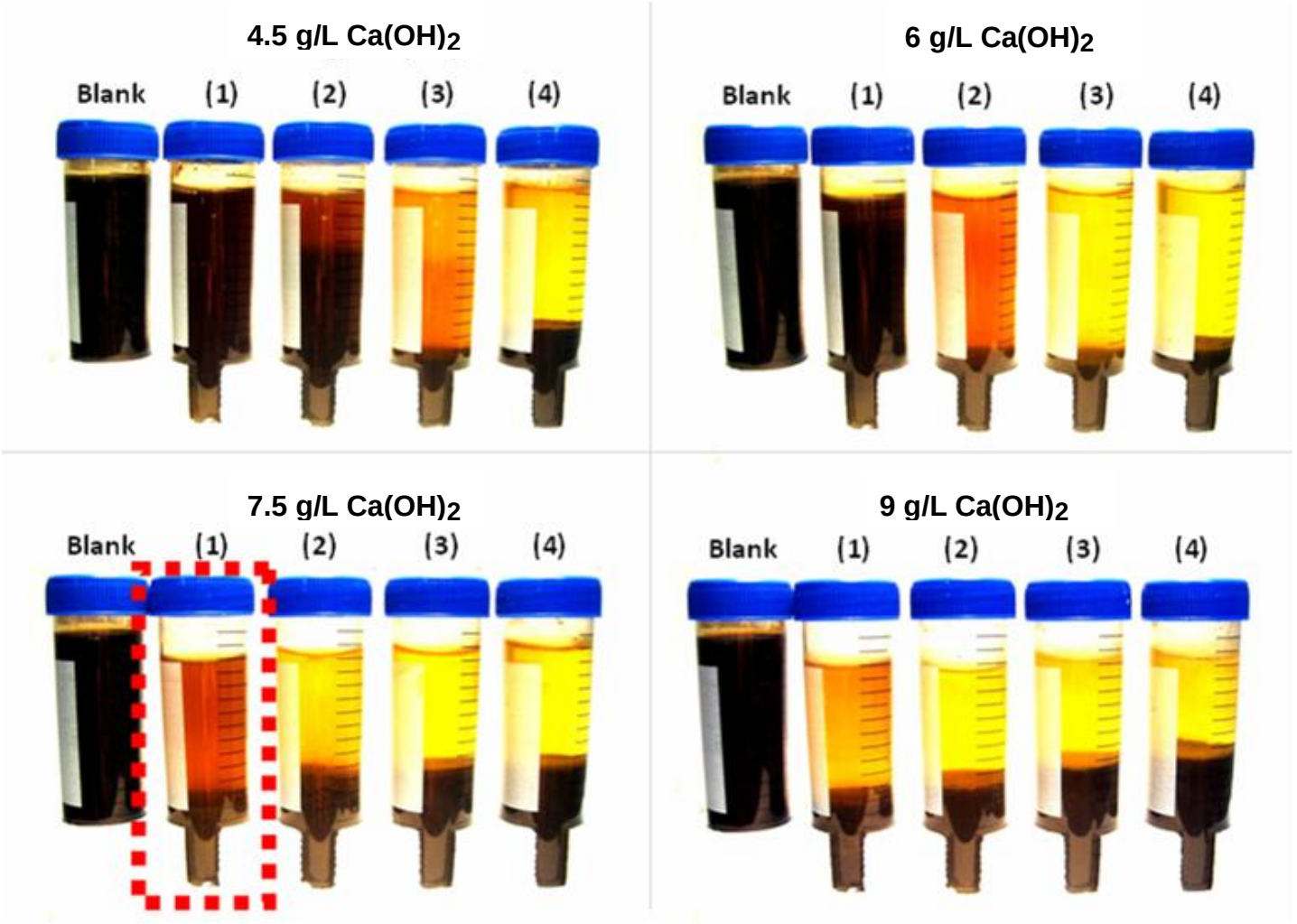


Figure 6: Chemically pretreated AD effluent. $Ca(OH)_2$ (4.5, 6, 7.5, 9 g/L) and $AlK(SO_4)_2 \cdot 12H_2O$ (1, 1.5, 2, 2.5 g/L) were used as coagulants in the pretreatments. The combination with 7.5 g/L $Ca(OH)_2$ and 1 g/L $AlK(SO_4)_2 \cdot 12H_2O$ (dashed rectangle) was selected as the optimal pretreatment method, judging by the turbidity, volume, TN and TP of the supernatant.



(1): 1 g/L $AlK(SO_4)_2 \cdot 12H_2O$

(2): 1.5 g/L $AlK(SO_4)_2 \cdot 12H_2O$

(3): 2 g/L $AlK(SO_4)_2 \cdot 12H_2O$

(4): 2.5 g/L $AlK(SO_4)_2 \cdot 12H_2O$

Bench culture of selected algal strain with chemically pretreated AD effluent

Algal community assemblages throughout the cultural period were similar in different nutrient concentrations with *Chlorella* being the dominant algal genus (>95%). In general, OD, TS, VS, and standard biovolume responded to the concentration of pretreated AD effluent had significant impact on algal growth rate (p's were < 0.001, < 0.001, 0.032, and 0.007, respectively; Appendix-Table 5). For OD responses, the optimal growth rate was from the 200 mg TN/L culture (k = 0.0673±0.0025 g/g/day). Moreover, the Hsu's MCB method (Appendix-Table 6; Figure 7) specified that the confidence interval (CI) of 200 mg TN/L culture (0, 0.0145) was the only one entirely above (not including) zero, which meant that it was significantly greater than the growth rates of 10, 40, 100, 300, and 400 mg TN/L cultures. For TS responses, the optimal growth rate was from the 200 mg TN/L culture (k = 0.0528± 0.0027 g/g/day). Its Hsu's MCB CI (0, 0.0121) indicated that it was significantly greater than the growth rates of other cultures. For VS responses, the optimal growth rate was from the 200 mg TN/L culture (k = 0.0538±0.0019 g/g/day); it was significantly greater than the growth rates of 10, 40, 300, and 400 mg TN/L cultures (whose Hsu's MCB CI's were all below zero), but had no significant difference from the growth rate of 100 mg TN/L culture (both CI's contained zero). And for standard biovolume responses, the optimal growth rate was also from the 200 mg TN/L culture (k = 0.056±0.0028 g/g/day). Its Hsu's MCB CI (0, 0.0165) indicated that growth rate of 200 mg TN/L culture was significantly different from the others.

Results of statistical test for mean growth rate (Appendix-Table 8) represented the difference between two treatments (nutrient concentrations of pretreated AD effluent). Algal growth rate from the medium of 200 mg TN/L was significantly higher than the other treatments, for all the responses (Figure 7). However, significance of differentiation between

two of the other concentrations varied.

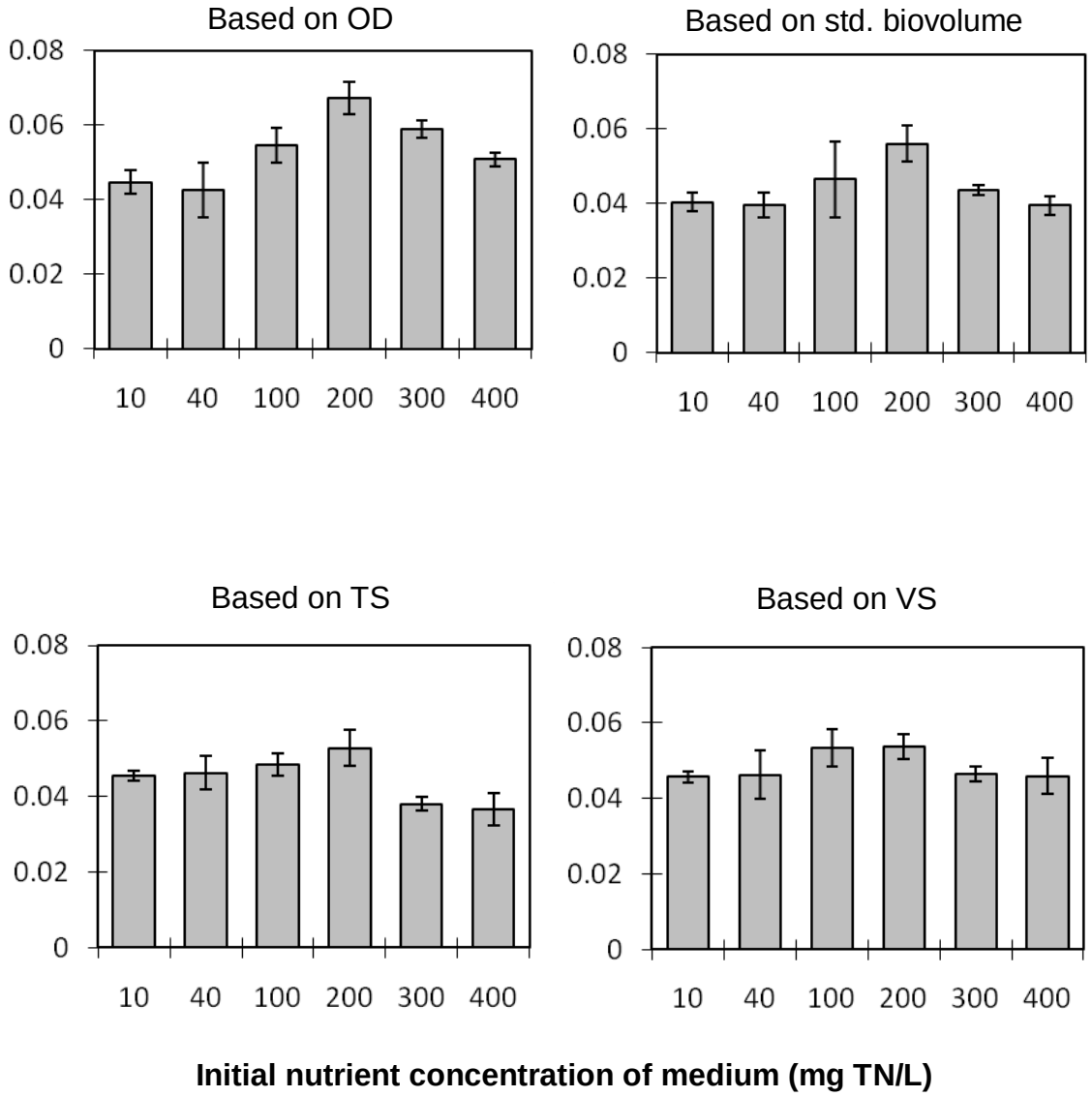


Figure 7: The growth rate of algae in different nutrient concentrations. Data were derived from three replications, with the measurements (responses) of optical densities (OD), standard biovolume, total solids (TS) and volatile solids (VS).

Chlorophyll analysis of the inoculum (Day-0) and final biomass (Day-20) demonstrated the chlorophyll content increase in all cultures (Figure 8). Among them, algae in 200 mg TN/L culture showed the highest content of chlorophyll (5.10±0.068 mg/L) in 20 days of

cultivation. Since chlorophyll content (especially chlorophyll *a*) is one of parameters to represent phytoplankton carbon (Redalje, 1983), a high chlorophyll content indicates high biomass accumulation.

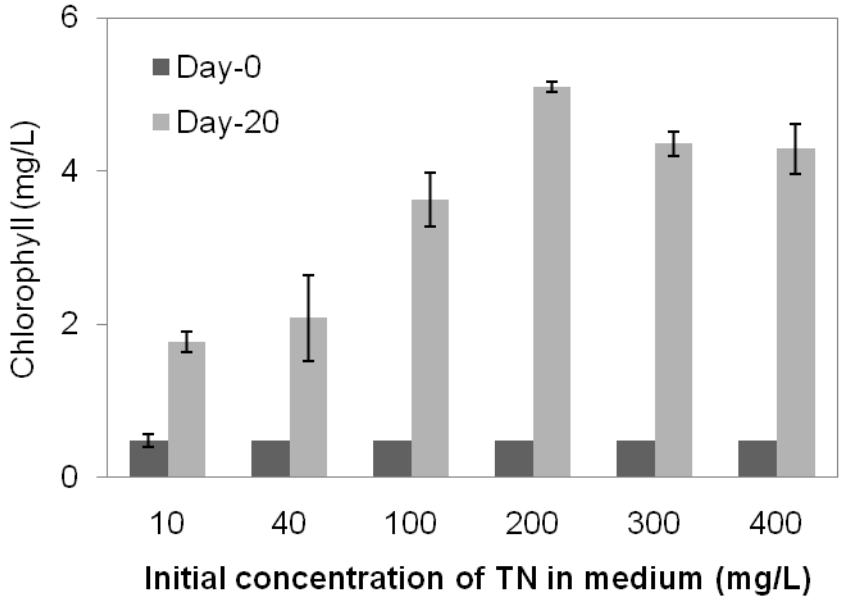


Figure 8: The increasing of chlorophyll content in different nutrient concentration before (Day-0) and after (Day-20) batch cultivation. Chlorophyll content is one of the indicators of algal growth. Data were acquired from 3 replications.

The concentrations of consumed TDN and TDP increased as the initial nutrient concentration increased (Table 3). 51.9-57.9% of TDP was consumed, and the percentage of consumed TDP was not influenced by initial nutrient concentration. However, the percentage of consumed TDN decreased as the initial nutrient concentration increased ($p < 0.001$, R-sq = 96.85%). The consumed TDN:TDP ratio of 10, 40, 100, 200, 300, and 400 mg TN/L cultures were 49.8, 71.8, 75.1, 76.8, 76.5, and 75.5 (Figure 9).

Table 3: The TDN and TDP uptake in different nutrient concentrations. Data were acquired by subtracting nutrient concentrations left in the media after 20 days of cultivation from the initial concentrations of each culture.

Initial AD conc. (mg TN/L)	TDN		TDP	
	conc. (mg/L)	% of initial	conc. (mg/L)	% of initial
10	19.3 ±0.6	93.5	0.39 ±0.05	56.0
40	36.8 ±1.2	98.1	0.51 ±0.11	56.5
100	55.3 ±0.6	74.0	0.74 ±0.05	54.6
200	117.0 ±4.6	72.0	1.52 ±0.04	57.9
300	139.3 ±2.1	61.9	1.82 ±0.09	51.9
400	169.3 ±7.6	58.0	2.24 ±0.06	52.1

The growth rate k was the target parameter to evaluate the effects of nutrient loadings on algal cultivation. Both low nutrient (10, 40 mg TN/L) and high nutrient (300, 400 mg TN/L) loadings had lower growth rates. The growth rates were peaked at the nutrient loadings of 100 mg TN/L (k = 0.0535) and 200 mg TN/L (k = 0.0538). In addition, there were no significant ($p > 0.05$) differences between the growth curves of 100 and 200 mg TN/L.

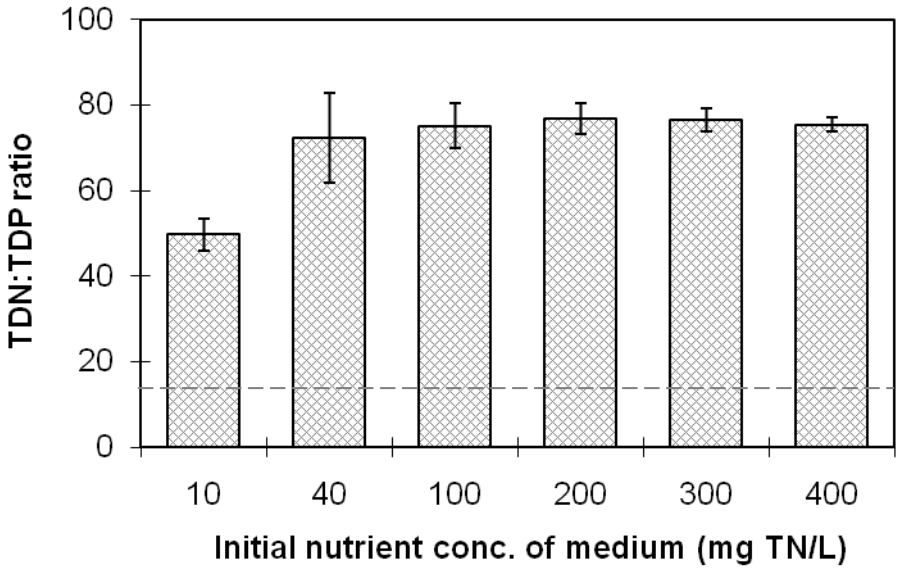
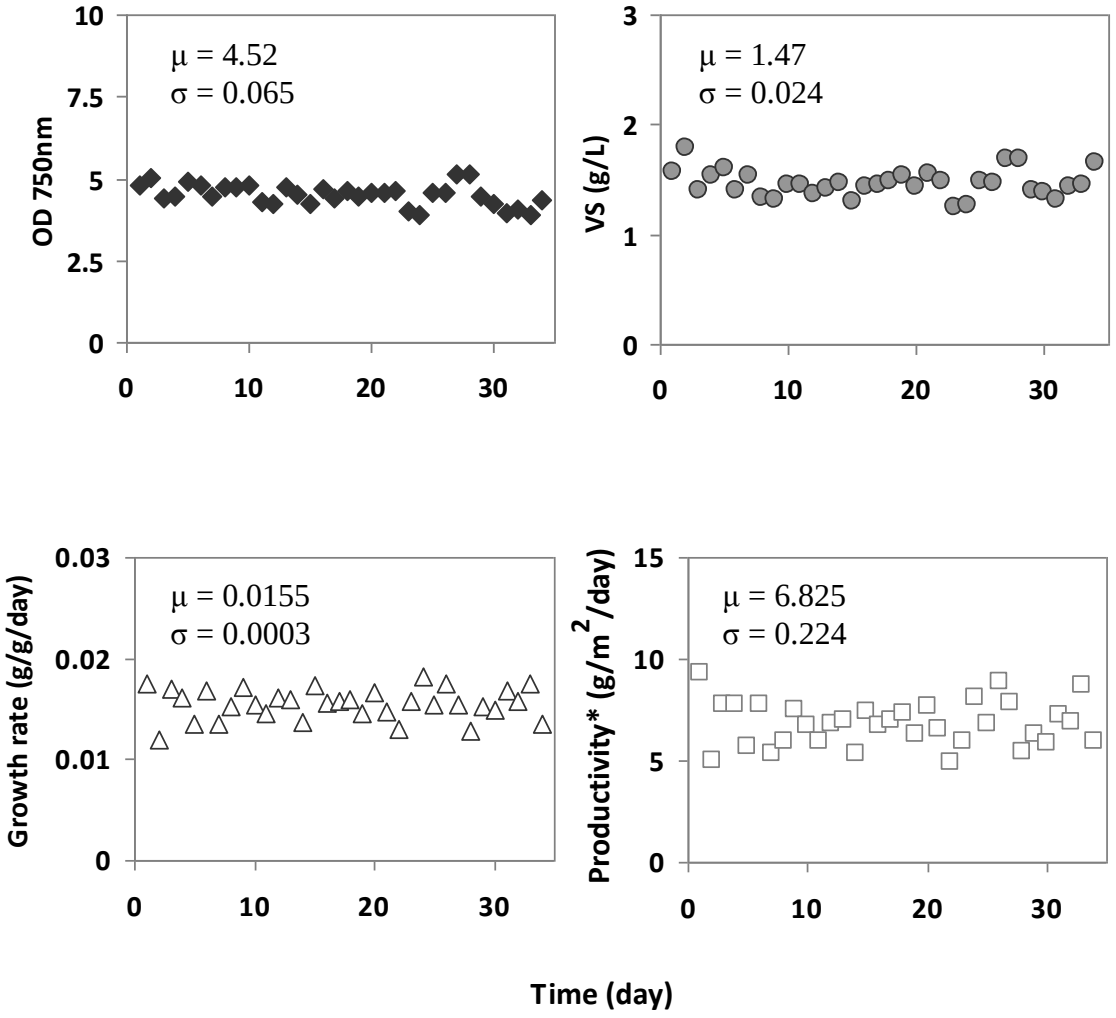


Figure 9: Consumed TDN:TDP ratio of cultures in different nutrient concentrations in 20 days. Data were acquired from three replications. The dash line represents the Redfield ratio (N:P = 16), which is much lower than all observed ratios.

Pilot algal culture in a semi-continuously fed raceway pond

In 35 days of cultivation (excluding the lag phase) following the semi-continuously fed and harvest strategy, the OD reading of the algal biomass in the pilot culture kept robust at 4.52 ± 0.065, and the VS of biomass was stable at 1.47 ± 0.024 g/L every day (Figure 10). The daily growth rate of the culture was 0.0155 ± 0.0003 g/g/day (based on VS). The daily productivity of the system was 6.825 ± 0.224 g/m^2 in a 30 cm deep pond.

Figure 10: Growth of algae in pilot-scale semi-continuously fed raceway pond in 35 days of cultivation, for the responses of OD and VS. Both growth rate and productivity were calculated based on VS. (*: in a 30 cm deep pond)



DISCUSSION

Effect of AD effluent on Algal Community Assemblage

Unfortunately, both 40 and 100 mg TN/L cultures were contaminated by bloodworms (Chironomids) a few days after the last feed (3 months). It was stated by EPA and other studies (Cairns and Dickson, 1976; Saether, 1975) that both mayflies and bloodworms were important freshwater indicator. Their larvae were more nearly restricted to and comprised a larger portion of the clean water association than any of the other groups. However, the 200 mg TN/L medium was not contaminated by invertebrates.

Over all, many factors (light, nutrient, dissolved organic matter, temperature, microorganisms, etc.) may have significant impacts on algal growth and community assemblage, and this study mainly focused on the effect of nutrient concentration. According to the results, filamentous cyanobacteria were sensitive to nutrient concentration, the densities of three observed filamentous cyanobacterial species dropped to nearly zero in the first 15 days of culture in 40, 100, and 200 mg TN/L AD effluent. Lee (1989) implied that excess nitrate-N in freshwater system facilitated the bloom of cyanobacteria. Because most nitrogen in AD effluent is in the form of ammonium-N (Mulbry et al., 2001; Kebede-Westhead et al., 2006), the increase of non-filamentous green algae and the decrease of filamentous cyanobacteria could be both related to the excess ammonium-N in AD effluent. The non-filamentous green algae could tolerate high ambient nutrient concentrations, particularly the *Chlorella* sp. Syrett (1962) reported that *Chlorella* and *Scenedesmus* could grow well in high concentration of nitrates or ammonium solutions, while many other planktonic algae cannot. Moreover, excessive free ammonia could inhibit both algal photosynthesis (Azov and Goldman, 1982; Abeliovich and Azov, 1976) and nitrate uptake (Ohmori et al., 1977). Hund (1997) found that increasing P concentration to 50mg/L and

above inhibited the growth of *Scenedesmus subspicatus*, however, P had much less effect on algal growth compared to excessive N. The *Chlorella* sp. cultured in 200 mg TN/L AD effluent for 5 months was selected as inoculum for the rest of this study because it was stable under the experimental conditions of high nutrient concentration.

Chemical pretreatment of AD effluent

Although both $Ca(OH)_2$ and $AlK(SO_4)_2 \cdot 12H_2O$ had significant impacts on the turbidity, volume, TN and TP of pretreated AD supernatant, the results denoted that the concentration of $Ca(OH)_2$ should be kept medium (7.5 g/L) to obtain relatively low turbidity. Meanwhile, the dosage of $AlK(SO_4)_2 \cdot 12H_2O$ should be kept low (1 g/L) for higher TP and appropriate N:P ratio. Total volume of supernatant was used to indicate the efficiency of chemical coagulation. The volume was not the highest under the optimal combination of two chemicals, but it can be improved using a screw press. The pretreatment reduced about 55% of TN and 93% of TP from the original AD effluent (Figure 5), which was understandable because most of N and P existed in dairy manure were in the form of organic particles (Mulbry et al., 2004) and the pretreatment separated large organic particles from relatively clear supernatant. After filtration with Millex-GS 0.22 mm membrane, about 80% of nitrogen remained in the filtrate (TDN), but only about 24% of phosphorus was detected as TDP. This demonstrated that most of nitrogen in AD effluent was soluble but most phosphorus was not.

Bench culture of selected algal strain with chemically pretreated AD effluent

Eutrophication of water bodies due to nutrient enrichment from agricultural non-point sources is the primary cause of excessive growth of algae in lakes and rivers (Paerl, 1988;

Oliver and Ganf, 2000; Vasconcelos and Pereira, 2001). However, even under eutrophic condition, other factors may also influence the growth rate of algae, such as light limitation, COD, biochemical oxygen demand (BOD), pH, temperature, turbulence, and N:P ratio (Paerl, 1988; Philips et al., 1997; Havens et al., 2003). Moreover, the growth of many freshwater planktonic algae may be inhibited in 0.01-0.001 mol/L solutions of nitrates or ammonium-N (Chu, 1942; ZoBell, 1935). Results from the presented study agreed that nutrient concentration had significant impact on algal growth rate. The overall pattern of growth rate presented a positive relationship between nutrient and algal growth in 10, 40, 100, and 200 mg TN/L cultures; however, it reversed to a negative relationship as the initial nutrient concentration increased (in 300 and 400 mg TN/L cultures). Therefore, the high contents of nitrogen and phosphorus in pretreated AD effluent may result in different growth pattern in comparison to the freshwater system. It has also been reported that high concentrations of inorganic nutrients may also result in decreasing photosynthesis and growth of *Scenedesmus obliquus*, *Chlorella pyrenoidosa*, *Anacystis nidulans*, *Plectonema boryanum*, and some marine algae (Azov and Goldman, 1982; Abeliovich and Azov, 1976). Moreover, stimulation can principally mask effects by toxic substances, whereas an inhibition can result in an overestimation of the toxicity (Hund, 1997).

All responses verified that the optimal growth rate of the selected algae, *Chlorella* sp., was observed in 200 mg TN/L pretreated AD effluent culture. Under the same initial nutrient concentration, growth rates based on TS, VS, and standard biovolume showed no significant differences from each other (Figure 7). Moreover, the growth rates based on OD also agreed with other responses in low initial nutrient concentrations (10, 40, and 100 mg TN/L of pretreated AD effluent; Figure 7). However, growth rates based on OD in high nutrient concentrations (200, 300, and 400 mg TN/L of pretreated AD effluent) are higher than the ones from other responses. Though the relationship between OD and algal concentration can

be established using a spectrometer, variations may occur due to the fact that the chlorophyll concentration in the algal cell varies according to the culture conditions and therefore affects this relationship (Lavens and Sorgeloos, 1996). For example, a culture under low light intensity will comparatively form more pigment and will eventually result in higher readings for OD (Vonshak, 1986). In this study, the turbidity of medium increased as the nutrient concentration increased; and with the accumulated algal biomass in the flasks, it is very possible that OD readings were positively curved due to the low light availability. Biggs (2000) reported that the nutrient concentrations (soluble inorganic nitrogen and TDP) were positively correlated with chlorophyll concentration of algal cell. Given the fact, it is also possible that high nutrient concentrations increased the content of chlorophyll and therefore affected the OD readings.

According to Hsu's MCB (Appendix-Table 6), estimation of population means (Figure 7) and z-test (Appendix-Table 8), the optimal growth rate (in 200 mg TN/L) was significantly higher than the growth rates of other cultures (based on OD, TS and standard biovolume). For the responses of VS, the difference in growth between 100 and 200 mg TN/L cultures was not significant. It is not clear whether nutrient concentration had a positive or negative impact on algal growth in 100 and 200 mg TN/L cultures according to the VS responses, but based on our previous study ("Effect of AD effluent on algal community assemblage"), the 200 mg TN/L culture was less likely to be contaminated by invertebrates (bloodworms). Therefore, the 200 mg TN/L of chemically pretreated AD effluent was selected as the optimal nutrient concentration for algal biomass accumulation.

Though there were only two data points of chlorophyll content, they showed that a higher biomass accumulation was occurred in 200 mg TN/L of pretreated AD effluent. Decreased chlorophyll contents were observed in higher nutrient loadings of 300 and 400 mg TN/L media, which could be caused by the inhibition of excessive nutrients (Azov and Goldman,

1982; Abeliovich and Azov, 1976; Ohmori et al., 1977; Hund, 1997).

About 65-82% of nitrogen in AD effluent is in the form of ammonium-N (Mulbry et al., 2001; Kebede-Westhead et al., 2006; Wilkie and Mulbry, 2002), which could be readily consumed by algae. De la Noue and Basseres (1989) reported 99.5% of ammonium-N and 88.2% of phosphate-P were removed in AD effluent with high initial concentration (102 mg TN/L and 8.31 mg TP/L). In this study, the selected algae consumed about 50-60% of initial TDP in all concentrations; however, the percentage of consumed TDN was not stable. One possible reason for the variation in nutrient uptake could be the toxicity or inhibition of high levels of ammonia to microalgae (Abeliovich and Azov, 1976).

Interestingly, the consumed N:P ratios were similar (about 72-76) in all cultures except for the 10 mg TN/L in which the ratio was 50 (Table 3), but all of them were much higher than the Redfield ratio 16:1 (Redfiled, 1958), which represents the stoichiometric ratio of N and P in phytoplankton. Rhee (1978) deemed that the optimal cellular N:P ratio might be species-specific. If this is so, the difference in this value would have great ecological importance. Moreover, Aslan and Kapdan (2006) implied the main reason for low removal performance at high nutrient concentrations could be the light limitation because of excess chlorophyll a concentrations in their study. In addition, they also reported the optimization of N:P ratio may enhance the nutrient removal capability of *Chlorella vulgaris* at high N and P concentrations.

However, if the cellular N:P ratio always falls into the range of the Redfield ratio (16:1) instead of being species-specific, one of the possibilities is that free ammonia was formed during the cultivation because the pH of medium was kept around 8.5, and with the help of high flow rate (150 rpm on orbital shaker), free ammonia was released into the atmosphere. And because only the N and P in the medium were measured to indirectly by subtracting remaining nutrient from the original concentration, the released ammonia would not be

detected. Moreover, bacterial consumption could be another reason that consumed N:P ratios were high. It has been reported that agricultural waste was a major source of nitrous oxide (N_2O) and nitric oxide (NO), accounting for about 6.2 and 5.5 Tg of global annual emission, respectively (Kroeze et al., 1999; Davidson and Kingerlee, 1997; Tenuta et al., 2000; Flessa et al., 1995); both of them were produced by microbial processes of nitrification and denitrification (Davidson, 1991). Because of the considerably large amount of bacteria in AD effluent (Carlson and Ingraham, 1983), along with the accumulating algal biomass in this study, the organic carbon source for denitrification was increased (Akiyama and Tsuruta, 2003) and oxygen could be consumed very fast even though the cultures were kept on an orbital shaker. Therefore, the denitrification process could have occurred and increased the N:P consumption ratios.

One of the preliminary experiments measured chemical contents of algal biomass cultured in different nutrient loadings to elucidate the effects of nutrient conditions on algal composition. The algal biomass from three different nutrient concentrations (40, 100 and 200 mg TN/L) were collected after 50 days of cultivation. The starch, protein and lipid contents of the algal biomass were measured. The starch and total sugar contents decreased as the initial nutrient concentration increased, while the protein content increased as the nutrient increased (Table 4). Syrett (1962) stated that the there was a remarkable change in the products of carbon assimilation with respect to the changes of ammonia-N in media, With the increase of ammonia-N, amino acids became more predominant, and sugar and sugar phosphates became less so (Holm-Hansen et al., 1959).

Table 4: Chemical contents of algal biomass which was cultured in different nutrient concentrations of AD effluent. Algal inocula were from local freshwater pond, and the AD effluent applied as nutrient source was not chemically pretreated. As the initial nutrient concentration increased, the protein content of algal biomass increase, but starch and sugar content decreased.

Content of algal biomass	Initial nutrient concentration (mg TN/L)		
	40	100	200
Lipid (%)	10.20±0.41	9.34±1.12	10.78±2.94
Protein (%)	19.42±0.03	21.49±0.07	26.10±0.01
Starch (%)	22.96±2.48	9.35±1.41	4.76±0.35
Total sugar (%)	17.21±0.49	10.40±1.38	4.79±3.12

Pilot algal culture in a semi-continuously fed raceway pond

The results of OD and VS in 35 days (after the lag phase) showed that the culture was relatively stable in the semi-continuously fed pond system (Figure 10). Daily growth rate (or productivity) of the culture also verified the robustness of the culture strategy in this study, which was 300 mL harvest of broth and 300 mL feed of chemically pretreated AD effluent daily in a 20 L pilot-scale raceway pond. Although the productivity was lower than some chemical defined cultivation (Li et al., 2007; Livansky and Doucha, 2000; Morita et al., 2000), it was actually moderate considering the complexity of AD effluent and the negative influence it might bring (Pizarro et al., 2006). In addition, the growth rate of the pilot-scale culture (0.0155±0.0003 g/g/day, based on VS) was lower than the bench-scale culture (0.0538±0.0019 g/g/day, based on VS), which was possibly because of the inhabitation of high ambience nutrient (Abeliovich and Azov, 1976), low light availability (Brody and Brody, 1962), and effect of other microorganisms (Carlson and Ingraham, 1983; Davidson, 1991).

Although the growth rate of algae might change when cultured at different scales, the semi-continuously harvest and feed strategy keeps the cultural system robust and the biomass growth fast (stable at exponential phase).

CONCLUSION

Experimental conclusions

Microalgae, as a type of non-food-based feedstock with high productivity, have become the exclusive focus of the research for sustainable biofuels. Moreover, they also have been applied to take up excess nutrients in the wastewater treatment industry for decades. This research was aimed to develop an integrated biofuel production and farm-waste treatment process from both ecological and engineering perspectives. A long-term cultivation of freshwater algae in AD effluent indicated that non-filamentous green algae (*Chlorella* sp. and *Scenedesmus* sp.) were able to tolerate high nutrient loadings. In particular, the community assemblage of a *Chlorella* sp. was stable in 200 mg TN/L AD effluent for more than 5 months.

Chemically pretreated AD effluent had been proven to increase the growth rate of algae by the preliminary experiment, as the process agglomerated suspended solids and intensified the light availability in the medium. In the kinetics study, an optimal growth rate of selected *Chlorella* sp. has been found in a bench culture with chemically pretreated (using 25 g/L $Ca(OH)_2$ and 3 g/L $AlK(SO_4)_2 \cdot 12H_2O$) AD effluent, which contained 200 mg/L of TN and 2.4 mg/L of TDP initially.

A pilot-scale cultivation of selected algae strain in a semi-continuously fed and harvested raceway pond was then conducted. The daily productivity was 6.825 ± 0.224 g/m^2 in a 30 cm deep pond (based on VS), although it was relatively low when compared with some chemical-defined intensive cultivation, the system kept a high robustness for 35 days.

Future research

Although a lot of literature has specifically measured and predicted the influence of some factors (such as nutrient loading, light availability, temperature, pH, etc.) on algal community assemblages in natural water systems, the algal communities cultured in extremely high nutrient loaded medium (such as industrial and agricultural wastewater) have not been fully explored yet. Therefore, further studies on the determinants of algal community change in AD effluent are needed. In addition, the determination of chemical compositions in AD cultured algal biomass is also essential for the detailed studies.

The consumed nitrogen and phosphorus were unbalanced when compared to the Redfield ratio, the high N:P ratio could be caused by many reasons, such as the loss of ammonia-N into the atmosphere due to a high pH, the luxury uptake of phosphorus and inhibition of excess ammonia-N. It is also possible that other microorganisms (e.g. bacteria) have played another important role in nutrient consumption (e.g. nitrification/denitrification). Therefore, given the complexity of AD effluent, accurate tracing and monitoring of rates of nutrient uptake and loss are also needed in the future, as well as the research on the relationship between algal and other microorganisms in AD effluent-based culture.

Moreover, the productivity of the pilot-scale study was relatively low, compared to some cultures in chemically defined media. It was possible that light availability was a crucial reason for a low growth rate; it was also possible that excess nutrients (especially ammonia-N) inhibited the photosynthetic capability of algae. These hurdles need to be determined and overcome so that our study can be more beneficial to both wastewater treatment and bioenergy industry.

APPENDIX

Table 5: Growth rate of algae in different nutrient concentrations (Mean ± Standard Error) and the p-value's of nutrient-growth relationship, for the responses of OD, TS, VS and standard biovolume.

Initial AD conc. (mg TN/L)	Growth rate (*k*, g/g/day)			
	OD	TS	VS	Std. Biovol.
10	0.0447 ±0.0018	0.0455 ±0.0007	0.0457 ±0.0008	0.0403 ±0.0014
40	0.0425 ±0.0042	0.0463 ±0.0026	0.0463 ±0.0037	0.0395 ±0.0020
100	0.0546 ±0.0027	0.0485 ±0.0017	0.0535 ±0.00528	0.0464 ±0.0059
200	**0.0673 ±0.0025**	**0.0528 ±0.0027**	**0.0538 ±0.0019**	**0.0560 ±0.0028**
300	0.0589 ±0.0013	0.0381 ±0.0010	0.0465 ±0.0012	0.0436 ±0.0008
400	0.0508 ±0.0010	0.0366 ±0.0024	0.0460 ±0.0027	0.0394 ±0.0014
p	< 0.001	< 0.001	0.032	0.007

Table 6: Intervals for level mean minus largest of other level means, for the responses of OD, TS, VS and standard biovolume, using Hsu's MCB (Multiple Comparisons with the Best) method*. For this study, highest growth rate was the best; therefore, the confidence interval (CI) entirely above zero was significantly better, the CI entirely below zero was significantly worse, and the CI's contained zero had no significant differences.

Initial AD conc. (mg TN/L)	Confidence Interval from Hsu's MCB			
	OD	TS	VS	Std. Biovol.
10	(-0.0237, 0)	(-0.0128, 0)	(-0.0131, 0)	(-0.0252, 0)
40	(-0.0339, 0)	(-0.0121, 0)	(-0.0122, 0)	(-0.0257, 0)
100	(-0.0185, 0)	(-0.0128, 0)	**(-0.0060, 0.0059)**	(-0.0165, 0)
200	**(0, 0.0145)**	**(0, 0.0121)**	**(-0.0059, 0.0060)**	**(0, 0.0165)**
300	(-0.0145, 0)	(-0.0201, 0)	(-0.0133, 0)	(-0.0204, 0)
400	(-0.0225, 0)	(-0.0217, 0)	(-0.0138, 0)	(-0.0247, 0)

*Family error rate = 0.1;

Critical value = 2.02.

Table 7: One-way ANOVA and Hsu's MCB analysis of nutrient effect on OD, TS, VS and Standard Biovolume

One-way ANOVA: VS versus C1

Source	DF	SS	MS	F	P
C1	5	0.0002754	0.0000551	3.17	0.032
Error	18	0.0003126	0.0000174		
Total	23	0.0005880			

S = 0.004167 R-Sq = 46.84% R-Sq(adj) = 32.07%

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.1
Critical value = 2.02

Level	Lower	Center	Upper
10	-0.013115	-0.007150	0.000000
40	-0.012215	-0.006250	0.000000
100	-0.005990	-0.000025	0.005940
200	-0.005940	0.000025	0.005990
300	-0.013265	-0.007300	0.000000
400	-0.013790	-0.007825	0.000000

One-way ANOVA: OD versus C1

Source	DF	SS	MS	F	P
C1	5	0.0017509	0.0003502	18.99	0.000
Error	18	0.0003319	0.0000184		
Total	23	0.0020828			

S = 0.004294 R-Sq = 84.07% R-Sq(adj) = 79.64%

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.1
Critical value = 2.02

Level	Lower	Center	Upper
10	-0.023671	-0.017525	0.000000
40	-0.033896	-0.027750	0.000000
100	-0.018496	-0.012350	0.000000
200	0.000000	0.008325	0.014471
300	-0.014471	-0.008325	0.000000
400	-0.022546	-0.016400	0.000000

Table 7 (cont'd)
One-way ANOVA: TS versus C1

Source	DF	SS	MS	F	P
C1	5	0.0007363	0.0001473	12.11	0.000
Error	18	0.0002189	0.0000122		
Total	23	0.0009552			

S = 0.003487 R-Sq = 77.09% R-Sq(adj) = 70.72%

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.1
Critical value = 2.02

Level	Lower	Center	Upper
10	-0.012816	-0.007825	0.000000
40	-0.012116	-0.007125	0.000000
100	-0.012791	-0.007800	0.000000
200	0.000000	0.007125	0.012116
300	-0.020116	-0.015125	0.000000
400	-0.021691	-0.016700	0.000000

One-way ANOVA: BioV versus C1

Source	DF	SS	MS	F	P
C1	5	0.0010254	0.0002051	8.02	0.000
Error	18	0.0004604	0.0000256		
Total	23	0.0014858			

S = 0.005057 R-Sq = 69.02% R-Sq(adj) = 60.41%

Level	N	Mean	StDev
10	4	0.038817	0.002508
40	4	0.038267	0.003384
100	4	0.047435	0.010209
200	4	0.056730	0.004863
300	4	0.043572	0.001342
400	4	0.039245	0.002455

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.1
Critical value = 2.02

Level	Lower	Center	Upper
10	-0.025151	-0.017913	0.000000
40	-0.025701	-0.018462	0.000000
100	-0.016533	-0.009295	0.000000
200	0.000000	0.009295	0.016533
300	-0.020396	-0.013157	0.000000
400	-0.024724	-0.017485	0.000000

Table 8: z-test of growth rate (P-value) among six treatments (with 10, 40, 100, 200, 300, and 400 mg TN/L of initial nutrient concentration). For those P-value's greater than 0.05, there was no significant difference between two treatments; for those P-value's less than 0.05, there was significant difference between two treatments.

Based on OD

	10	40	100	200	300
40	0.924				
100	0.000	0.000			
200	0.000	0.000	0.000		
300	0.000	0.000	0.000	1.000	
400	0.000	0.000	1.000	1.000	1.000

Based on TS

	10	40	100	200	300
40	0.402				
100	0.533	0.729			
200	0.001	0.002	0.024		
300	1.000	1.000	1.000	1.000	
400	1.000	1.000	1.000	1.000	0.784

Based on VS

	10	40	100	200	300
40	0.373				
100	0.002	0.005			
200	0.000	0.000	0.446		
300	0.602	0.795	1.000	1.000	
400	0.634	0.725	0.999	1.000	0.601

Based on standard biovolume

	10	40	100	200	300
40	0.885				
100	0.026	0.016			
200	0.000	0.000	0.000		
300	0.000	0.000	1.000	1.000	
400	0.805	0.582	1.000	1.000	1.000

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