GROWTH CONDITION OPTIMIZATION AND BACTERIAL CONTROL FOR MASS PRODUCTION OF MICROALGAE

by

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Renhe Qiu, titled Growth Condition Optimization and Bacterial Control for Mass Production of Microalgae and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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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.

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ABSTRACT

Microalgae are considered as one of the most promising future energy feedstocks because of some advantages, such as the simple cellular structure, short production cycle, high lipid content, and fast growth. However, relatively high production costs due to low lipid productivity, high nutrients demand, and high water/energy consumption are some of the major obstacles impeding commercial production of algal biofuels. Microalgae strain *Chlorella sorokiniana* DOE1412 was used in this dissertation due to its robust growth and high productivity. This work focuses on two area that can reduce the production costs: optimizing environmental growth conditions, and a bacteria removal strategy for recycled water.

Cultivation of microalgae for biofuel production is highly influenced by numerous environmental conditions, including physical conditions (e.g., light and temperature) and chemical conditions (e.g., nutrients, salinity, and pH). These environmental conditions not only affect the accumulation of biomass but also influence the biochemical composition of microalgal biomass. The pH is one of the most critical environmental conditions in microalgal cultivation since it determines the solubility and availability of CO_2 and nutrients, and has a significant influence on microalgal metabolism. Here, cell growth and lipid content of *Chlorella sorokiniana* DOE1412 were first evaluated at different pH values in flask cultivation. Culture pH was manipulated by CO_2 addition. The optimal pH for DOE1412 is approximately 6.0 when only accounting for cell growth and lipid production and not considering the CO_2 efficiency. A flat panel airlift photobioreactor (PBR) was used for scale-up cultivation at five different pH levels (6.5, 7, 7.5, 8 and 8.5). Data of pH values and CO_2 addition was collected by a data logger. Biomass productivity increased with decreasing pH. By taking into account not only the cell growth and lipid production

but also CO_2 addition, the lowest value of CO_2 addition was achieved at pH 8 (2.01 g CO_2/g biomass). The fatty acid profiles and biodiesel properties, such as iodine value (IV), saponification value (SV), cetane number (CN), degree of unsaturation (DU), long-chain saturated factor (LCSF), and cold filter plugging point (CFPP), were determined as a function of pH. The calculated CN of biodiesel, which theoretically could be produced from the algae cultivated at pH 6.5, 7 and 7.5, satisfied the US standard ASTM D6751; among them, the pH 6.5 products met the European standard EN 14214. Finally, protein content in microalgal biomass increased with increasing pH, while C/N ratio in cells decreased.

Microalgae grown in open systems are prone to biological contaminations, including bacteria, zooplankton, virus, and other algae. Cultivation water recovered from large-scale open raceway ponds contains bacteria that can affect culture health. Developing an inexpensive and effective strategy to control bacterial contamination in recycled cultivation water is essential for making algal production financially and environmentally sustainable. We tested several bacterial deactivation strategies, including ozonation, chlorination, chloramination and the addition of hydrogen peroxide, on artificial recycled water samples containing mixtures of microalgae *Chlorella sorokiniana* DOE1412 and *Escherichia coli* strain DH5α. Disinfectant decay, bacterial deactivation and algal survival curves were obtained for each disinfectant at different doses to determine bacteria removal rate, minimum contact time, microalgae survival rate, disinfectant residual, and concentration-time (CT) value. These data were used to compare the efficiency of different bacterial deactivation methods. Results showed that chlorination with an initial dosage of 0.5 mg/L is the most economic and successful method for selectively deactivating bacteria. A 5-log reduction of *E. coli* cells was achieved after 1 min of contact time, while the microalgae survival rate was 58%. No chlorine was detected after 5 min of exposure. The other methods

investigated showed higher CT value or lower microalgae survival rate for 5-log deactivation of *E. coli* in artificial recycled water samples. The effectiveness of chlorination was also confirmed when tested on the authentic recycled water samples.

CHAPTER 1: Introduction

1.1. Microalgal biomass

The recognition that fossil fuel supplies are finite and expected to decline over the next few decades, as well as the volatile prices of petroleum, combined with the fast-growing concern about global warming has led to significant interest in developing alternative energy sources. Biofuels are considered to be one of the most viable alternative sources of energy since they are renewable, sustainable and environmentally friendly. Moreover, compared with other renewable sources (e.g., wind, tidal and solar), biofuels allow solar energy to be stored and used directly in existing engines and transport infrastructures (Scott et al., 2010). However, the development of biofuels still requires additional research to assure that a technically and economically viable biofuel resource costs about the same as petroleum fuels, requires low land use, enables air quality improvement and requires minimal water use (Brennan and Owende, 2010).

Microalgae specifically have received notable attention and have been proposed as one of the most promising future energy feedstocks (Schenk et al., 2008; Wijffels and Barbosa, 2010; Chen et al., 2015). They are a group of unicellular or simple multi-cellular photosynthetic microorganisms that primarily use water, CO_2, and sunlight to produce biomass and O_2. There are both prokaryotic microalgae and eukaryotic microalgae (Singh and Cu, 2010). Examples of prokaryotic microalgae include cyanobacteria (*Cyanophyceae*), and eukaryotic microalgae include green algae (*Chlorophyta*), red algae (*Rhodophyta*) and diatoms (*Bacillariophyta*) (Mata et al., 2010; Singh and Cu, 2010). The nutrients required for growing microalgae are carbon (typically CO_2), nitrogen, phosphorus, mineral salts, trace elements, and silicon (for diatom) (Borowitzka and Moheimani, 2013). Most of those nutrients are available from municipal, industrial and

agricultural wastewater. The main advantages of microalgae over other feedstocks for biofuels are as follows:

(1) Microalgae biomass can be harvested batch-wise nearly all year round, therefore, oil productivity has the potential to exceed the yield of the best oilseed crops, e.g. biodiesel yield of 58,700 L/ha for microalgae (30% oil by wt.) compared with 1190 L/ha for canola, 1892 L/ha for Jatropha, 2689 L/ha for coconut and 5950 L/ha for oil palm. This means that the land area needed to produce algal biofuels is significantly less than that needed using other biofuels feedstocks (Chisti, 2007).

(2) Microalgae grow at a very fast rate; they typically double in periods as short as 3.5 h. They store their energy by making lipids. Depending on species and growth conditions, the common lipid content ranges from 20% to 50% dry weight (Chisti, 2007). The lipid can be extracted and converted into gasoline, diesel or jet fuel.

(3) Microalgae cultivation consumes less water than terrestrial crops, despite the fact that they grow in aqueous media, thereby greatly reducing freshwater use (Dismukes et al., 2008).

(4) Microalgae can tolerate brackish water and marginal land, and therefore cultivating algae avoids land use change, and minimizes associated environmental impacts (Searchinger et al., 2008), without compromising agricultural products derived from terrestrial crops (Day et al., 2012).

(5) Microalgae can fix CO_2 efficiently from industrial exhaust gases during photosynthesis, providing a very promising alternative for the mitigation of CO_2, the most prominent greenhouse gas (Hamasaki et al., 1994; Brown, 1996; Benemann, 1997; Doucha et al., 2005; Wang et al., 2008; Borkenstein et al., 2011).

(6) Apart from obtaining nutrients from growth medium, microalgae have the ability to utilize nutrients (especially nitrogen and phosphorus) from polluted municipal, industrial and agricultural wastewater, which provides a promising option for the bioremediation of wastewater (Cantrell et al., 2008; Mulbry et al., 2008; de Godos et al., 2010; Rawat et al., 2011).

(7) Microalgae cultivation does not require herbicides or pesticides application (Rodolfi et al., 2009).

(8) Depending on the species, microalgae have the potential of producing valuable co-products, including a large range of fine chemicals and bulk products, such as fats, proteins, carbohydrates, natural dyes, pigments, antioxidants, high-value bioactive compounds, and other fine chemicals and biomass (Spolaore et al., 2006; Li et al., 2008b; Raja et al., 2008; Milledge, 2011). Co-production of fuels and chemicals improves the economics of microalgal biofuel production.

(9) The biochemical composition of algal biomass can be manipulated by varying growth conditions. Hence the oil yield can be significantly enhanced (Qin, 2005).

(10) Microalgae can use energy from the sun through photosynthesis and produce renewable hydrogen through their metabolism (Ghirardi et al., 2000).

Although microalgal biofuels hold great promise, the production cost of algal biofuels is currently not competitive with petroleum-based products. Many major technical challenges need to be tackled before production will be ready for commercial scale (Greenwell et al., 2010). Further research efforts required to make microalgal biofuels cost-effective and sustainable include: studying the molecular biology of microalgae; selecting and bioengineering microalgae strains

optimized to regional climate conditions; developing large-scale microalgae cultivation systems; optimizing cultivation conditions (e.g. nutrient, temperature, light, pH, salinity and mixing); developing efficient techniques for lipid extraction, biomass harvesting and downstream processing; utilizing recycled water or wastewater; reducing and controlling operating costs and energy consumption; and utilizing residues to make valuable co-products (Singh et al., 2011; Chen et al., 2015). Nevertheless, given the vast potential of microalgae as the most efficient primary producers of biomass, there is little doubt that they will eventually become one of the most important alternative energy sources.

1.2. Microalgae cultivation systems

A photobioreactor (PBR) is a closed system used to cultivate algae that are not directly exposed to the atmosphere. It can be illuminated by artificial light, solar light or by both. Because microalgae are cultivated in a closed system, culture conditions are easier to control, and there is less contamination and evaporative losses when compared with open pond systems (Posten and Schaub, 2009). Moreover, a PBR has much higher biomass productivity and cell density, due to better stirring mechanisms and maximized sunlight capture. Biomass productivity per unit area of algae grown in PBRs can be twice that of open pond systems, while the cell density can be 30 times higher (Chisti, 2007). Higher cell density can reduce the cost of harvesting. Flat panel, tubular and column bioreactors are the three main types of PBRs. Flat panel PBRs have received significant attention for cultivation of photosynthetic microorganisms. Advantages of these PBRs include: a large illumination surface area, suitability for outdoor cultivation, high biomass productivities, relatively inexpensive, easy to clean, readily tempered, and low oxygen buildup

(Ugwu et al., 2008). However, there are some limitations as well including ability to scale up, short light penetration depth, and poor temperature regulation (Gupta et al., 2015).

Unlike PBRs, a raceway pond is an open system. It is made of a closed-loop flow channel with a typical depth of about 0.3 m (Chisti, 2007). Open raceway ponds are the most commonly and widely used culture systems for large-scale algal biomass production due to their low initial cost and low energy requirements. One disadvantage of open raceway ponds is that microalgae grown in open systems are prone to biological contaminations, including bacteria, zooplankton, virus, and other algae (Wang et al., 2013).

1.3. Environmental conditions of microalgae cultivation

Cultivation of microalgae for biofuel production is highly influenced by numerous environmental conditions, including physical conditions (e.g., light and temperature) and chemical conditions (e.g., nutrients, salinity, and pH) (Hu et al., 2008; Singh and Dhar, 2011). These environmental conditions not only affect profoundly the accumulation of biomass but also influence the biochemical composition of microalgal biomass.

1.3.1 Light

Being the basic energy source for photoautotrophic organisms, the availability and intensity of light are one of the key parameters affecting photosynthetic activity (Richardson et al., 1983). With an increase in light intensity, the growth rate of microalgae increases until light saturation (usually around 200 to 400 μmol photons m^{-2} s^{-1}) is reached (Radakovits et al., 2010). Moreover, if the light intensity increases much above saturation, photoinhibition often becomes evident leading to declining biomass productivity (Richmond, 2000). The chemical composition of microalgal biomass can also be modulated by light intensity. High light alters fatty acid

synthesis to produce more saturated and monounsaturated fatty acids, while low light promotes production of polyunsaturated fatty acids (Fabregas et al., 2004; Khotimchenko and Yakovleva, 2005).

1.3.2 Temperature

Although microalgae can survive at a variety of temperatures, the optimal temperature for growth is generally from 20 to 30 °C (Singh et al., 2012). Within this narrow range, a rise in temperature leads to exponential increases in cell growth. Temperatures above the optimal range may kill microalgae cells, while temperatures below the optimal range may not, except for temperatures below freezing. Lower temperatures can reduce the respiratory biomass losses during the dark period (Raven and Geider, 1988). Therefore, high biomass productivity can be achieved by increasing the temperature to the optimal value as quickly as possible in the morning (to maintain high productivity) and followed by rapidly decreasing the temperature at night (to minimize biomass losses). However, for outdoor and open microalgal cultivation systems, the ability to control temperatures is often limited and is determined by atmospheric temperature, solar irradiance, and humidity. Moreover, temperature has been found to have a major effect on the fatty acid composition of microalgae. High temperatures have been shown to favor the formation of saturated fatty acids in many species (Renaud et al., 2002).

1.3.3 Nutrients

Nutrients supplied to microalgal cultivation include macronutrients (e.g., nitrogen, phosphorus, carbon, and sulfur) and micronutrients (e.g., iron, zinc, copper, calcium, and magnesium). Carbon, nitrogen and phosphorus are the most important nutrients for growth and metabolism of microalgal cells (Wijffels and Barbosa, 2010).

Nutrient limitation may cause morphological and physiological changes of microalgae cells, and therefore affect the growth rate and biomass production (Cheng and He, 2014). Nitrogen is the most critical for the biosynthesis of nucleic acids, proteins, and photosynthetic pigments. Nitrogen deprivation limits the synthesis of photosynthetic proteins and pigments, therefore increasing the lipid content of biomass. This has been observed for a variety of microalgae species (Li et al., 2008a; Converti et al., 2009; Rodolfi et al., 2009). Phosphorus is involved in a number of cellular metabolic processes. The effect of phosphorus limitation on lipid content appears to be algae strain dependent. Both positive (Khozin-Goldberg and Cohen, 2006; Xin et al., 2010) and negative (Reitan et al., 1994) effects have been observed.

1.3.4 Salinity

Different microalgae species can tolerate different ranges of salinity (Kirst, 1990). Within the tolerance range, it has been found that high salinity increases lipid content, which can contribute to increases in biofuel yield (Takagi et al., 2006; Bartley et al., 2013). Excess salinity inhibits photosynthesis, thus reduces the yield of biomass (Takagi et al., 2006; Cho et al., 2007; Rao et al., 2007). Also, the composition of lipid can also be altered by salinity. Low salinity tends to increase the percentage of unsaturated fatty acids, whereas high salinity results in higher production of saturated fatty acids (Chen et al., 2008).

1.3.5 pH

The pH of the growth medium is one of the most important environmental conditions in microalgal cultivation since it determines the solubility and availability of CO_2 and nutrients, and has a significant influence on microalgal metabolism (Chen and Durbin, 1994). Each microalgal species has an optimal pH range for biomass and lipid production, which is narrow and strain-specific (Goldman et al., 1982; Khalil et al., 2010; Moheimani and Borowitzka, 2011; Moheimani,

2013). Notably, pH is the major determining factor of relative concentrations of the carbonaceous species in water(Azov, 1982). The pH of microalgal cultures rises gradually during the day due to the uptake of inorganic carbon by algae. Higher pH limits the availability of carbon from CO_2, which, in turn, inhibits cell growth (Azov, 1982; Chen and Durbin, 1994). Also, it is reported that adjusting the culture to high pH can suppress undesired biological contaminants (Bartley et al., 2014).

Many researchers control pH by adding buffers, which is not cost-effective or realistic at a large scale. Also, it can be a potential pitfall, because the effect of buffers on microalgal metabolism is not clear yet. Another commonly used method for controlling pH in algal cultivation is CO_2 injection. As the cells consume carbon, the pH rises and additional CO_2 is add as required to maintain a constant pH. The CO_2 is typically added as a gas and can be supplied from flue gas.

1.4. Water recycling in microalgae cultivation

Production of microalgae consumes large quantities of water if recycling is not part of the processing scheme. It has been reported that about 1 tonne of water is needed to make 1 kg of biomass (Guieysse et al., 2013). Because microalgae are aquatic species, it is necessary to use a significant amount of water to survive and proliferate. Water is required for the microalgal photosynthesis process. Life-cycle assessments of algae cultivation indicate that the economics of algal mass production would be improved significantly if the water footprint is reduced (Yang et al., 2011). Using recycled cultivation water after harvesting could not only reduce the demands of fresh water and nutrients but also minimize the energy consumption (Farooq et al., 2015). However, regardless of the type of raceway used for cultivation - open raceway ponds or photobioreactors - biological contaminants, such as bacteria, zooplankton, virus, and other algae, will inevitably be

brought into the culture by water or gas (Wang et al., 2013). The harvested water could contain different types of biological contaminants depending on the algae species, growth conditions and growth phase when harvested (Loftus and Johnson, 2017). Without a pretreatment to eliminate the biological contamination in recycled cultivation water, the competition for nutrients could inhibit the algal growth and reduce the lipid productivity (Zhang et al., 2012). Moreover, some contaminants cause algal cells to lyse by attaching to their surface or releasing alga-lytic substances (Shi et al., 2006; Kim et al., 2007; Chen et al., 2014).

1.4.1 Filtration and centrifugation

Because of the relatively small diameter of microalgal cells, membrane filtration has been deployed to effective remove biological organisms with large volumes of water. However, this approach results removal of the larger algal cells; but only partial bacterial removal. It is also time-consuming for heavy contamination and costly at commercial scale (Rathore and Shirke, 2011).

Some researchers focused on using high-speed centrifugation to control biological contamination. It has been reported that a significantly lower bacterial load was achieved after centrifugation (4200 rpm) (Erkelens et al., 2014). However, the energy-intensive nature of centrifugation makes it impractical to be implemented in large scale culture systems (Rodolfi et al., 2003; Erkelens et al., 2014).

1.4.2 Changes of the environmental conditions

Adjusting some environmental conditions to an optimal range, such as high salinity and high pH, could help to eliminate biological contamination. For example, conditions can be optimized to maintain algae growth but limit growth of biological contaminants. Post et al. increased the salinity of *Dunaliella salina* culture above 20% (w/v) NaCl to prevent amoeba and

ciliates (Post et al., 1983). Reducing the pH of algae culture to 3.0 also has been reported to effectively remove flagellates (Wang et al., 2013). The problem with this approach is that it has to be optimized for every algal species, requires a complex process control system, and involves the addition of chemicals (acids, bases, salt) that will accumulate in a recycle system.

1.4.3 Chemical additives

There are some reports suggesting to use chemicals to kill biological contaminants. Pesticides, such as Trichlorphone, Decamethrin, Tralocythrin, and Buprofezin, have been used to kill zooplankton in the algae culture (Wang et al., 2013). Chemical compounds, such as quinine sulphate and ammonia bicarbonate were found sufficient to eliminate protozoa predators and have less damage to algae cells (Moreno-Garrido and Canavate, 2001; Mendez and Uribe, 2012). In addition, disinfectants, such as ozone, chlorine and hydrogen peroxide, have been investigated to deactivate bacterial cells in algal cultures (González-López et al., 2013). Ozone, one of the most potent oxidizing agents available in the industry, is very effective against bacteria, viruses, fungi, and algae by breaking through the cell membrane and oxidizing all essential components. Chlorine, the most dominant disinfectant for water disinfection, can also be used for removal of most microorganisms and is relatively inexpensive. Meanwhile, hydrogen peroxide is also active against different types of microorganisms, including bacteria, fungi, viruses, spores, and yeast. González-López et al. found that ozonation was the most effective method to remove bacteria in recycled water recovered from marine microalgae *Nannochloropsis gaditana* culture (González-López et al., 2013).

1.5. Dissertation objectives and format

This dissertation has a general objective to reduce the production costs of microalgal biomass products in two areas: 1) optimizing environmental growth conditions, such as pH and salinity; and 2) developing a bacteria removal strategy for recycled water. Microalgae strain *Chlorella sorokiniana* DOE1412 was used in this dissertation due to its robust growth and high productivity.

The focus of the first study presented in this dissertation (Appendix A) is to investigate the pH effects on cell growth, lipid production, and CO_2 addition when culturing *Chlorella sorokiniana* DOE1412 in closed reactors. Both Erlenmeyer flasks and flat panel photobioreactor (PBR) cultivation systems were used. The pH was controlled by injection of CO_2 into the culture, instead of adding buffers. In flat panel PBR cultivation, the overall CO_2 addition to PBR was monitored by a datalogger. The optimal pH was approached by taking into account not only the cell growth and lipid production but also CO_2 addition. The fatty acid profiles and biodiesel properties, such as iodine value (IV), saponification value (SV), cetane number (CN), degree of unsaturation (DU), long-chain saturated factor (LCSF), and cold filter plugging point (CFPP), were determined as a function of pH. Protein content in microalgal biomass and C/N ratio in cells were also investigated.

The focus of the second study presented in this dissertation (Appendix B) is to investigate different bacterial deactivation strategies on the recycled water of microalgae. Two types of recycled water samples were used in this study: authentic recycled water samples and artificial recycled water samples. The authentic samples were obtained from experimental 8000 L outdoor Algae Raceway Integrated Design (ARID) raceway cultivation system. The artificial recycled water samples were made by mixing the *Chlorella sorokiniana* DOE1412 cultures and *E. coli* DH5α cultures. Ozonation, chlorination, chloramination and the addition of hydrogen peroxide

were tested on artificial recycled water samples. The effectiveness of bacterial deactivation methods was evaluated by disinfectant decay, bacterial deactivation and algal survival curves. Some parameters such as initial disinfectant dose, disinfectant residual, bacteria removal rate, minimum contact time, microalgae survival rate, and concentration-time (CT) value were obtained to compare different deactivation methods. The most effective method was also tested on the authentic recycled water samples.

Chapter 2 presents the culture conditions and some detailed methods used in this dissertation; It also presents some additional results not included in peer-review articles (Appendix A and B). Chapter 3 gives a summary of the conclusions of this dissertation and some recommendations for future work in this area.

CHAPTER 2: Detailed methods and additional results

2.1 Culture conditions

Freshwater microalgae strain *Chlorella sorokiniana* DOE1412 and *Escherichia coli* strain DH5α were selected in this study. Three different growth medium, BG-11, Pecos (PE-001A) and Luria broth (LB), were used for cultivation. The medium recipes are shown in Table 1-4.

Table 1 BG-11 medium recipe.

Component	Stock Solution	Amount	Final Concentration
$NaNO_3$	30 g/200 mL dH_2O	10 mL/L	1.5 g/L
K_2HPO_4	0.8 g/200 mL dH_2O	10 mL/L	40 mg/L
$MgSO_4 \cdot 7H_2O$	1.5 g/200 mL dH_2O	10 mL/L	75 mg/L
$CaCl_2 \cdot 2H_2O$	0.72 g/200 mL dH_2O	10 mL/L	36 mg/L
Citric Acid$\cdot H_2O$	0.12 g/200 mL dH_2O	10 mL/L	6 mg/L
Ferric Ammonium Citrate	0.12 g/200 mL dH_2O	10 mL/L	6 mg/L
$Na_2EDTA \cdot 2H_2O$	0.02 g/200 mL dH_2O	10 mL/L	1 mg/L
Na_2CO_3	0.4 g/200 mL dH_2O	10 mL/L	20 mg/L
BG-11 Trace Metal Solution	-	1 mL/L	-

Table 2 PE-001A medium recipe.

Component	Stock Solution	Amount	Final Concentration
$(NH_2)_2CO$	4 g/200 mL dH_2O	5 mL/L	0.1 g/L
$MgSO_4 \cdot 7H_2O$	2.4 g/200 mL dH_2O	1 mL/L	12 mg/L
$NH_4H_2PO_4$	5 g/200 mL dH_2O	1 mL/L	25 mg/L
Potash	7.5 g/200 mL dH_2O	2 mL/L	75 mg/L

$FeCl_3$	0.63 g/200 mL dH_2O	1 mL/L	3.15 mg/L
Na_2CO_3	4 g/200 mL dH_2O	1 mL/L	20 mg/L
EDTA	0.218 g/200 mL dH_2O	4 mL/L	4.36 mg/L
BG-11 Trace Metal Solution	-	1 mL/L	-

Table 3 BG-11 trace metal solution recipe.

Component	Final Concentration
H_3BO_3	2.86 g/L
$MnCl_2 \cdot 4H_2O$	1.81 g/L
$ZnSO_4 \cdot 7H_2O$	0.22 g/L
$Na_2MoO_4 \cdot 2H_2O$	0.39 g/L
$CuSO_4 \cdot 5H_2O$	79 mg/L
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg/L

Table 4 LB medium recipe.

Component	Final Concentration
Yeast Extract	5 g/L
Tryptone	10 g/L
NaCl	10 g/L

For bench top cultivation, strains were cultured in the 1 L Erlenmeyer flasks at room temperature (25 °C). The culture volume was 500 mL. The artificial light source consisted of four 61 cm long 54 watt fluorescent light tubes illuminating the top of the flasks, with a light intensity of 200 μmol photons m^{-2} s^{-1} on the flask surface. The light cycle was 12 h-on/12 h-off. A CO_2/air mixture (5/95, v/v) was continually injected into each flask at 1 LPM. The injection of CO_2 was adjusted by a pH controller when a constant pH was required. All the gasses were saturated with

water before entering each flask to decrease water evaporation. The experiment setup is shown in Fig. 1.

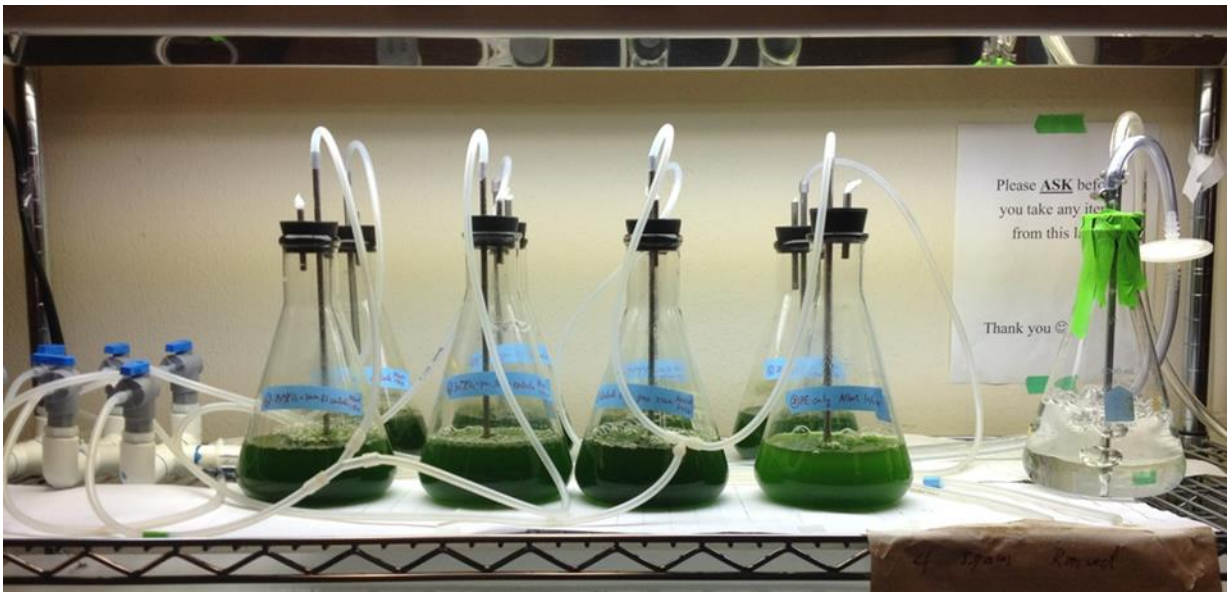

Fig. 1. Experimental setup for flask cultivation.

For flat panel photobioreactor cultivation, two acrylic panel bioreactors with dimensions of 122 (H) × 91 (L) × 10 cm (W) were used. The working volume was 90 L. The air was continually injected into the PBR via three air bubbling stones. Air flow was kept constant at 6 LPM to ensure the culture was well mixed. CO_2 was injected via a micro bubble diffuser with a maximum rate of 0.3 LPM. The flow rate of CO_2 was controlled by a data logger to maintain the desired culture pH. The PBR was illuminated by eight 61 cm long 54 watt fluorescent light tubes on the side of bioreactors with 250 μmol photons m^{-2} s^{-1} light intensity. The experiment setup is shown in Fig. 2.

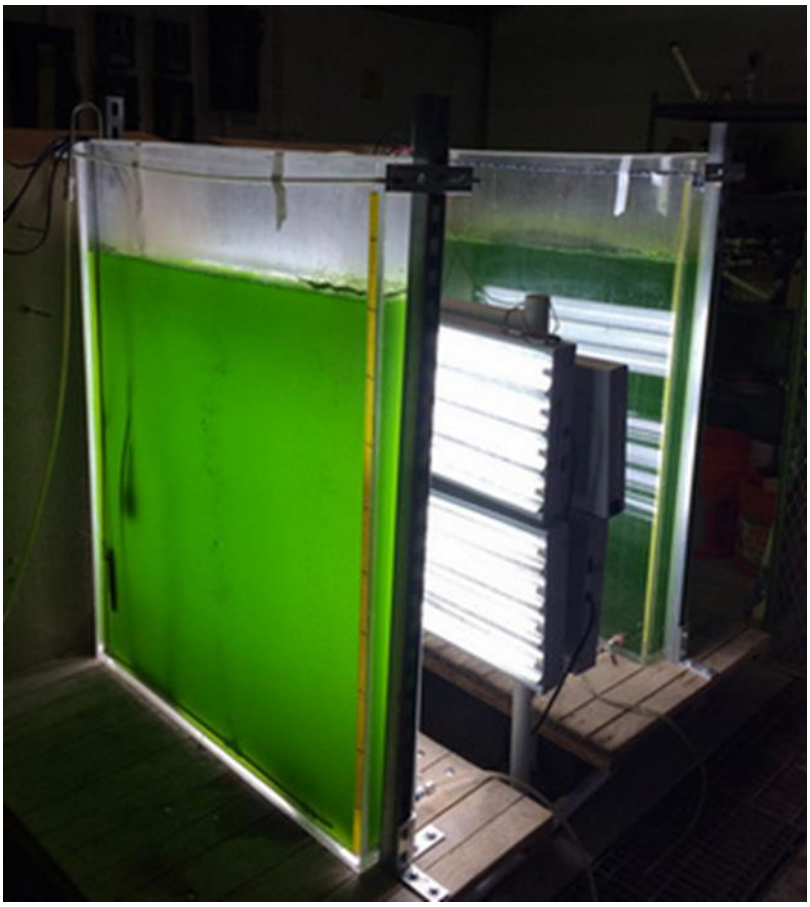

Fig. 2. Experimental setup for flat panel PBR cultivation.

For microalgal growth on plates, BG-11 agar plates supplemented with 1.5% (w/v) agar were prepared by the method reported by Allen (Allen, 1968). As for *E. coli* cultures, LB agar plates were used, which also contained 1.5% (w/v) agar. Plates can be used for cell counting up to a month after preparation. A 0.2 mL aliquot from diluted cultures was spread on the surface of agar plates. Colonies were counted when the number of colonies is about 100 or below 100 per plate. LB agar plates were incubated for 24 h at 37 °C before counting. BG-11 agar plates were placed at ambient temperature for 120 h with illumination (200 μmol photons m^{-2} s^{-1}, 12 h-on/12

h-off) on the plate surface. Examples of agar plates with algal and *E. coli* colonies are shown in Fig. 3.

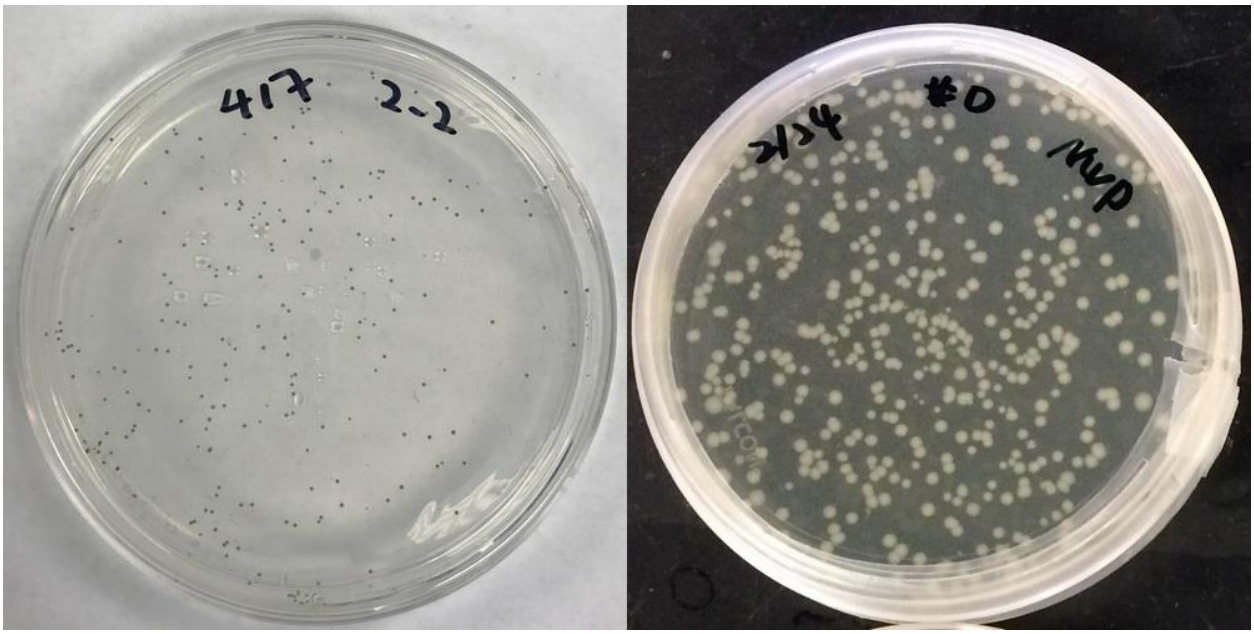

Fig. 3. Agar plates with algal (left) and *E. coli* (right) colonies.

2.2 Determination of disinfectants concentration

2.2.1 Ozone

The concentration of dissolved ozone was measured using the indigotrisulfonate (ITS) ozone residual test (Rakness et al., 2010). Indigo solution was required to be prepared on the day of sampling, which can be made directly by adding 77 mg potassium indigotrisulfonate, 10 g sodium dihydrogen phosphate, 7 mL concentrated phosphoric acid and diluting with deionized water to 1 L. Freshly made indigo solution should be stored in cold-dark conditions until use and between uses. The dark blue indigo solution will quickly be decolorized when ozone is present. A spectrophotometer was used to determine the extent of decolorization by measuring the absorbance

at 600 nm. The extent of decolorization during ozonation was correlated with the concentration of dissolved ozone. The ozone concentration can be calculated by the equation below:

$$C = \frac{\left[(ABS_{Blank} \times TV_{Blank}) \times \left(\frac{IV_{Sample}}{10}\right)\right] - (ABS_{Sample} \times TV_{Sample})}{f \times SV \times b}$$

where:

C: Concentration of dissolved ozone, mg/L

ABS_{Blank}: Measured absorbance at 600 nm of the blank, cm^{-1}

TV_{Blank}: Total volume of blank solution (10 mL Indigo solution + ~10 mL deionized water), mL

IV_{Sample}: Volume of Indigo solution that is added to sample flask, mL

ABS_{Sample}: Measured absorbance at 600 nm of the reacted sample, cm^{-1}

TV_{Sample}: Total volume of the reacted sample, mL

F: Sensitivity coefficient, 0.42 L/mg-cm

SV: Reacted sample volume, mL

b: Path length of sectrophotometer's chamber (1 cm in this case), cm

2.2.2 Chlorine

The concentration of free chlorine was determined by using the DPD (N,N-diethyl-p-phenylenediamine) colorimetric method (Hach Method 8021) (Rice et al., 2017). In this method, DPD was oxidized by free chlorine, showing a pink color with its optical intensity proportional to the free chlorine concentration. The absorbance of the sample was quantified using a digital portable colorimeter (DR/890, Hach Company, Loveland, CO) at 515 nm. The calibration curve

was pre-programmed in the colorimeter. This method was accurate for the concentration of free chlorine 0-2.00 mg/L. 10 mL of sample was required for each measurement.

2.2.3 Monochloramine

The concentration of monochloramine was measured by the MonochlorF reagent (Hach Company, Loveland, CO) based on the indophenol method (Hach Method 10171) (Lee et al., 2007). MonochlorF reagent was a mixture of sodium nitroferricyanide, sodium hydroxide and phenol. In this method, monochloramine can react with a phenol or a substituted phenol to form an intermediate, which coupled with excess substituted phenol to form a green-colored indophenol. The absorbance of the sample was measured using a digital portable colorimeter (DR/890, Hach Company, Loveland, CO) at 655 nm, with was proportional to the concentration of monochloramine in the sample. The calibration curve was pre-programmed in the colorimeter. This method can be used to measure monochloramine up to 4.50 mg/L (as Cl_2). 10 mL of sample was needed for each measurement.

2.2.4 Hydrogen peroxide

The concentration of hydrogen peroxide was determined by using the peroxytitanic colorimetric method (Boltz and Howell, 1978). In this method, hydrogen peroxide can react with titanium (IV) in acid solution to form a yellow-colored peroxytitanic acid. The absorbance of peroxytitanic acid at 407 nm was proportional to the amount of hydrogen peroxide present in the sample. An ultraviolet-visible spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, Inc., Waltham, MA) was used to measure the absorbance. A standard curve was prepared by using a fresh solution of hydrogen peroxide of known concentration to prepare dilutions of five different concentrations, as shown below:

$$C = 62.1 \times ABS - 0.907 \quad (R^2 = 0.999)$$

where:

C: Concentration of hydrogen peroxide, mg/L

ABS: Measured absorbance at 407 nm of the reacted sample, cm^{-1}

2.3 Additional results

Different microalgae species can tolerate different ranges of salinity (Kirst, 1990). To investigate this stain specific characteristic, microalgae DOE1412 were cultivated on Pecos (PE-001A) medium at different salinities. Salinity was adjusted by addition of Na_2SO_4. Fig. 4 shows the growth curves. DOE1412 can be cultivated in medium with a salinity of up to 10 g/L without any noticeable decrease in growth. Algal culture crushed when salinity of the medium reached 30 g/L. When optimizing nutrient additives, the PE-001A medium needed regulation to make sure salinity was not higher n than 10 g/L.

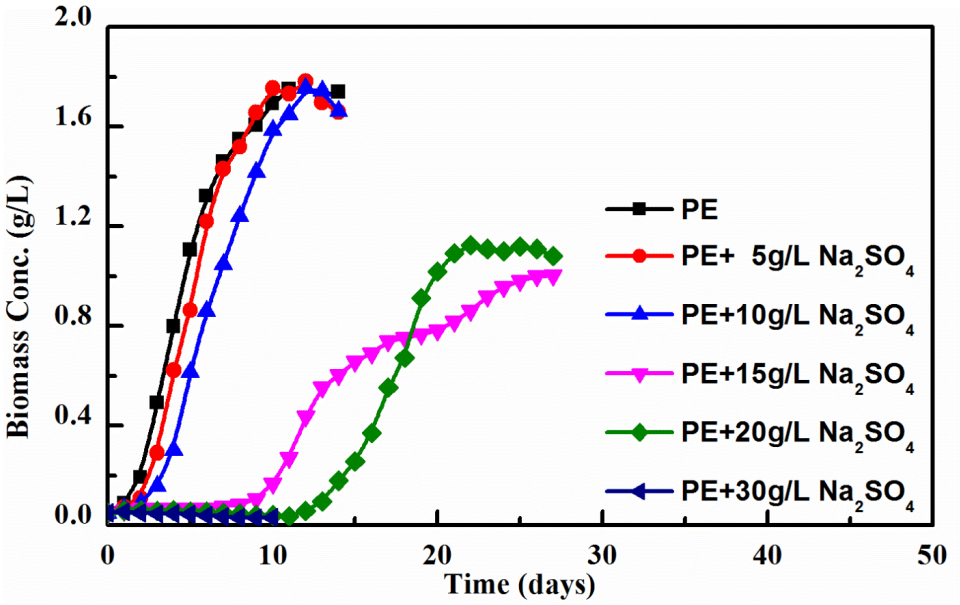

Fig. 4. Growth curves for *Chlorella sorokiniana* DOE1412 grown at different salinities in 500 mL flasks. Data are the average of duplicates.

Fig. 5 shows the lipid content of DOE1412 grown on PE-001A medium at different salinities. Lipid content appeared to be influenced by salinity. It generally decreased with increasing salinity. Microalgae *Chlorella sorokiniana* are freshwater strains, therefore, it makes sense that the highest lipid content was observed at the lowest salinity.

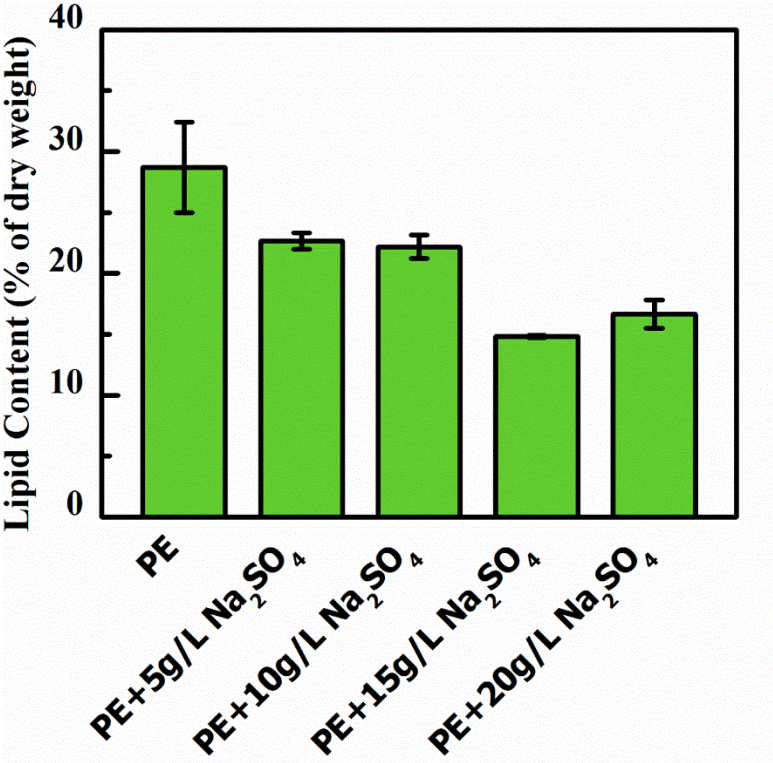

Fig. 5. Lipid content for *Chlorella sorokiniana* DOE1412 grown at different salinities in 500 mL flasks. Data are shown as means ± S.D., n=2.

As shown in Fig. 6, The fatty acid profiles appeared to be unaffected by salinity: At different salinities, *Chlorella* sp. mostly accumulated 16 and 18 carbon fatty acids, which are

suitable for biodiesel production. The primary components were C16:0 and C18:2 regardless of medium salinity.

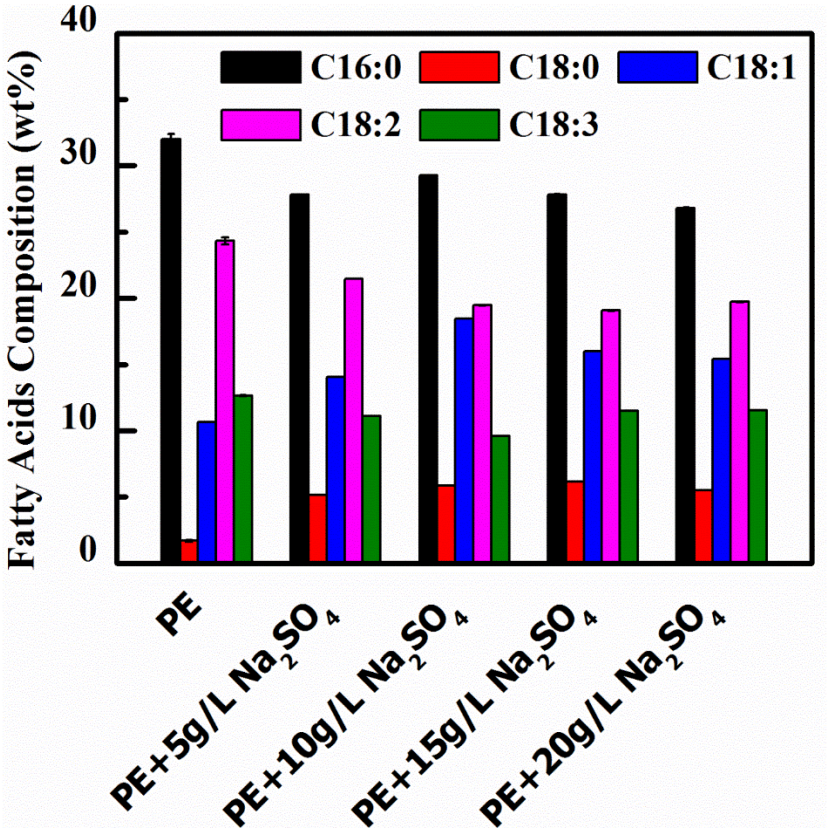

Fig. 6. Fatty acid profiles for *Chlorella sorokiniana* DOE1412 grown at different salinities in 500 mL flasks. Data are shown as means ± S.D., n=2.

CHAPTER 3: Conclusions

This main objective of this dissertation is to reduce the production costs of microalgal biomass in two area: optimizing environmental growth conditions and bacteria removal strategy for recycled water. On the one hand, nutrients can be utilized more efficiently at optimal growth conditions. Culture pH, a critical environmental condition for algae cultivation, was optimized in flask cultivation for fast cell growth and high lipid productivity. Moreover, the costing of CO_2 is a significant economic consideration. Culture pH was investigated for efficient CO_2 utilizing in scale-up cultivation. On the other hand, water usage is significant for algae cultivation. Water recycling can be implemented to reduce the costs of water supply. Pretreatment methods, necessary for eliminating bacterial contamination in recycled water, were investigated for deactivating bacteria not only efficiently but also cost-effectively.

Based upon the work done in this dissertation, the following recommendations are provided for future work:

- Investigate the balance between biomass growth, lipid production and CO_2 addition at a larger scale or in an outdoor cultivation system.
- Develop a model of microalgal cell growth and CO_2 addition in flat panel PBRs in order to predict productivity potential and cost on different environmental conditions (e.g., pH, temperature, and nutrients).
- Use flue gas as carbon source to cultivate microalgae
- Develop a deactivation method for removing *Vampirovibrio chlorellavorus*, the predatory bacteria of *Chlorella* sp, from recycled water.

- Develop optimized deactivation method for scaled-up system
- Implement deactivation technique in using impaired water sources to grow algae

The complete methods, results, discussion and conclusions of this study are presented in the papers appended to this dissertation. For general information purposes, a brief description of the most important findings along with the author's contribution is provided below: *Appendix A: Effects of pH on cell growth, lipid production and CO2 addition of microalgae Chlorella sorokiniana*

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In this work, cell growth and lipid content of *Chlorella sorokiniana* DOE1412 were first evaluated at different pH in flask cultivation. Culture pH was manipulated by CO_2 addition. The optimal pH for DOE1412 is approximately 6.0 when only accounting for cell growth and lipid production and not considering the CO_2 efficiency. A flat panel airlift photobioreactor (PBR) was used for scale-up cultivation at five different pH levels (6.5, 7, 7.5, 8 and 8.5). Data of pH values and CO_2 addition was collected by a data logger. Biomass productivity increased with decreasing pH. By taking into account not only the cell growth and lipid production but also CO_2 addition, the lowest value of CO_2 addition was achieved at pH 8 (2.01 g CO_2/g biomass). The amount of lipid was similar regardless of pH. Biodiesel produced at low pH would meet the diesel standards. Protein content in microalgal biomass increased with increasing pH, while C/N ratio in cells decreased. This study shows how much CO_2 is required to cultivate algae in 90 L flat panel PBRs and provides further evidence that there is a balance between biomass growth, lipid production, and CO_2 addition.

The author of this dissertation designed and built experimental setup, performed the cultivation experiments, analyzed the biomass product, and wrote the manuscript.

Appendix B: Specific deactivation of bacteria in recycled water of microalgae Chlorella sorokiniana cultures

(To be submitted to Chemosphere)

In this work, ozonation, chlorination, chloramination and the addition of hydrogen peroxide were investigated on artificial recycled water samples containing mixtures of microalgae *Chlorella sorokiniana* DOE1412 and *Escherichia coli* strain DH5α. Chlorination with a dosage of 0.5 mg/L is the most economic and successful sterilization method: 5-log reduction of *E. coli* cells was achieved after 1 min of contact time, while the microalgae survival rate was 58%. No chlorine was detected after 5 min of exposure. The other methods investigated showed higher CT value for 5-log deactivation of *E. coli* in artificial recycled water samples, or resulted in the destruction of both the algae and the bacteria. The effectiveness of chlorination was also confirmed when tested on the authentic recycled water samples.

The author of this dissertation prepared the recycled water samples, designed and performed the deactivation experiments, analyzed the decay kinetics, prepared the cell count plates, conducted the cell counting, and wrote the manuscript.

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APPENDIX A: Effects of pH on cell growth, lipid production and CO_2 addition of microalgae *Chlorella sorokiniana*

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Abstract

Microalgae have emerged as one of the most promising alternative energy feedstocks. Some advantages include the simple cellular structure, short production cycle, high lipid content, and fast growth. However, high production costs due to high CO_2 usage and low lipid productivity have been some of the major challenges impeding the commercial production of algal biodiesel. Here, cell growth and lipid content of *Chlorella sorokiniana* DOE1412 were first evaluated at different pH in flask cultivation. Culture pH was manipulated by CO_2 addition. The optimal pH for DOE1412 is approximately 6.0 when only accounting for cell growth and lipid production and not considering the CO_2 efficiency. A flat panel airlift photobioreactor (PBR) was used for scale-up cultivation at five different pH levels (6.5, 7, 7.5, 8 and 8.5). Data of pH values and CO_2 addition was collected by a data logger. Biomass productivity increased with decreasing pH. By taking into account not only the cell growth and lipid production but also CO_2 addition, the lowest value of CO_2 addition was achieved at pH 8 (2.01 g CO_2/g biomass). The fatty acid profiles and biodiesel properties, such as iodine value (IV), saponification value (SV), cetane number (CN), degree of unsaturation (DU), long-chain saturated factor (LCSF), and cold filter plugging point (CFPP), were determined as a function of pH. CN of biodiesel produced at pH 6.5, 7 and 7.5 satisfied the US standard ASTM D6751; among them, the pH 6.5 products met the European standard EN 14214. Finally, protein content in microalgal biomass increased with increasing pH, while C/N ratio in cells decreased.

Keywords: Microalgae, Biodiesel, *Chlorella sorokiniana*, pH, Lipid, Carbon dioxide.

Highlights

- A culture pH of approximately 6.0 is optimal for *Chlorella sorokiniana* DOE1412 biomass productivity in flask cultivation.
- Culturing algae at pH 8 is most cost-effective with a ratio of 2.01 g CO_2 per g biomass observed during cultivation in 90 L photobioreactors.
- Biodiesel produced at lower pH satisfies higher diesel standards.
- Biomass made at higher pH has higher protein content and lower carbon to nitrogen ratio.

A.1. Introduction

Microalgae can fix CO_2 efficiently from industrial exhaust gasses during photosynthesis, providing a very promising alternative for the mitigation of CO_2, the most prominent greenhouse gas (Hamasaki et al., 1994; Brown, 1996; Benemann, 1997; Doucha et al., 2005; Wang et al., 2008; Borkenstein et al., 2011). Microalgae have the ability to utilize nutrients (especially nitrogen and phosphorus) from polluted municipal, industrial and agricultural wastewater, which allows for use of non-potable water for growth and provides a promising option for the bioremediation of wastewater (Cantrell et al., 2008; Mulbry et al., 2008; de Godos et al., 2010; Rawat et al., 2011). However, the production cost of microalgal biodiesel is currently not competitive with petroleum-based products. Many significant technical challenges need to be tackled before this pathway is ready for commercial-scale implementation (Greenwell et al., 2010). Some example research and development areas include: studying the molecular biology of microalgae; selecting and bioengineering microalgae strains optimized to regional climate conditions; developing large-scale microalgae cultivation systems; optimizing cultivation conditions (e.g., nutrient, temperature, light, pH, salinity, and mixing); developing efficient techniques for lipid extraction, biomass harvesting,

and downstream processing; reducing and controlling operating costs and energy consumption; and utilizing residues to make valuable co-products (Singh et al., 2011; Chen et al., 2015). This work specifically focuses on optimizing the pH and the amount of CO_2 required for efficient algal growth.

Chlorella sorokiniana DOE1412, a member of the Trebouxiophyceae class, also known as UTEX B 3016, was the microalgae strain used in this study. It has been reported as an important microalga with potential for biodiesel production, accumulating high amounts of lipids and proteins (Griffiths and Harrison, 2009; Neofotis et al., 2016). Moreover, it can grow robustly at temperatures of up to 40 °C and in impaired water (Doucha et al., 2005; Ugwu et al., 2007).

Microalgae cultivation can be optimized by regulating a variety of environmental parameters, such as light, temperature, nutrients, salinity, temperature, and pH (Hu et al., 2008; Singh and Dhar, 2011). These parameters not only affect the accumulation of biomass profoundly but also influence the biochemical composition of microalgal biomass. The pH is one of the most critical environmental conditions in microalgal cultivation since it determines the solubility and availability of CO_2 and nutrients, and has a significant influence on microalgal metabolism (Chen and Durbin, 1994). Each microalgal species has an optimal pH range for biomass and lipid production, which is narrow and strain specific (Goldman et al., 1982; Khalil et al., 2010; Moheimani and Borowitzka, 2011; Moheimani, 2013). Specifically, pH is critical in determining the relative concentrations of the carbonaceous species in water (Azov, 1982). The pH of microalgal cultures rises gradually during the day due to the uptake of inorganic carbon by microalgae. Higher pH limits the availability of CO_2, thus, inhibiting cell growth (Azov, 1982; Chen and Durbin, 1994). On the other hand, algal cultivation at high pH can suppress undesired biological contaminants (Bartley et al., 2014).

Methods for controlling pH include CO_2 injection, buffer addition, and acid/base adjustment. The former two are more commonly used in algae cultivation (Spolaore et al., 2006; Moheimani, 2013; Bartley et al., 2014). However, the presence of buffers can be a potential pitfall for research because, to date, effects of buffers on microalgal metabolism are not entirely clear. Also, it is not cost-effective or realistic at large scale. The present study focuses on regulating pH by controlling and measuring the amount of CO_2 injected during cultivation. CO_2 addition is a significant economic consideration for biodiesel production. However, carbon balances are not typically performed on algal bioreactors and many economic studies do not provide detailed analyses of the costs associated with producing and transporting CO_2 (Davis et al., 2011). A common simplifying assumption is based on co-locating an algal production system with a facility that produces waste CO_2, without considering the potential negative effects associated with toxic substances in flue gas on algal growth, the availability of land near the flue gas, or the possibility for scalability. More recently, the need to understand the costing for CO_2 was highlighted by some studies (Quinn et al., 2013; Venteris et al., 2014). CO_2 and land were identified as two barriers for the large-scale potential of microalgae (Quinn et al., 2013).

In this study, both Erlenmeyer flasks and flat panel photobioreactor (PBR) cultivation systems were used. Moreover, real-time data of temperature, dissolved oxygen, pH and CO_2 flow rate were monitored during PBR cultivation. An optimal culturing pH range was determined by taking into account not only the cell growth and lipid production but also CO_2 addition. The implications of this research demonstrate the tradeoffs between growth and CO_2 addition that can be implemented in the field to enhance the economical and sustainable viability of algal cultivation.

A.2. Materials and methods

A.2.1 Strains and growth medium

Chlorella sorokiniana DOE1412 (Unkefer et al., 2017) was obtained from the National Alliance for Advanced Biofuels and Bioproducts (NAABB) cultivation team. Currently it is available from UTEX. The seed culture (500 mL in a 1 L Erlenmeyer flask) was continuously shaken on a platform at 120 rpm under a 12:12 h light/dark period at ambient temperature. Culture medium was replaced every 14 days. Algae cells were resuspended in fresh medium after old medium was removed by centrifugation.

The standard growth medium used for freshwater *Chlorella* sp. is BG-11 (Rippka et al., 1979). For this work, an optimized medium PE-001A was used. *Chlorella sorokiniana* DOE1412 grew as well on the PE-001A medium as on the BG-11 medium; however, the PE-001A medium recipe is ten times less expensive (Lammers et al., 2017). The PE-001A medium consists of the following: 100 mg/L $(NH_2)_2CO$, 12 mg/L $MgSO_4 \cdot 7H_2O$, 25 mg/L $NH_4H_2PO_4$, 75 mg/L Potash, 3.15 mg/L $FeCl_3$, 20 mg/L Na_2CO_3, 4.36 mg/L EDTA and 1 mL/L trace metal solution. The trace elements in the solution consist of the following: 2.86 g/L H_3BO_3, 1.81 g/L $MnCl_2 \cdot 4H_2O$, 0.22 g/L $ZnSO_4 \cdot 7H_2O$, 0.39 g/L $Na_2MoO_4 \cdot 2H_2O$, 79 mg/L $CuSO_4 \cdot 5H_2O$ and 49.4 mg/L $Co(NO_3)_2 \cdot 6H_2O$. Based on pH titration of PE-001A medium, a pKa = 6.2 is observed between pH 6 and pH 9.

A.2.2 Flask cultivation

Chlorella sorokiniana DOE1412 was cultured in a 1 L Erlenmeyer flask using sterile PE-001A medium at room temperature (25 °C). The culture volume was 500 mL. The artificial light source consisted of four 61 cm long 54 watt fluorescent light tubes (FLT5464, Hydrofarm, Inc., Petaluma, CA) illuminating the top of the flasks, with a light intensity of 200 μmol photons m^{-2} s^{-}

[1] on the flask surface, measured by a quantum meter (MQ-200, Apogee Instruments, Logan, UT). The light cycle was 12 h-on/12 h-off. The initial biomass concentration was approximately 0.06 g/L. Cultures were grown at four different pH levels (6, 7, 8 and 9) using a pH controller to adjust injection of CO_2. Air at a flow rate of 1 L/min (LPM) was added to improve culture mixing. For the control group, a CO_2/air mixture (5/95, v/v) was continually injected into each flask at 1 LPM. All the gasses were saturated with water before entering each flask to decrease water evaporation.

A.2.3 PBR cultivation

Airlift flat-panel PBRs were used for scale-up cultivation. They were built using 6.35 mm thick transparent acrylic panels with dimensions of 122 (H) × 91 (L) × 10 cm (W). The working volume was 90 L. The air was continually injected into the PBR via three air bubbling stones (Discard-A-Stones 12526, Lee's Aquarium & Pet Products, San Marcos, CA) placed at the bottom of the PBR to achieve proper mixing of the microalgae culture. An air compressor (3Z355H, W. W. Grainger, Inc., Lake Forest, IL) was used to maintain the proper injection pressure. Air flow was kept constant at 6 LPM to ensure the culture was well mixed. CO_2 was injected discontinuously (0.3 LPM when it was on) via a micro bubble diffuser (1PMBD075, Pentair Aquatic Eco-Systems, Inc., Apopka, FL), controlled by a data logger (CR1000, Campbell Scientific, Inc., UT, Logan, UT) to maintain the desired culture pH. The pH (HI1006-2005, Hanna Instruments, Inc., Woonsocket, RI), dissolved oxygen (DO6400/T, Sensorex, Garden Grove, CA) and temperature (109SS-L, Campbell Scientific Inc., Logan, UT) probes were placed in the PBR and scanned every second. The data logger was also connected to a digital mass flow meter (TopTrak 820, Sierra Instruments, Monterey, CA), to monitor CO_2 addition. The 10-minute averaged data were stored. The PBR was illuminated by eight 61 cm long 54 watt fluorescent light

tubes (FLT5464, Hydrofarm, Inc., Petaluma, CA) providing 250 μmol photons m^{-2} s^{-1} on the surface.

Chlorella sorokiniana DOE1412 was grown in 4 L Erlenmeyer flasks provided with a 8 LPM flow rate of a CO_2 /air mixture (5/95, v/v) each, at room temperature (25 °C), for seven days before being transferred to the PBRs. The initial biomass concentration was approximately 0.3 g/L. Cultures were grown at five different pH levels (6.5, 7, 7.5, 8 and 8.5), in duplicates, in the PBRs.

A.2.4 Biomass concentration

The biomass concentration, in dry weight, was determined by measuring the optical density of samples at wavelength 750 nm (OD_{750}) using an ultraviolet-visible spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, Inc., Waltham, MA). Daily samples were taken from the reactors during the daylight hours. They were diluted by appropriate ratios to ensure that the measured OD_{750} values were in the linear range of 0.1-0.7. The relationship between OD_{750} and biomass concentration was determined experimentally. Biomass was centrifuged, washed, and dried. A linear regression relationship was obtained:

$$\text{Biomass concentration} = \text{conversion factor} \times OD_{750} \times \text{dilution factor} \quad (1)$$

The conversion factor was validated periodically for all the treatments.

A.2.5 Lipid content

Algae samples were harvested by a centrifuge (J2-21, Beckman Coulter, Inc., Brea, CA) at relative centrifugal force (RCF) of 4400×g for 10 min. The pellets were dried at 37 °C in a forced

air oven (414005-114, VWR International LLC, Radnor, PA) to determine the dry biomass weight, then ground to a fine powder using a mortar and pestle.

The lipids were extracted using microwave-assisted extraction (Eskilsson and Bjorklund, 2000). The powdered algae were transferred to MARSXpress vessels (0.3-0.5 g per vessel) of the CEM Microwave Accelerated Reaction System (MARS, CEM Corp., Matthews, NC). Ten mL of pre-mixed chloroform-methanol (2:1 by volume) were added to each vessel and held at room temperature for at least 2 h. The suspensions were then heated via microwave to 70 °C and held at 70 °C for 60 min (Patil et al., 2013). Vessel contents were cooled to room temperature. The samples were then filtered with glass fiber and dried with a nitrogen evaporator (N-Evap 112, Organomation Association Inc., Berlin, MA). The lipid content was determined by weight (lipid/biomass, x %).

A.2.6 Fatty acid profiles

A transesterification reaction is necessary to convert the lipid obtained from microwave-assisted extraction into fatty acid methyl esters (FAMEs). Transesterification was done by reacting the extracted lipid with 3M methanolic HCl (Galella et al., 1993). The lipids and solvent were heated for 10 min at 57 °C to convert them to FAMEs (Gong and Jiang, 2011).

The FAMEs were solubilized in dichloromethane (dilution factor of 5) and analyzed with an Agilent 7890A gas chromatograph (GC) coupled to an Agilent 5975C inert XL mass selective detector (MSD) with Triple-Axis Detector (Agilent Technologies, Santa Clara, CA). A 1 μL sample was injected in pulsed splitless mode by an Agilent 7693 autosampler (Agilent Technologies, Santa Clara, CA). Separation of compounds was accomplished on a fused silica capillary column (Omegawax 250, Supelco, Bellefonte, L × I.D. 30 m × 0.25 mm, d_f 0.25 μm).

The oven temperature program was 2 min at 90 °C, increasing at 3 °C min^{-1} to 240 °C, where the temperature was maintained for 15 minutes. Helium was used as carrier gas at a constant flow rate of 1.0 mL/min. The solvent delay was 5 min. The mass spectrometry (MS) source temperature was set to 230 °C; the quadrupole temperature was set to 150 °C, and the transfer line temperature was 180 °C. Spectra were acquired in positive (70 eV) full scan mode from 50 to 600 m/z at 1.4 spectra/s scan speed.

A.2.7 Biodiesel properties

The biodiesel properties such as iodine value (IV), saponification value (SV), cetane number (CN), degree of unsaturation (DU), long-chain saturated factor (LCSF), and cold filter plugging point (CFPP) were determined by empirical equations. CN was calculated using Eq. 2 (Krisnangkura, 1986):

$$\mathrm{CN} = 46.3 + \frac{5458}{\mathrm{SV}} - 0.225 \times \mathrm{IV} \quad (2)$$

where SV in mg KOH/g and IV in g I_2/100g were calculated from fatty acid profiles (Francisco et al., 2010):

$$\mathrm{SV} = \sum_i \frac{560 \times \mathrm{N_i}}{\mathrm{M_i}} \quad (3)$$

$$\mathrm{IV} = \sum_i \frac{254 \times \mathrm{D_i} \times \mathrm{N_i}}{\mathrm{M_i}} \quad (4)$$

N_i is the percentage of each component, M_i is the molecular mass, and D_i is the number of double bonds.

DU was determined by an empirical equation, taking into account the weight percentage of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) (Ramos et al., 2009):

$$DU = MUFA + (2 \times PUFA) \quad (5)$$

LCSF and CFPP were obtained based on Eqs. 6 and 7 (Ramos et al., 2009):

$$LCSF = (0.1 \times C16) + (0.5 \times C18) + C20 + (1.5 \times C22) + (2 \times C24) \quad (6)$$

$$CFPP = (3.1417 \times LCSF) - 16.477 \quad (7)$$

where C16, C18, C20, C22 and C24 are the weight percentage of each saturated fatty acid.

A.2.8 Total carbon (TC) and total nitrogen (TN) content

TC and TN content in microalgae biomass was quantified by the Arizona Laboratory for Emerging Contaminants (ALEC, Tucson, AZ), using an elemental combustion analyzer ECS-4010 (Costech Analytical Technologies, Inc., Valencia, CA). All the biomass samples were dried and ground to a fine powder prior to analysis. The system used helium carrier gas to bring the combustion gasses to GC separation and a thermal conductivity detector (TCD). Sulfanilamide, NIST 1632b coal, and LECO samples including soil, corn gluten, and cysteine were used as calibration standards. Triplicate measurements were made on each sample.

A.2.9 Statistical analysis

One-way analysis of variance (ANOVA) was used for statistical analysis. The analyses were conducted using JMP software (JMP Pro 12.2.0, SAS Institute Inc., Cary, NC). Results were reported at 95% confidence intervals.

A.3. Results and discussion

A.3.1 Effects of pH on cell growth and lipid content in 500 mL flasks

Fig. A1 shows the growth curves of DOE1412 in flask cultivation at different pH. Data are the average of duplicates. The pH of the control group (continuous addition of 5% CO_2 in air) was 5.8 ± 0.1 during the entire growth period. After a one day lag phase, the growth curves were observed to be close to linear for the first four days. The growth and productivity data are shown in Table 1. The linear growth rates for pH 5.8, 6, and 7 were statistically the same (one-way ANOVA: $p > 0.05$), with the highest average rate (0.353 g/L-day) observed at pH 7. The growth rates decreased with increasing pH from 7 to 9. Also, the R^2 value for the linear regression was much closer to 1 at higher pH, due to CO_2 limitation. The effect of pH was investigated using CO_2 addition by Moheimani (Moheimani, 2013) for an unidentified species of *Chlorella*. The highest specific growth rate observed for microalgae grown in a 5 L open glass aquarium PBR, was at pH 7. Since their reactors were operated in a semi-continuous mode by harvesting three times a week, the data obtained was for short growth periods of 3-4 days, which is similar to our linear growth period.

The biomass productivity were calculated for a two-week culture period (Table A1), which is after stationary phase was reached. The maximum biomass productivity observed was at pH 6 (0.140 g/L-day), which was statistically the same as the productivity at pH 5.8 (one-way ANOVA: $p > 0.05$). Also, we found that biomass productivity decreased with increasing pH from 6 to 9. The productivity was significantly lower at pH 9 (0.071 g/L-day), almost half of the value for pH 6. Thus, our observed optimal pH for DOE1412 biomass productivity in batch culture was approximately pH 6.

The pKa of the PE-001A medium is 6.2 based on titration experiments. When microalgae DOE1412 were suspended in the medium, the pKa range of bicarbonate buffering system was stable. The dissolved inorganic carbon is mainly found in three forms in the system, including aqueous carbon dioxide (CO_2(aq)), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}), where CO_2(aq) and HCO_3^- can be utilized by most of the microalgae for photosynthesis. However, some algae species only use HCO_3^- to complement CO_2, while others can efficiently utilize HCO_3^- alone (Silva et al., 2013). The distribution of inorganic carbon species is pH-dependent. At pH 9, the predominant form is HCO_3^-, while both CO_2(aq) and HCO_3^- are equally present at pH 6. As the algae grow, the total dissolved inorganic carbon decreases, and pH slowly increases. After additional CO_2 is added to the system, the pH immediately drops to the desired value, and the equilibrium between the carbon species is reestablished accordingly. Based on the results of this study, DOE1412 appeared to have the ability to use HCO_3^- as a sole carbon source, however the algal growth rate was significantly lower than when both CO_2(aq) and HCO_3^- are present in the media. Lam and Lee (Lam and Lee, 2013) found that *Chlorella vulgaris* preferred to use CO_2(aq) at acidic conditions instead of HCO_3^- at alkaline conditions, which agrees with the findings of this study.

Fig. A2 shows the lipid content of DOE1412 grown in flasks at different pH. Lipid content appeared to be unaffected by pH (one-way ANOVA: $p > 0.05$) with an average value of 29.5% by mass. Moheimani (Moheimani, 2013) found similar results in that the lipid content of *Chlorella* sp. remained constant at 23 ± 2%. Since the species of *Chlorella* is different in the two studies, it is not surprising that the actual percentage lipid is different.

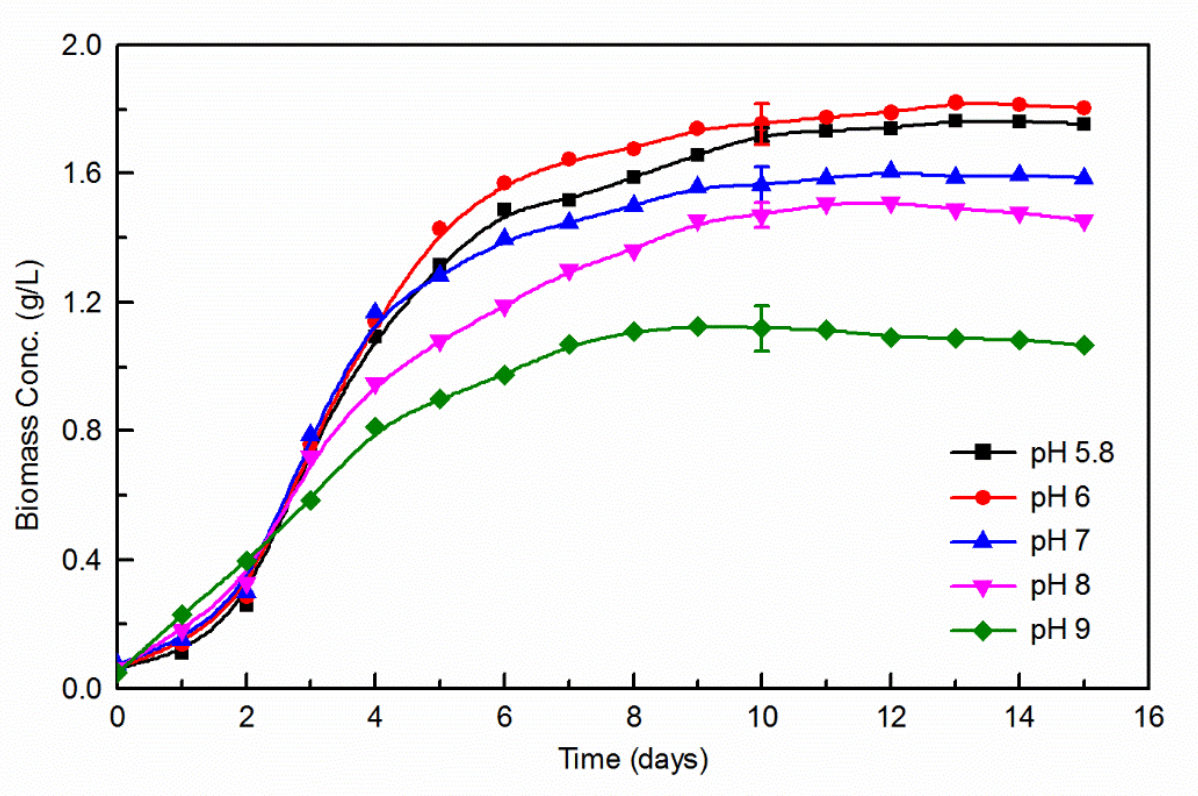

Fig. A1. Growth curves for *Chlorella sorokiniana* DOE1412 grown at different pH in 500 mL flasks. Data are the average of duplicates. ± S.D. lines at t = 10 day are shown.

Table A1 Growth and productivity summary at different pH in flasks.

pH	Linear growth rate (g/L-day)	R^2	Biomass productivity (g/L-day)
5.8	0.345 ± 0.004	0.96	0.138 ± 0.002
6	0.348 ± 0.005	0.96	0.140 ± 0.004
7	0.353 ± 0.004	0.96	0.119 ± 0.005
8	0.269 ± 0.010	0.97	0.109 ± 0.003
9	0.193 ± 0.007	0.99	0.071 ± 0.004

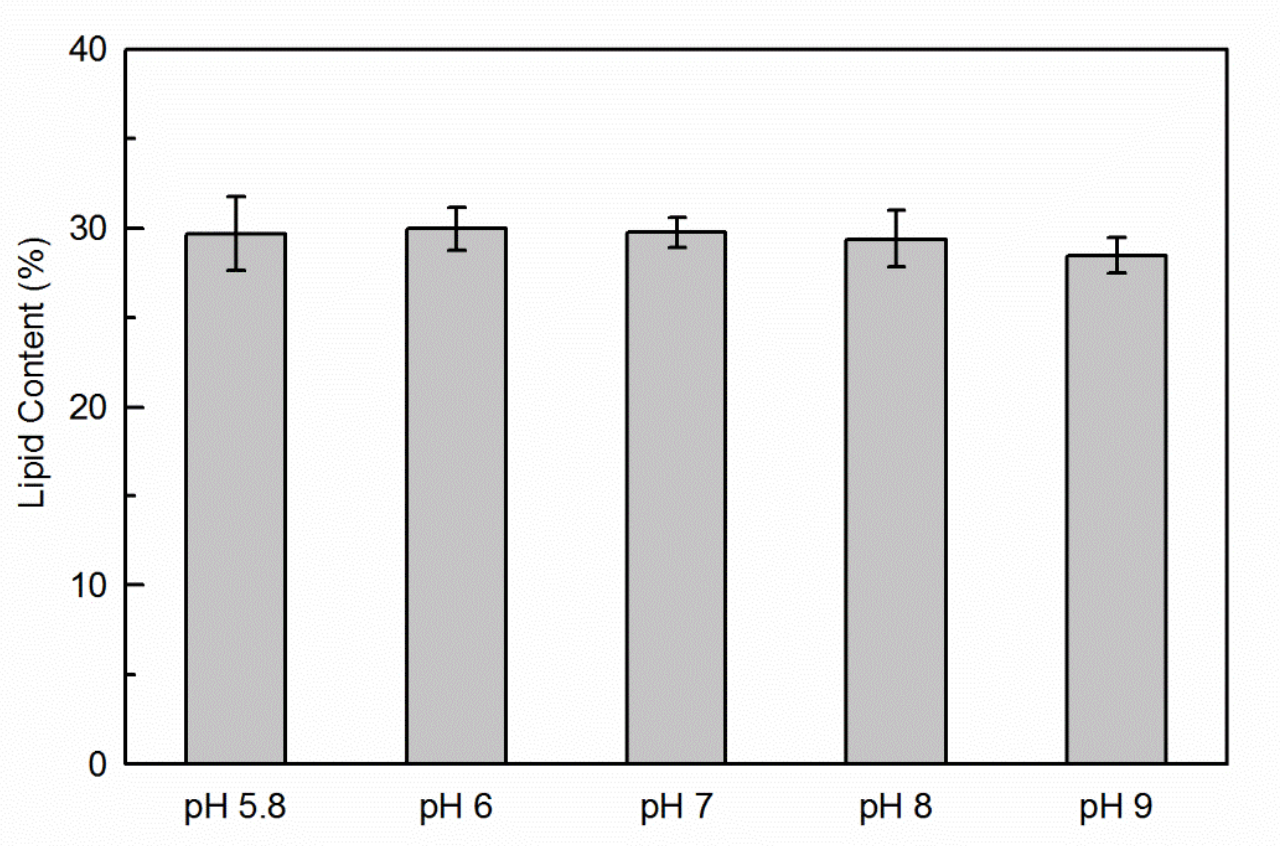

Fig. A2. Lipid content for *Chlorella sorokiniana* DOE1412 grown at different pH in 500 mL flasks. Data are shown as means ± S.D., n=4.

A.3.2 Effects of pH on cell growth, CO_2 addition and lipid content in 90 L airlift PBRs

After determining the optimal pH range in easy to control bench scale flasks, the cultivation system was scaled up to 90 L PBRs. At the larger scale, it is easier to monitor multiple parameters simultaneously including pH, temperature, DO, and CO_2 addition. Our focus was CO_2 addition as a function of pH. The scaled-up PBR reactors were designed specifically to monitor CO_2 addition, using a data logger to control pH. Based on bench scale results, the pH range investigated was from 6.5 to 8.5. Using a pH below 6.5 is not cost-effective due to the extremely high amount of CO_2 addition (44.24 L/day), more than 2.5 times the amount required at pH 7. On the other extreme, pH 9, biomass productivity was significantly lower than what we observed at pH 8.

Fig. A3 shows the culture pH and cumulative CO_2 addition during a 24-hour period, from 1:00 am to 0:59 am when the desired culture pH was 7. No initial pH adjustment of medium was performed. It took less than 1 minute to reach the desired pH. During the daytime (7 am to 7 pm, 6~18 h in Fig.3), pH remained at 6.98 ± 0.01. During the nighttime, it was 6.99 ± 0.07. Both CO_2 injection and respiration during the dark growth phase lowered the pH in the PBR, which caused larger pH fluctuations. With no photosynthesis during the dark period, CO_2 addition was much less than during the daytime as expected. The small amount of addition might be due to CO_2 loss to the atmosphere.

Fig. A4 shows the growth curves of DOE1412 and CO_2 addition during PBR cultivation at different pH values. Data are the average of duplicates. DOE1412 exhibited a longer linear growth phase with lower growth rates in these larger reactors, which were light limited. The linear growth rates calculated using the first ten days of data were 0.091 g/L-day for pH 6.5, 0.074 g/L-day for pH 7, 0.068 g/L-day for pH 7.5, 0.061 g/L-day for pH 8, and 0.023 g/L-day for pH 8.5. Biomass

growth rates decreased with increasing pH from 6.5 to 8.5, with a significant drop in rate at a pH of 8.5 (about a quarter of the rate at pH 6.5).

The biomass productivity and CO_2 addition as a function of pH are shown in Table A2. Biomass productivity was determined by the average biomass accumulation before the culture reached the stationary phase. It generally increased with decreasing pH, where the productivity at pH 6.5 > pH 7 > pH 7.5 > pH 8 > pH 8.5. CO_2 addition (g CO_2/g biomass) was calculated using the density of CO_2 at standard temperature and pressure (1.96 g/L). The cost of CO_2 was estimated using the current cost of existing pipeline CO_2 supplies for low pressure delivery ($15/tonne) (Davis et al., 2016). At low pH, more CO_2 was added to the PBR, providing more carbon to the DOE1412 culture. Although excess CO_2 led to higher biomass productivity, CO_2 was utilized less efficiently. The CO_2 addition (g CO_2/g biomass) decreased with increasing pH from 6.5 to 8, while the value at pH 8.5 was slightly higher the value at pH 8. The CO_2 addition was highest at 13.32 g CO_2/g biomass at pH 6.5, costing $200/tonne biomass, which might not be economically viable. The lowest value was 2.01 g CO_2/g dry biomass at pH 8, costing $30/tonne biomass, which was the most cost-effective culture pH for this algal species, in term of CO_2 addition.

Similar to the flask results, the lipid content was not a function of pH in the PBRs; no significant differences were found (See Fig. 5A. one-way ANOVA: $p > 0.05$). Overall, the lipid content was slightly lower at the larger scale (25.7% by mass), which is often observed. These values were similar to the observations of Moheimani (Moheimani, 2013).

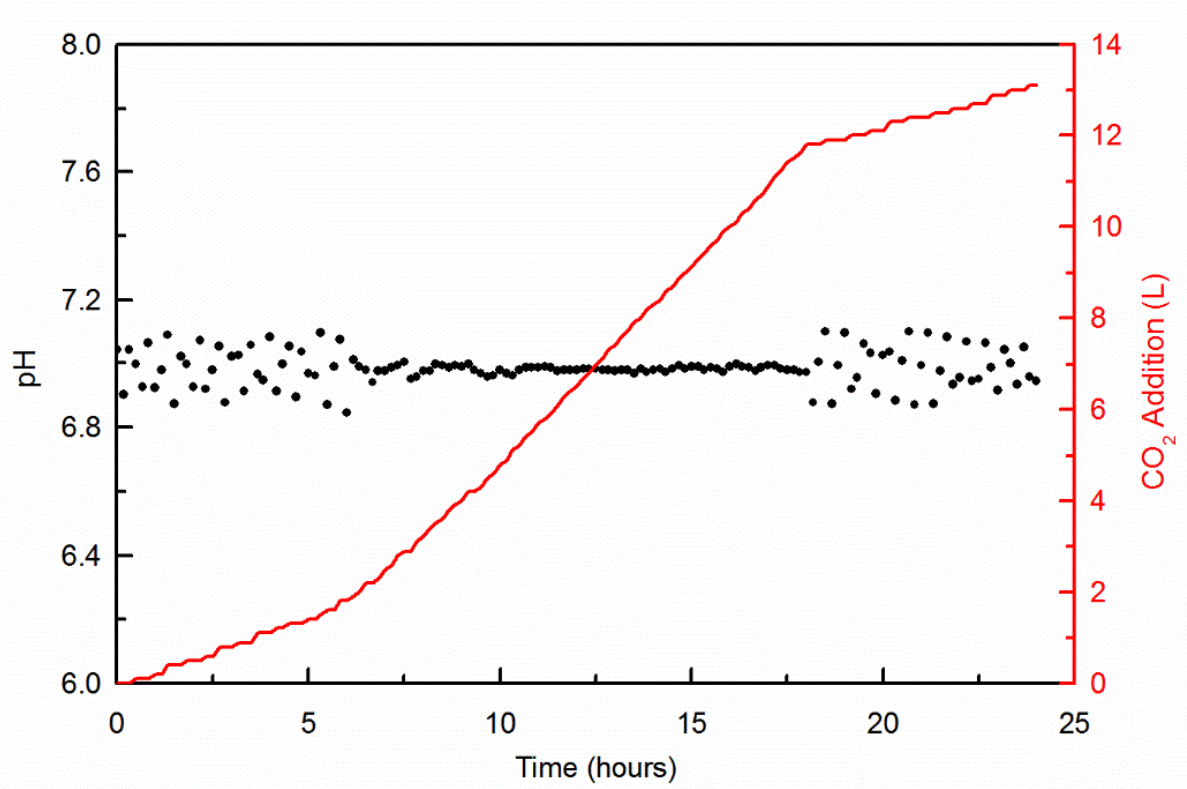

Fig. A3. pH and CO_2 addition in a 90 L PBR when pH was maintained at 7 within 24 hours for *Chlorella sorokiniana* DOE1412 culture (Light: 6~18 h; Dark: 0~6 & 18~24 h).

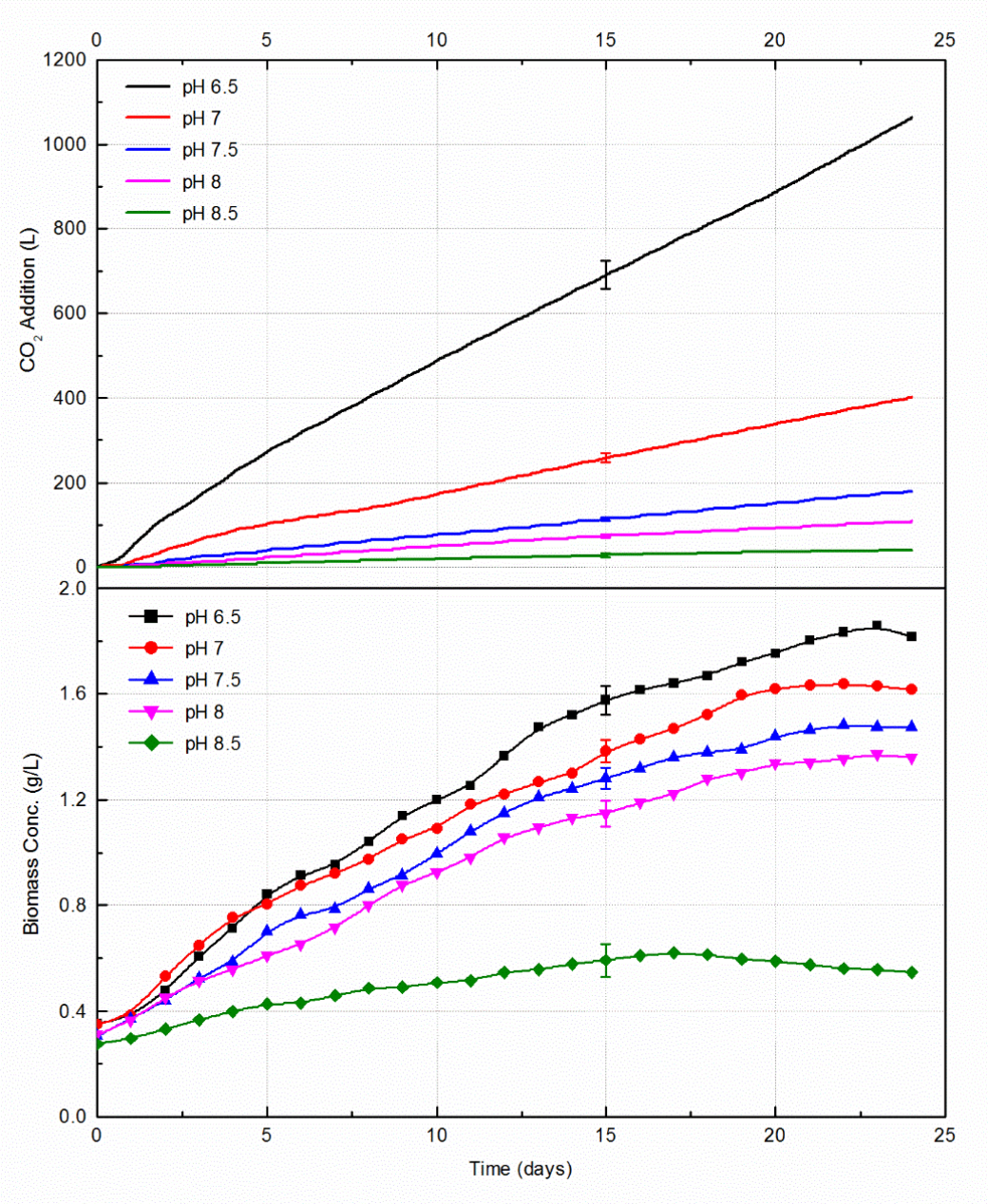

Fig. A4. Growth curves for *Chlorella sorokiniana* DOE1412 and CO_2 addition for cultures at different pH in a 90 L PBR. Data are the average of duplicates. ± S.D. lines at t = 15 day are shown.

Table A2 Biomass productivity, CO_2 addition, and CO_2 cost estimate for cultures at different pH in a 90 L PBR.

pH	Biomass productivity (g/day)	CO_2 addition			CO_2 cost estimation ($/tonne biomass)
		(L/day)	(L CO_2/g biomass)	(g CO_2/g biomass)	
6.5	6.51	44.24	6.79	13.32	200
7	5.85	17.15	2.93	5.75	86
7.5	5.07	7.71	1.52	2.98	45
8	4.62	4.73	1.02	2.01	30
8.5	1.84	1.98	1.08	2.12	32

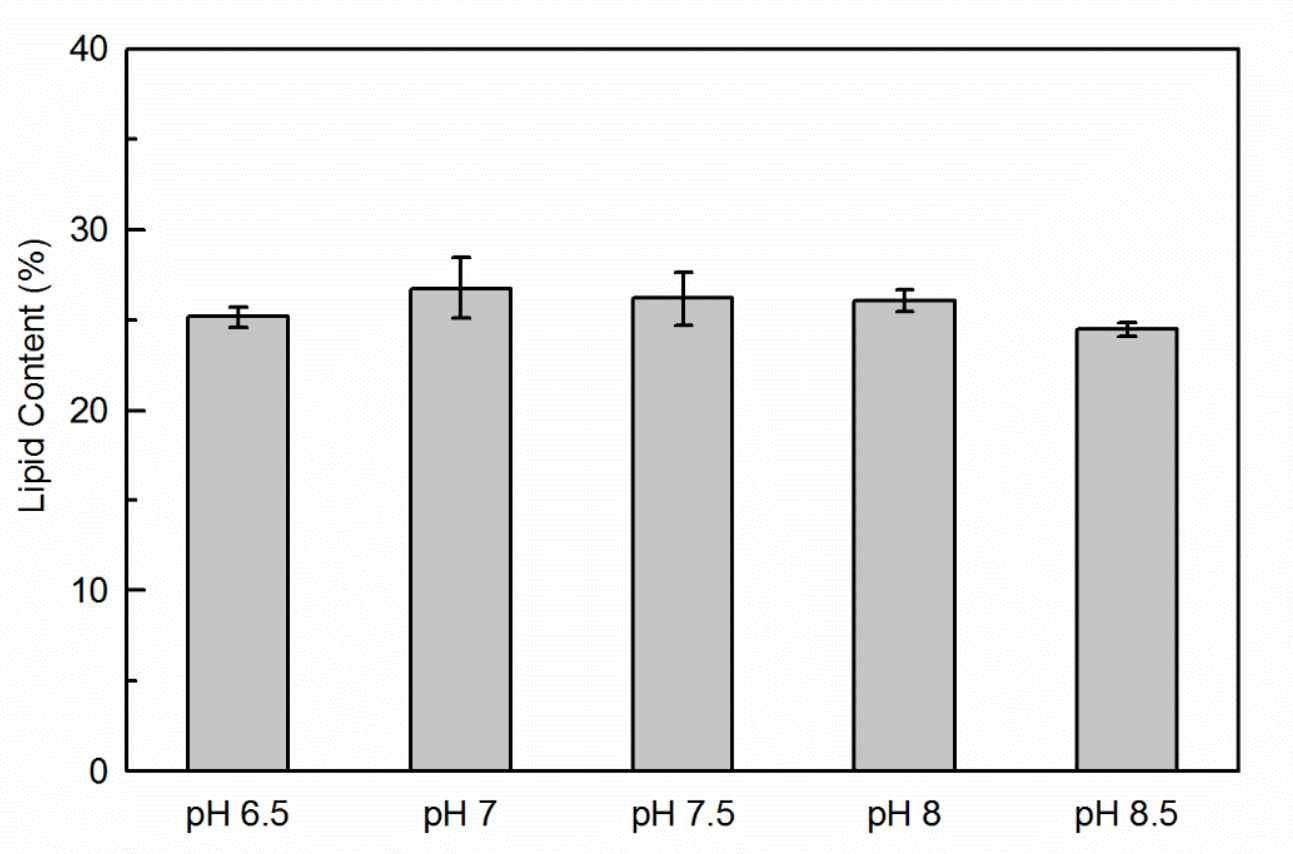

Fig. A5. Lipid content for *Chlorella sorokiniana* DOE1412 grown at different pH in a 90 L PBR. Data are shown as means ± S.D., n=4.

A.3.3 Effects of pH on fatty acid profiles and biodiesel properties in a 90 L airlift PBR

It is well known that fatty acid profile determines the quality of produced biodiesel, especially when the microalgae strain is able to produce PUFAs. As shown in Table A3, DOE1412 mostly accumulated 16 and 18 carbon fatty acids, which are suitable for biodiesel production. The primary components were C16:0 and C18:2. Neofotis et al. investigated the changes in the fatty acid profile of DOE1412 during a growth period of 16 days and found similar results: C16:0 and C18:2 became predominant species after a week of growth (Neofotis et al., 2016). The fatty acid profiles appeared to be influenced by culture pH. The fractions of SFA and MUFA decreased with increasing cultivation pH from 6.5 to 8.5, while the fraction of PUFA increased. Generally, unsaturated fatty acids, consisting of MUFAs and PUFAs, have lower melting points than SFAs. They are excellent for cold weather biodiesel production, but require an oxidative stabilizer to be used safely. It has been reported that MUFAs are most favorable for biodiesel production, because of the melting points that allow for flow in cold climates, while limiting the amount of oxidizer addition (Hoekman et al., 2012). In this study, the highest fraction of MUFA (16.51%), consisting of C16:1 and C18:1, was observed when *Chlorella* was cultivated at pH 6.5.

Based on the fatty acid profiles, this study also estimated several important properties of biodiesel, shown in Table A4. The CN is a prime dimensionless indicator of the ignition quality of diesel, determined by the ignition delay time in a diesel engine. Higher CN leads to better ignition quality and better engine performance (Gopinath et al., 2009). For the two common biodiesel standards, ASTM D6751 (United States) and EN 14214 (Europe), the minimum CN values are 47 and 51, respectively. In this work, the estimated CN values vary from 41 to 51, higher CN values are achieved when the algae are cultivated at lower pH. Fatty acids produced at cultivation pH values of 6.5, 7 and 7.5 satisfied the US standard, among them, pH 6.5 met the

European standard. It was reported that increasing DU led to decreasing CN (Knothe et al., 2003), which is also observed in this study. The IV is a crude measure of fatty acids unsaturation, which relates to the oxidative stability of diesel (Hoekman et al., 2012). EN 14214 defines a maximum IV specification of 120 mg I_2/100 g, while ASTM D6751 does not include this specification. In this study, fatty acids produced at pH 6.5, 7 and 7.5 met the European standard, while pH 8 products were just 0.6% higher than the threshold. Finally, the CFPP is an indicator of diesel flow performance at low temperature. Better cold-weather performance is observed at lower CFPP values. Both ASTM D6751 and EN 14214 do not mention a CFPP limit; however, each country can specify certain temperature limits based on geographic location and time of year (Knothe, 2006). With CFPP ranges from -5.5 °C to -2.2 °C, the biodiesel that would be produced regardless of culture pH are predicted to have decent cold-weather performance.

Overall, biodiesel that would be produced from *Chlorella* cultivated at lower pH generally could meet the diesel standard. Furthermore, since biodiesel is often blended in the United States; a 20% blend of biodiesel and petroleum diesel should be compatible with all diesel engines (Balat, 2011). Biodiesel produced from DOE1412 cultivated at pH 6.5-8.5 can satisfy both US and European standards by blending it with a relatively high CN diesel.

Table A3 Fatty acid profiles at different pH in a 90 L PBR (% of total FAMEs). Data are shown as means ± S.D., n=4.

Fatty acid	pH 6.5	pH 7	pH 7.5	pH 8	pH 8.5
Palmitic (C16:0)	39.52±0.36	37.63±0.25	35.92±0.58	34.27±0.28	29.24±0.70
Palmitoleic (C16:1)	3.46±0.26	2.55±0.10	2.87±0.44	3.72±0.08	2.12±0.05
Hexadecadienoic (C16:2)	6.82±0.75	7.13±0.28	7.69±0.50	7.62±0.68	10.93±0.81
Hexadecatrienoic (C16:3)	2.97±0.14	4.90±0.27	5.66±0.23	7.19±0.39	8.95±0.42
Stearic (C18:0)	1.20±0.17	1.46±0.08	1.59±0.26	1.49±0.16	1.14±0.07
Oleic (C18:1)	13.06±0.20	12.72±0.54	11.93±0.42	9.16±0.23	8.11±0.06
Linoleic (C18:2)	24.98±0.50	23.39±0.31	21.70±0.75	22.41±0.64	21.85±0.25
Linolenic (C18:3)	5.16±0.17	7.55±0.03	9.62±0.08	11.28±0.39	15.25±0.33
Not identified (NI)	2.84±0.19	2.69±0.27	3.04±0.44	2.88±0.37	2.44±0.97
SFA	40.72±0.19	39.09±0.17	37.50±0.33	35.75±0.14	30.37±0.76
MUFA	16.51±0.07	15.27±0.64	14.80±0.05	12.88±0.16	10.22±0.10
PUFA	39.93±0.30	42.97±0.03	44.67±0.13	48.50±0.10	56.97±1.81

Table A4 Comparison of biodiesel properties as a function of cultivation pH to ASTM and EN biodiesel standards.

Property	pH 6.5	pH 7	pH 7.5	pH 8	pH 8.5	ASTM D6751	EN 14214
CN	51	49	48	46	41	≥ 47	≥ 51
SV (mg KOH/g)	204	205	204	205	205	-	-
IV (g I_2/100 g)	97	106	112	121	140	-	≤ 120
DU (wt %)	96	101	104	110	124	-	-
LCSF (wt %)	4.6	4.5	4.4	4.2	3.5	-	-
CFPP (°C)	-2.2	-2.4	-2.7	-3.4	-5.5	-	-

A.3.4 Effects of pH on protein content and carbon to nitrogen (C/N) ratio in a 90 L PBR

For microalgal biomass, the protein content can be as high as 50% by weight, with a low C/N ratio of about 10 (Elser et al., 2000). Lourenco et al. provided a practical method for determining protein content suitable for microalgae, using a nitrogen-to-protein convention factor of 4.78 (Lourenco et al., 2004). Templeton and Laurens (Templeton and Laurens, 2015) reported a new conversion factor (4.08) determined from new data along with literature. In this work, the factor of 4.78 was used to calculate protein content, because it was more commonly used in other studies (Laurens, 2015). TC, TN, and protein content as well as C/N ratio of the DOE1412 biomass produced at different pH in PBRs are shown in Table 5. Data are the average of triplicates. TC content appeared to be unaffected by pH (one-way ANOVA: $p > 0.05$). At pH 8, 2.01 g CO_2 were added to produce each gram of biomass (Table 2).The resulting biomass consisted of 48.12% C by mass. According to the carbon mass balance, 87.8% of CO_2 added was efficiently fixed by microalgae at pH 8. Protein content, which was converted from TN content, increased with increasing pH. The values ranged from 15.5% to 30.7%, while C/N ratio in the cells decreased. Ogbonda et al. (Ogbonda et al., 2007) also found a significant difference in protein production of *Spirulina* sp. at various pH, with the most protein synthesized at pH 9.

As mentioned by Chisti (Chisti, 2007), using biomass after lipid extraction to produce methane by anaerobic digestion can improve the economics of microalgal biodiesel production in a refinery approach. As a substrate for anaerobic digestion, microalgal biomass with lower protein content and higher C/N ratio can diminish the ammonia toxicity and enhance methane production (Sialve et al., 2009). In this work, the highest C/N ratio of biomass produced was 15.1. The C/N ratio of lipid extracted biomass at different pH should be lower than the value shown in Table 5, because the extracted lipid is much more carbon-rich according to the lipid profile. Therefore,

biomass after lipid extraction, regardless of culture pH, has a low C/N ratio compared to the optimal C/N ratio (20-35) for anaerobic digestion. A microalgal biomass co-digestion with a substrate rich in carbon is necessary for increasing the anaerobic production of methane from lipid extracted *Chlorella* biomass.

Table A5 TC, TN, and protein content in biomass produced at different pH in PBRs. Data are shown as means ± S.D., n=3.

	pH 6.5	pH 7	pH 7.5	pH 8	pH 8.5
TC content (wt %)	48.79±0.36	48.64±0.28	48.18±0.31	48.12±0.26	48.53±0.17
TN content (wt %)	3.24±0.03	4.19±0.07	4.62±0.04	4.89±0.02	6.42±0.02
Protein content (wt %)	15.5±0.1	20.0±0.3	22.1±0.2	23.4±0.1	30.7±0.1
C/N ratio	15.1±0.2	11.6±0.2	10.4±0.1	9.84±0.05	7.56±0.03

A.4. Conclusion

The main purpose of this work was to investigate the pH effects on cell growth, lipid production and CO_2 addition when culturing *Chlorella sorokiniana* DOE1412. By manipulating pH through CO_2 injection, pH 6 seems most beneficial for growth and lipid accumulation. At a larger scale, even though the growth rate was lower, pH 8 was more cost effective in CO_2 demand requiring 2.01 g CO_2 per g biomass. The amount of lipid was similar regardless of pH. Biodiesel produced at low pH would meet the diesel standards. The biomass made at higher pH contains more protein with a lower C/N ratio. In conclusion, this project shows how much CO_2 is required to cultivate algae in 90 L flat panel PBRs and provides further evidence that there is a balance between biomass growth, lipid production and CO_2 addition.

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APPENDIX B: Specific deactivation of bacteria in recycled water from microalgae *Chlorella sorokiniana* cultures

(Submitted to Algal Research)

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Abstract

Recycling cultivation water after biomass harvesting is expected to reduce the water requirement, nutrient demand, and energy consumption for algal biofuels and bioproducts production. Cultivation water recovered from large-scale open raceway ponds contain bacteria that can affect culture health and potentially decrease productivity. Developing an inexpensive and effective strategy to eliminate bacterial contamination in recycled cultivation water or spent medium is essential for making algal production financially and environmentally sustainable. Ideally, the method does not harm the algae. We tested several bacterial deactivation strategies, including ozonation, chlorination, chloramination and the addition of hydrogen peroxide, on artificial recycled water samples containing mixtures of microalgae *Chlorella sorokiniana* DOE1412 and *Escherichia coli* strain DH5α. Disinfectant decay, bacterial deactivation and algal survival curves were obtained for each disinfectant at different doses to determine bacteria removal rate, minimum contact time, microalgae survival rate, disinfectant residual, and concentration-time (CT) value. These data were used to compare the efficiency of different bacterial deactivation methods. Results showed that chlorination with an initial dosage of 0.5 mg/L is the most economic and successful bacterial deactivation method: a 5-log reduction of *E. coli* cells was achieved after 1 min of contact time, while the microalgae survival rate was 58%. No chlorine was detected after 10 min of exposure. The other methods investigated showed higher CT value or lower microalgae survival rate for 5-log deactivation of *E. coli* in artificial recycled water samples. The effectiveness of chlorination was also confirmed when tested on the authentic recycled water samples.

Keywords: Microalgae, *Chlorella sorokiniana*, Bacterial deactivation, Water recycling.

Highlights

- Chlorination is the most effective and economic method to deactivate *E. coli* in recycled water from *Chlorella* cultures
- Chlorination shows less negative impact on algal cells than ozonation at the same dose of 0.5 mg/L
- Chlorination is efficient to deactivate bacteria for recycled medium obtained from outdoor raceway

B.1. Introduction

Microalgae have received considerable interest in recent years as one of the most promising third generation biomass feedstocks, thanks to their fast growth, high yields, short production cycle, and their ability to fix greenhouse gases and remediate wastewater (Chisti, 2007; Wang et al., 2008; Rawat et al., 2011; Chiu et al., 2015; Lammers et al., 2017). Furthermore, microalgal biomass can be used to make a wide range of products, including biofuels, fertilizer, anti-oxidants, cosmetics, pharmaceuticals, animal feed, and human nutraceuticals (Borowitzka, 2013; Unkefer et al., 2017). However, most of these algae-based biofuels and bioproducts remain economically unviable due to the high capital and operating costs (Laurens et al., 2017). Life-cycle assessments of algae cultivation indicated that the water footprint and nutrient usage are significant for the mass production of microalgae (Yang et al., 2011). Recycling cultivation spent medium after harvesting the algal biomass not only reduces the demands of fresh water and nutrients but also minimizes the energy consumption (Farooq et al., 2015).

Open raceway ponds are the most commonly and widely used culture systems for large-scale algal biomass production due to their low initial cost, and low energy requirements. However,

microalgae grown in open systems are prone to biological contaminations, including bacteria, zooplankton, virus, and other algae (Wang et al., 2013). The harvested water from open raceway ponds contains different types of biological contaminants, depending on the algae species, growth conditions and growth phase when harvested (Loftus and Johnson, 2017). Based on previous reports and our research on the decline and death of *Chlorella* sp. cultures in open raceway ponds, predatory bacteria is the primary biological contamination for *Chlorella* sp (Soo et al., 2015; Park et al., 2018). Without a pretreatment to eliminate the bacterial contamination in recycled cultivation water, the competition between algae and bacteria for nutrients could inhibit the algal growth and reduce the lipid productivity (Zhang et al., 2012). Moreover, some bacteria cause algal cells to lyse by attaching to their surface (Shi et al., 2006), or release alga-lytic substances (Kim et al., 2007; Chen et al., 2014).

Many strategies have been reported to overcome the challenges of bacterial contamination in recycled cultivation water of microalgae. Membrane filtration has been deployed to remove bacterial cells, but this approach results in partial bacteria removal only, is time-consuming for heavy contamination and costly at commercial scale (Rathore and Shirke, 2011). Some researchers focused on using high-speed centrifugation to control bacterial contamination, but the energy-intensive nature of centrifugation makes it impractical to be implemented in large scale culture systems (Rodolfi et al., 2003; Erkelens et al., 2014). Some disinfection methods, such as chlorination, the addition of hydrogen peroxide and heating, also have been investigated to deactivate bacterial cells in algal cultures (González-López et al., 2013). González-López et al. (2013) found that ozonation was the most effective method to remove bacteria in recycled water recovered from marine microalgae *Nannochloropsis gaditana* culture.

The selection of pretreatment strategy is highly dependent on algae species (Fret et al., 2017). In this study, freshwater microalgae *Chlorella sorokiniana* DOE1412 (also labeled as UTEX B 3016), one of the most promising algal strains isolated by the National Alliance for Advanced Biofuels and Bioproducts (NAABB) for biofuels production, was selected due to its robust growth and high productivity (Griffiths and Harrison, 2009; Neofotis et al., 2016; Qiu et al., 2017). *Escherichia coli*, one of the most common types of bacteria, was chosen as the target bacteria in this study. *E. coli* is often used as an indicator of the effectiveness of a wastewater treatment strategy (Elmund et al., 1999). The focus of this work is to develop a pretreatment method to deactivate bacterial cells while avoiding substantial impact on microalgal culture. Oxidizing agents are often used as disinfectants to remove bacterial contamination, because they can pass through cell walls/membranes, then react with intracellular components, which leads to cell destruction (Denyer and Stewart, 1998). Ozone, chlorine, chloramine, and hydrogen peroxide are the most common oxidizing agents for water treatment (Zhou and Smith, 2001; Abbaszadegan and Margolin, 2007). Thus, we compared the effectiveness of these four bacterial deactivation methods (ozonation, chlorination, chloramination, and the addition of hydrogen peroxide) on pretreatment of recycled cultivation water for DOE1412. The optimal pretreatment method was determined, and tested on the authentic recycled water which was recovered from *Chlorella sorokiniana* DOE1412 cultures grown in an outdoor open pond raceway.

The residual concentration is also important for water reuse and regulation. Maximum residual disinfectant level goals (MRDLGs) and maximum residual disinfectant levels (MRDLs) for three disinfectants (chlorine, chloramines and chlorine dioxide) have been set by the U.S. EPA. Therefore, the amount of disinfectant was monitored to assure that the residual amount was lower than EPA standards. Concentration-time (CT) values are defined as the product of the disinfectant

concentration and the contact time. They are used to characterize the deactivation efficiency of certain disinfectants, and are typically expressed in units of mg-min/L.

B.2. Materials and methods

B.2.1 Algal and bacterial strains

The culture of freshwater microalgae *Chlorella sorokiniana* DOE1412 was obtained from NAABB, funded by the US Department of Energy (Neofotis et al., 2016). The stock culture (500 mL in a 1 L Erlenmeyer flask) was maintained in sterile BG-11 liquid medium on a platform continuously shaken at 120 rpm. The BG-11 medium consists of the following: 1.5 g/L $NaNO_3$, 40 mg/L K_2HPO_4, 75 mg/L $MgSO_4 \cdot 7H_2O$, 36 mg/L $CaCl_2 \cdot 2H_2O$, 6 mg/L citric acid$\cdot H_2O$, 6 mg/L ferric ammonium citrate, 1 mg/L $Na_2EDTA \cdot 2H_2O$, 20 mg/L Na_2CO_3 and 1 mL/L trace metal solution (Rippka et al., 1979). The trace elements in the solution consist of the following: 2.86 g/L H_3BO_3, 1.81 g/L $MnCl_2 \cdot 4H_2O$, 0.22 g/L $ZnSO_4 \cdot 7H_2O$, 0.39 g/L $Na_2MoO_4 \cdot 2H_2O$, 79 mg/L $CuSO_4 \cdot 5H_2O$ and 49.4 mg/L $Co(NO_3)_2 \cdot 6H_2O$ (Rippka et al., 1979). The algae cells were grown with a light intensity of 120-125 μmol photons m^{-2} s^{-1} at ambient temperature. The light cycle was 12 h-on/12 h-off. Old culture medium was replaced by fresh BG-11 medium every 14 days.

Escherichia coli strain DH5α, one of the most common strains for routine cloning applications (Kostylev et al., 2015), was obtained from the School of Plant Sciences at the University of Arizona. *E. coli* was inoculated from a frozen 20% glycerol stock into 5 mL of sterile Luria broth (LB) in snap-cap culture tubes (17 mm × 100 mm). The LB liquid medium consists of 5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl. The culture was grown at 37 °C overnight with continuous shaking (200 rpm).

B.2.2 Preparation of recycled water samples

Two types of recycled water samples were used in this study: artificial recycled water samples and authentic recycled water samples. The artificial recycled water samples were made by mixing the *Chlorella sorokiniana* DOE1412 cultures and *E. coli* DH5α cultures. Both cultures were harvested by centrifugation, then washed and resuspended in sterile deionized water. Each sample (250 mL) consisted of approximately 1×10^6 CFU/mL DOE1412 cells, and about the same amount of *E. coli* cells. The mixtures were continuously stirred at 100 rpm on magnetic stir plates.

The authentic samples were obtained from experimental 8000 L outdoor Algae Raceway Integrated Design (ARID) raceway cultivation system (Waller et al., 2012). *Chlorella sorokiniana* DOE1412 biomass was harvested when the ash-free dry weight (AFDW) reached a final concentration of ~0.4 g/L. The spent medium samples were collected after harvesting the pond using a spiral plate centrifuge (Evodos type 10, Evodos B.V., Raamsdonksveer, Netherlands) (40 Hz, ~1100 × g, 750 L/h). The supernatant contained DOE1412 cells (~1×10^6 colony-forming unit per milliliter, CFU/mL) and bacterial cells. The amount of bacterial cells and the specific types of bacteria in the cultures were not determined, because the type and amount varies greatly depending on the time of year and cultivation conditions. The bacterial cells monitored grew on LB plates.

B.2.3 Bacterial deactivation methods

B.2.3.1 Ozonation

Ozonation was carried out with a concentrated ozone stock solution (~50 mg/L) prepared by continuously bubbling ozone gas with a diffuser into a 1 L stirred liquid-jacketed vessel filled with ultra-pure water. The ultra-pure water was chilled in advance and maintained at 1 °C to

improve ozone solubility. The ozone gas was produced from pure oxygen using a pilot scale ozone generator (Modular 8HC, Xylem Wedeco, Herford, Germany). The in-gas and off-gas ozone concentration, gas pressure and gas flow rate were continuously monitored. The desired ozone dose of 0.25 to 1 mg/L was achieved by adding ozone from a stock solution. The dissolved ozone concentration of the stock solution and the residual ozone concentration of samples were measured using the indigotrisulfonate (ITS) ozone residual test (Rakness et al., 2010).

B.2.3.2 Chlorination

A sodium hypochlorite stock solution (~60 g/L Cl_2) was used for chlorination. Sodium hypochlorite stock solution was then added into the culture sample to achieve the desired free chlorine dose of 0.25 to 1 mg/L. The free chlorine concentration of the stock solution and the residual free chlorine concentration of samples were determined with a digital portable colorimeter (DR/890, Hach Company, Loveland, CO) by using the DPD (N,N-diethyl-p-phenylenediamine) colorimetric method (Hach Method 8021) (Rice et al., 2017).

B.2.3.3 Chloramination

Monochloramine stock solution (~250 mg/L) was prepared daily for chloramination by slowly adding sodium hypochlorite solution (~60 g/L Cl_2) to ammonium chloride solution with a Cl_2/NH_3 molar ratio of 1:1.4. The ammonium chloride solution was pre-adjusted to pH 8 using an ultra-high concentration (200 g/L) of sodium hydroxide, and chilled to 5 °C. A slight excess of ammonia was maintained to reduce the potential for breakpoint chlorination (Mitch and Sedlak, 2002). Monochloramine is the dominant chloramine at this condition. The monochloramine stock solution was then added into the culture sample to achieve the desired monochloramine dose of 0.25 to 1 mg/L. The concentration of monochloramine was measured by the MonochlorF reagent

(Hach Company, Loveland, CO) based on the indophenol method (Hach Method 10171) (Lee et al., 2007).

B.2.3.4 Hydrogen peroxide addition

Hydrogen peroxide (H_2O_2) stock solution (~500 g/L) was diluted and then added into the water sample to achieve the desired hydrogen peroxide dose of 1 to 5 mg/L. The concentration of hydrogen peroxide was measured using the peroxytitanic colorimetric method (Boltz and Howell, 1978). Absorbance was measured by using an ultraviolet-visible spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, Inc., Waltham, MA) at 407 nm.

B.2.4 The effectiveness of deactivation methods

The effectiveness of bacterial deactivation was evaluated by both disinfectant decay kinetics and cell counting. Three samples were collected at each contact time (1, 2, 5, 10, 20, 30, 40, 50, 60, 75, 90, 105 and 120 min). Sampling was terminated after no residual disinfectant was detected. One of the samples was used to measure the concentration of residual disinfectant. The amount of this sample was decided by the method used, from 0.25 mg/mL to 5 mg/mL. The other two samples collected (1 mL each) were used for cell counting on agar plates, in parallel with the residual disinfectant measurement. Both cell count samples were immediately mixed with 1 mL of 100 mg/L sodium thiosulfate solution to quench any residual disinfectant. These diluted samples were labeled as S0. Based on experimental results, 100 mg/L sodium thiosulfate does not affect the growth of algae and *E. coli* on agar plates.

Serial dilutions (10-fold, 100-fold and 1000-fold; labeled as S1, S2 and S3) of the culture S0 were done in sterile deionized water. A 0.2 mL aliquot from each culture (S0, S1, S2 and S3)

was spread on the surface of agar plates. For microalgal growth on plates, BG-11 agar plates supplemented with 1.5% (w/v) agar were prepared by the method reported by Allen (1968). LB agar plates, which were used for *E. coli* cultures, also contained 1.5% (w/v) agar. LB agar plates were incubated for 24 h at 37 °C before counting. BG-11 agar plates were placed at ambient temperature for 120 h with illumination (200 μmol photons m^{-2} s^{-1}, 12 h-on/12 h-off) on the plate surface. Triplicates were performed for each cell count sample.

Colonies were counted when the number of colonies is about 100 or below 100 per plate. Cell count results were expressed as CFU/mL. All cell count data shown in the figures were log transformed (means ± S.D., n=3). When the cell count of 0.2 mL culture S0 was zero, the CFU/mL of the original sample should be less than 10, therefore, the cell count detection limit of this method was 10 CFU/mL. All the data in this study were calculated based on the worst case scenario when the detection limit was reached. The disinfectant decay curves were presented by the concentration of disinfectant residual over contact time. The CT values were calculated by integrating the areas under disinfectant decay curves over a given contact time. The integration was estimated by summing trapezoidal regions created by the sampling time points.

B.3. Results and discussion

B.3.1 Ozonation on artificial recycled water

Ozone is one of the most potent oxidizing agents available in the industry. It is very effective against bacteria, viruses, fungi, and algae by breaking through the cell membrane and oxidizing all essential components. The effectiveness of ozonation depends on dosage, contact time, and the susceptibility of the target organisms (von Gunten, 2003). Three different doses of ozone (0.25, 0.5 and 1 mg/L) were applied to the artificial recycled water with 120 min of contact

time. The typical ozone dose of 1 mg/L is sufficient to deactivate most bacteria, while 120 min is long enough for excess contact (Dyas et al., 1983). At a concentration of only 0.25 mg/L ozone, a 5-log reduction of the *E. coli* cells was not achieved after 120 min of contact. Both 0.5 and 1 mg/L ozone deactivated *E. coli* cells by a 5-log reduction, and resulted in log reductions of 1.4 and 2.8 for microalgal cells, respectively.

Fig. B1 shows the ozone decay curve and concentration of *E. coli* and microalgal cells as determined by plate counts in artificial recycled water treated with 0.5 mg/L ozone. The coefficient of variation was less than 2.0% for the cell count $\log_{10}$ (CFU/mL) data. After 1 min of contact time, the *E. coli* cells in the water sample were deactivated by a 5-log reduction, and the $\log_{10}$ (CFU/mL) for microalgae decreased from 6.0 to 4.6. Meanwhile, the ozone concentration in the recycled water had a significant drop from 0.50 to 0.14 mg/L. The cell density of microalgae remained relatively stable after 1 min, while the ozone concentration kept decreasing. After 20 min of exposure, no ozone residual was detected in the culture. The total log reductions of *E. coli* and microalgal cells using 0.5 mg/L ozone were 5.0 and 1.4, respectively. Hence, 99.999% of the *E. coli* cells were efficiently deactivated by 0.5 mg/L of ozone, while only 3.8% of microalgal cells survived. CT value for 5-log deactivation of *E.coli* was 0.32 mg-min/L with 0.5 mg/L ozone. A dose of 0.5 mg/L ozone was also tested on water samples consisted of DOE1412 cells (~1×10^6 CFU/mL) and *E. coli* cells (~1×10^6 CFU/mL) separately. Results are provided in the supporting information (Fig. BS1 and BS2). The disinfectant decay, bacterial deactivation and algal survival curves are similar compared to what observed in Fig. B1. CT values are not comparable due to different test conditions.

The results demonstrated that the required dose and contact time of ozone for deactivation vary with different target microorganisms and suspension medium. The study by González-López

et al. (2013) achieved less deactivation of bacteria with a higher dose of 95 mg/L ozone: the bacterial load in the recycled water was reduced from 1.8×10^6 CFU/mL to 1.9×10^3 CFU/mL after 5 min of contact time, a 3-log reduction. While in this study a 5-log reduction was achieved. In addition to working with a different algal species. González-López et al. (2013) used culture medium instead of deionized water to suspend cultures in their investigation. Although the authors did not comment on this extensively, the culture medium may have contained components that deplete the ozone which could also account for the differences in results.

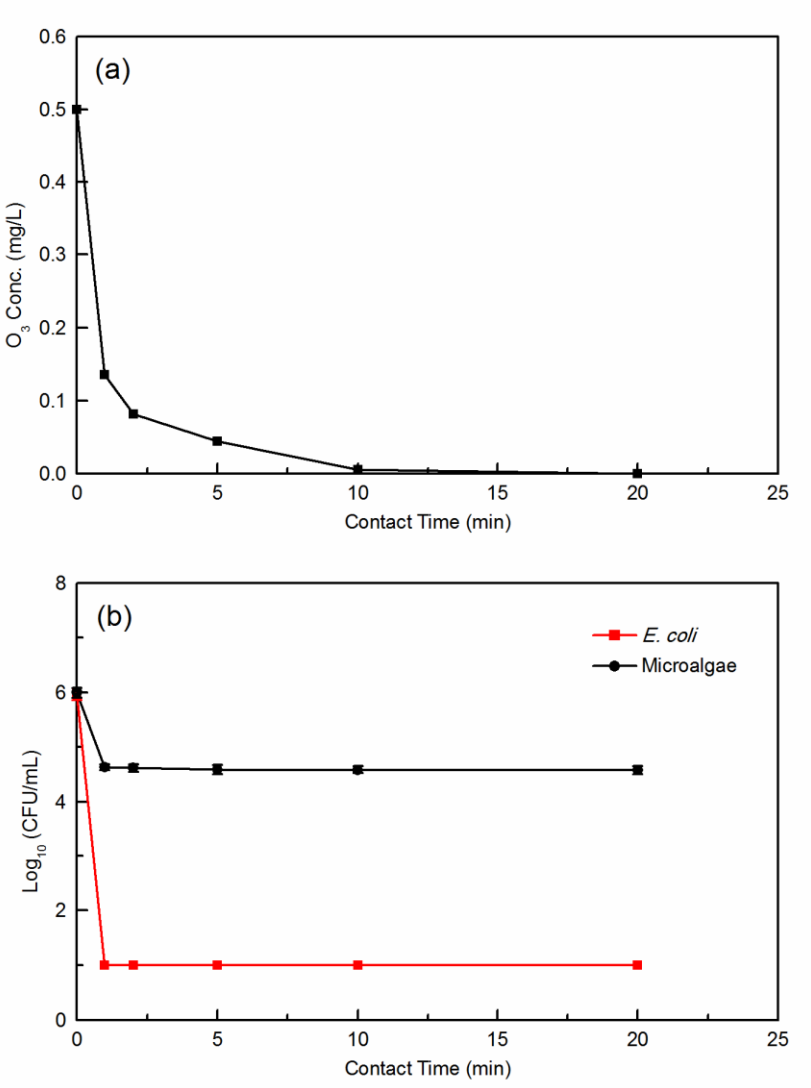

Fig. B1. (a) Ozone decay curve, and (b) live concentration of *E. coli* and *Chorella sorokiniana* in artificial recycled water treated with 0.5 mg/L ozone. Cell count data are shown as means ± S.D., n=3.

B.3.2 Chlorination on artificial recycled water

Chlorine is the most dominant disinfectant for water disinfection. It is relatively inexpensive and can be used for removal of most microorganisms. Three different doses of chlorine (0.25, 0.5 and 1 mg/L) were applied to the artificial recycled water with 120 min of contact time. A dose of chlorine above 1 mg/L may cause ocular and respiratory irritation, coughing, dyspnea and headaches, while 120 min is long enough to guarantee excess residence time (Evans, 2005). Results showed that 0.25 mg/L chlorine was only able to deactivate 16.6% of the *E. coli* cells after 120 min of contact, while 1 mg/L chlorine removed both *E. coli* and microalgal cells in the water sample by a 5-log reduction. A dose of 0.5 mg/L chlorine achieved a 5-log reduction of *E. coli* cells and kept a significant amount of microalgal cells intact.

Fig. B2 shows the chlorine decay curve and concentration of viable *E. coli* and microalgal cells in artificial recycled water treated with 0.5 mg/L chlorine. The coefficient of variation was less than 2.0% for the cell count $\log_{10}$ (CFU/mL) data. After 1 min of contact time, all the *E. coli* cells in the recycled water were removed, while the microalgal cells only had a log reduction of 0.09. The chlorine residual was 0.14 mg/L at 1 min, then gradually decreased to zero at 10 min. After a 10 min residence time, the total log reductions of *E. coli* and microalgal cells using 0.5 mg/L chlorine were 5.0 and 0.24, respectively. A 5-log reduction of *E. coli* cells was achieved while 58% of microalgal cells remained intact after chlorination. Chlorine preferentially killed *E. coli* over microalgae in the artificial recycled water. CT value for 5-log deactivation of *E.coli* was 0.32 mg-min/L with 0.5 mg/L chlorine. A dose of 0.5 mg/L chlorine was also tested on water samples consisted of DOE1412 cells (~1×10^6 CFU/mL) and *E. coli* cells (~1×10^6 CFU/mL) separately. Results are provided in the supporting information (Fig. BS3 and BS4). The chlorine residual remained as high as 0.37 mg/L after 10 min of contact with the water sample (~1×10^6

CFU/mL *E. coli* cells). The chlorine residual levels are below EPA standards in drinking water throughout. Other than that, the disinfectant decay, bacterial deactivation and algal survival curves are similar compared to what observed in Fig. B2. CT values are not comparable due to different test conditions.

González-López et al. (2013) reported that chlorination (1 mg/L, 5 min) decreased the bacterial load 1000-fold, but it showed a negative impact on algal growth when treated water was reused. More specifically, the algal biomass final concentration was < 0.1 g/L when grown on water pretreated with chlorine compared to the control experiments in which the final concentration was ~ 0.8 g/L. González-López et al. (2013) mentioned that the reason could be the composition change of the culture medium by chlorination. A 5-log reduction of bacterial cells was achieved in this study and the negative effect on algal growth was not observed. Potential reasons for the varied results are a low concentration of disinfection by-products (DBPs) due to low dose, zero chlorine residual detected in this study, or variation in algal species. González-López et al. (2013) used the marine algae *Nannochloropsis* sp. in their study. Similar to the freshwater algae *Chlorella* sp. used in this study, the algal cells have rigid cell walls. However the structure of their cell wall is different: *Nannochloropsis* sp. cell walls contain cellulosic polyssacharides and non-hydrolyzable biopolymer algaenans (Scholz et al., 2014), while *Chlorella* sp. have a diverse structure which varies at the species level. The cell wall of *Chlorella sorokiniana* was composed mainly of glucosamine (Takeda, 1991). González-López et al. (2013) did not report residual chlorine concentrations in their investigation.

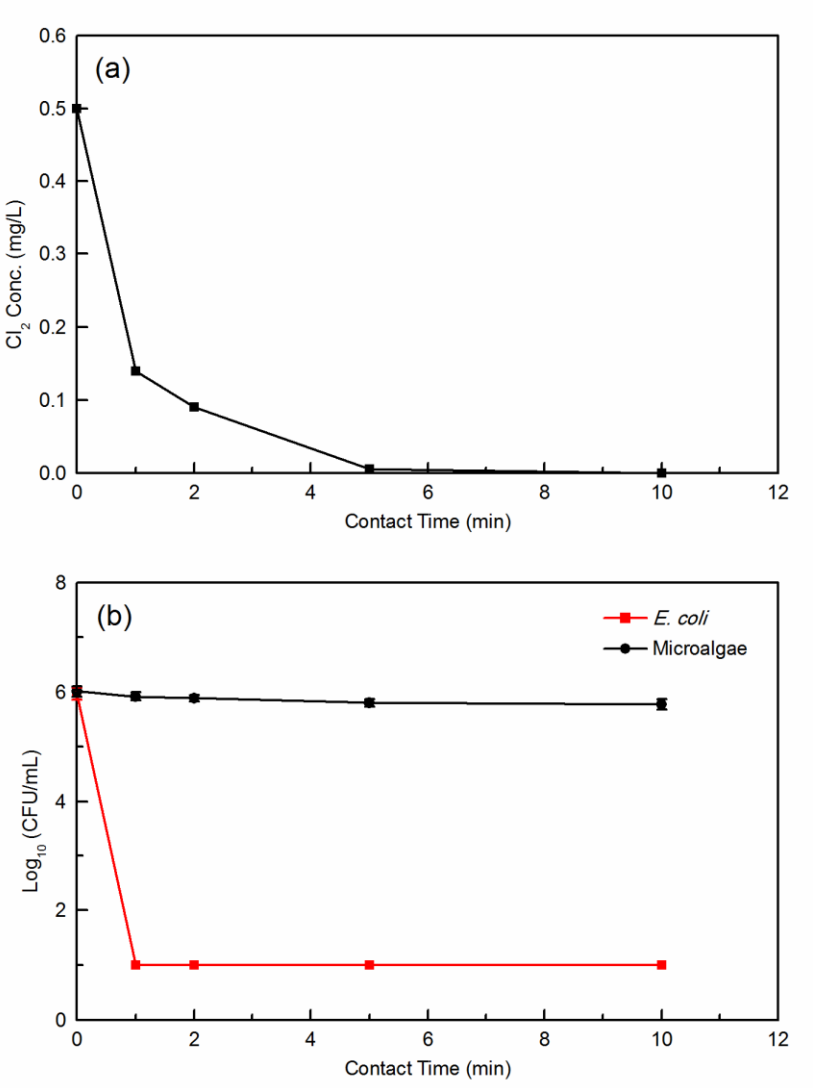

Fig. B2. (a) Chlorine decay curve, and (b) live concentration of *E. coli* and *Chorella sorokiniana* in artificial recycled water treated with 0.5 mg/L chlorine. Cell count data are shown as means ± S.D., n=3.

B.3.3 Chloramination on artificial recycled water

Chloramine is another disinfectant used to eliminate bacterial contamination, which can be used as an alternative to chlorine in water treatment. The use of chloramine at water utilities in the United States has increased in recent years due to its better stability in the distribution system and lower levels of regulated disinfectant by-products (Seidel et al., 2005). There are three inorganic forms: monochloramine, dichloramine, and trichloramine. Monochloramine is the most effective form of chloramine and is known to be stable and persistent. Three different doses of monochloramine (0.25, 0.5 and 1 mg/L) were applied to the artificial recycled water with 120 min of contact time. The same doses and contact time were chosen for comparison to chlorination. Similarly to results observed for chlorine, a dose of 0.25 mg/L monochloramine appeared not high enough, which only achieved a log reduction of 0.1 for *E. coli* cells after 120 min. The *E.coli* and microalgal cell counts of culture S0 were both zero at 1 mg/L monochloramine dosage and 120 min of contact. A dose of 0.5 mg/L monochloramine was appropriate for the artificial recycled water sample.

Fig. B3 shows the monochloramine decay curve and concentration of viable *E. coli* and microalgal cells in artificial recycled water treated with 0.5 mg/L monochloramine. The coefficient of variation was less than 4.5% for the cell count $\log_{10}$ (CFU/mL) data. After 90 min of contact time, the amount of *E. coli* cells in the recycled water was reduced by 5-log. There was no monochloramine residual detected at 120 min. After a 120 min contact time, the microalgal cells had a log reduction of 2.6, which means 99.8% of microalgae were deactivated by monochloramine. CT value for 5-log deactivation of *E.coli* was 12.8 mg-min/L with 0.5 mg/L monochloramine. A dose of 0.5 mg/L monochloramine was also tested on water samples consisted of DOE1412 cells (~1×10^6 CFU/mL) and *E. coli* cells (~1×10^6 CFU/mL) separately. Results are

provided in the supporting information (Fig. BS5 and BS6). The amount of microalgal cells was reduced by 5-log after 120 min in the water sample (~1×10^6 CFU/mL DOE1412 cells). The long time required is not ideal compared to ozonation and chlorination. Other than that, the disinfectant decay, bacterial deactivation and algal survival curves are similar compared to what observed in Fig. B3. CT values are not comparable due to different test conditions. Monochloramine appeared to be less effective than chlorine in this study, which was also observed by Butterfield (1948). This is because monochloramine is a relatively weak oxidant, and it oxidizes organics at a slower rate than chlorine (Lee and von Gunten, 2010).

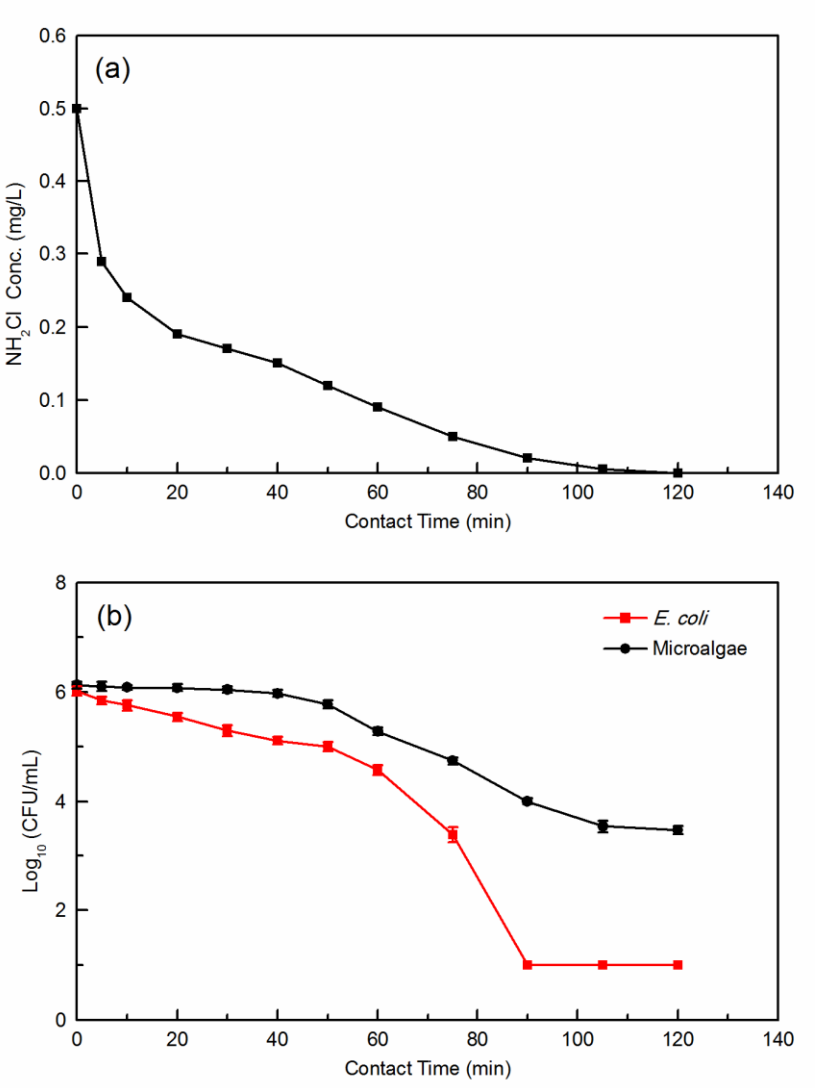

Fig. B3. (a) Monochloramine decay curve, and (b) live concentration of *E. coli* and *Chorella sorokiniana* in artificial recycled water treated with 0.5 mg/L monochloramine. Cell count data are shown as means ± S.D., n=3.

B.3.4 Hydrogen peroxide addition on artificial recycled water

Hydrogen peroxide is active against different types of microorganisms, including bacteria, fungi, viruses, spores, and yeast. Four different doses of hydrogen peroxide (0.5, 1, 2 and 5 mg/L) were applied to the artificial recycled water with 120 min of contact time. Higher doses of hydrogen peroxide were tested because the total log reduction of *E. coli* was only 0.15 after 120 min of contact with an initial dose of 0.5 mg/L H_2O_2. Based on the results, even with a dose of 5 mg/L H_2O_2, the cell density of *E. coli* remained above 4.0×10^5 CFU/mL after 120 min.

Fig. B4 shows the H_2O_2 decay curve and variation of *E. coli* and microalgal loads in artificial recycled water treated with 5 mg/L H_2O_2. The coefficient of variation was less than 2.0% for the cell count $\log_{10}$ (CFU/mL) data. With 120 min of contact, the total log reductions of *E. coli* and microalgal cells were 0.37 and 0.18, respectively. This means that 43% of *E. coli* and 66% of the microalgae were still active after 120 min. Moreover, the H_2O_2 residual was 1.9 mg/L at 120 min. A dose of 5 mg/L H_2O_2 was also tested on water samples consisted of DOE1412 cells (~1×10^6 CFU/mL) and *E. coli* cells (~1×10^6 CFU/mL) separately. Results are provided in the supporting information (Fig. BS7 and BS8). The disinfectant decay, bacterial deactivation and algal survival curves are similar compared to what observed in Fig. B4. CT values are not comparable due to different test conditions. According to the study conducted by González-López et al. (2013), H_2O_2 seemed only effective at deactivating bacteria in recycled water at doses of > 5% (v/v), which is unsafe and corrosive to the skin.

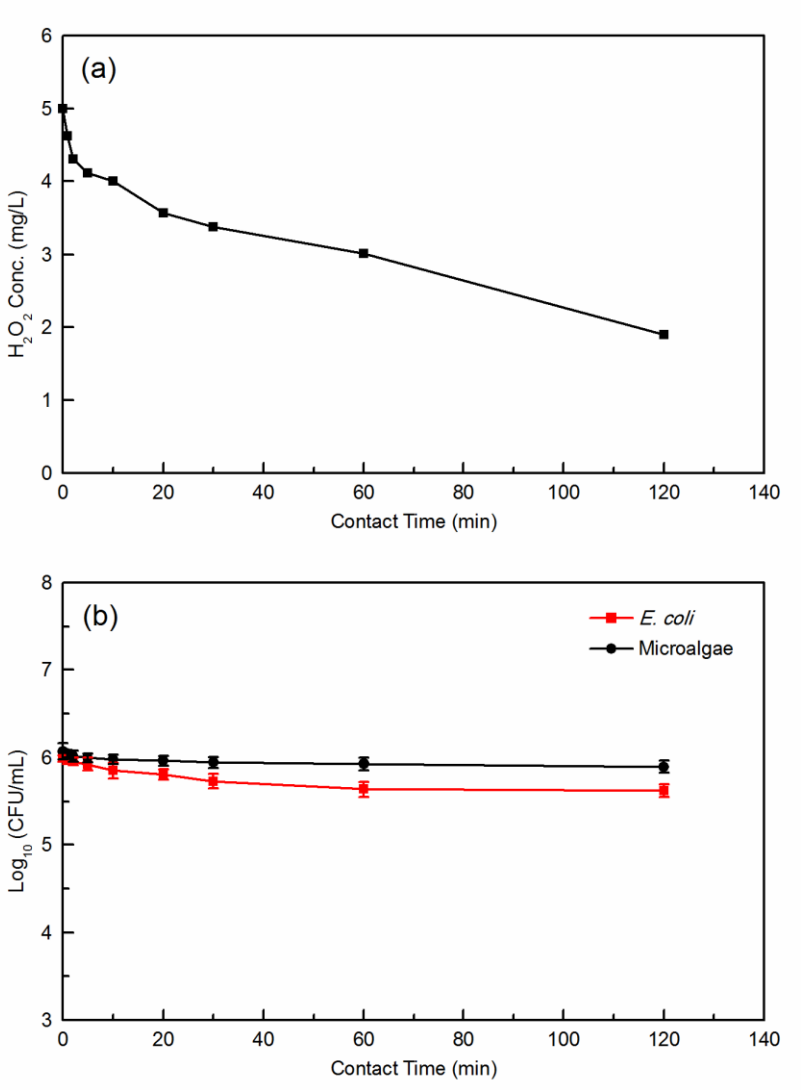

Fig. B4. (a) H_2O_2 decay curve, and (b) live concentration of *E. coli* and *Chorella sorokiniana* in artificial recycled water treated with 5 mg/L H_2O_2. Cell count data are shown as means ± S.D., n=3.

B.3.5 Comparison of bacterial deactivation methods on artificial recycled water

The comparative data for different deactivation methods on artificial water samples is shown in Table B1. Based on the *E. coli* deactivation results on the artificial recycled water samples, both ozonation (0.5 mg/L) and chlorination (0.5 mg/L) were highly effective bacterial deactivation methods: they were able to reduce *E. coli* cells by 5-log in the water samples after 1 min of contact time, with the same CT value (0.32 mg-min/L) for 5-log deactivation. Moreover, no residual concentration was detected after 20 min of contact for 0.5 mg/L ozonation, and no residual chlorine after 10 min for 0.5 mg/L chlorination. Compared to ozonation and chlorination, bacterial deactivation by chloramination (0.5 mg/L) was significantly less effective; CT value for 5-log deactivation of *E.coli* was 40 times higher than when the same dose of ozone and chlorine was used. Lastly, the results show that even the highest dose of hydrogen peroxide tested in this study (5 mg/L) was not able to deactivate *E. coli* by 5-log in the artificial recycled water sample after 120 min.

Table B1 Comparative data for different deactivation methods on artificial water samples

	Dose (mg/L)	Contact time (min)	CT value[a] (mg-min/L)	Algae survival rate	No residual time (min)
Ozone	0.5	1	0.32	3.8%	20
Chlorine	0.5	1	0.32	58%	10
Monochloramine	0.5	90	12.8	0.2%	120
Hydrogen peroxide	5	> 120	N/A	N/A	>120

[a] CT value for 5-log deactivation of *E. coli* in the artificial recycled water sample

The major advantages of using chlorine over ozone for treatment of water containing bacteria and the microalgae *Chlorella sorokiniana* DOE1412 are survival rate of the algae and cost. Approximately 58% of the algae survived when treated with chlorine (0.5 mg/L), compared to 3.8% when treated with the same concentration of ozone. Therefore, if there is residual oxidant in the recycled medium, the effect to the algal cells in the pond would be minimal. Furthermore, chlorine is the cheapest of the four disinfectants tested in this study. It can be purchased directly as liquid sodium hypochlorite, solid calcium hypochlorite, or chlorine gas. On the other hand, ozone needs to be produced on-site, and requires a significant capital investment and high maintenance expenditures. Plumlee et al. (2014) provided a cost estimate of ozone for water reclamation: a 10 million gallon per day (MGD) ozone facility operation with a 3 mg/L ozone dose would require a capital investment of $6.8M and $0.061M annual operations and maintenance (O&M) costs. In comparison, a study conducted by the Water Environment Research Foundation in 1995 showed estimated capital costs of $3.1M (including costs associated with the dechlorination process and the Uniform Fire Code requirements) and annual O&M costs of $0.16M for a 10 MGD chlorine facility with a 5 mg/L chlorine dose (Darby et al., 1995). After scaling the costs from 1995 to 2014 using the Chemical Engineering Plant Cost Index, the estimated capital costs of the chlorine facility would be $4.7M (including costs associated with optional process). Thus, the capital cost of the chlorine facility is at a minimum 31% less than the capital cost of the ozone facility which produces a lower amount or dose.

B.3.6 Test on authentic recycled water

Since chlorination was the most economic and successful bacterial deactivation method for mixtures of *E. coli* and *C. sorokiniana*, a dose of 0.5 mg/L were applied to the authentic recycled

water recovered from ARID open raceway pond. Fig. B5 shows the chlorine decay curve and concentration of viable bacteria and microalgal cells. The coefficient of variation was less than 3.0% for the cell count $\log_{10}$ (CFU/mL) data. The initial cell density was 7.9×10^3 CFU/mL of unidentified bacteria and 1.5×10^6 CFU/mL of microalgae DOE1412. After 2 min of contact time, the bacterial load in the authentic recycled water sample decreased to less than 10 CFU/mL, achieving a 3-log reduction. The chlorine residual was 0.12 mg/L at 1 min, 0.04 mg/L at 2 min, and zero detected after 10 min. With 10 min of contact, the total log reduction of microalgal cells was 0.25, which means 57% of microalgal cells remained intact. CT value for 3-log deactivation of bacteria was 0.39 mg-min/L with 0.5 mg/L chlorine. Overall, bacterial deactivation using chlorine showed a similar deactivation performance on the authentic recycled water samples as was observed in the artificial recycled water samples.

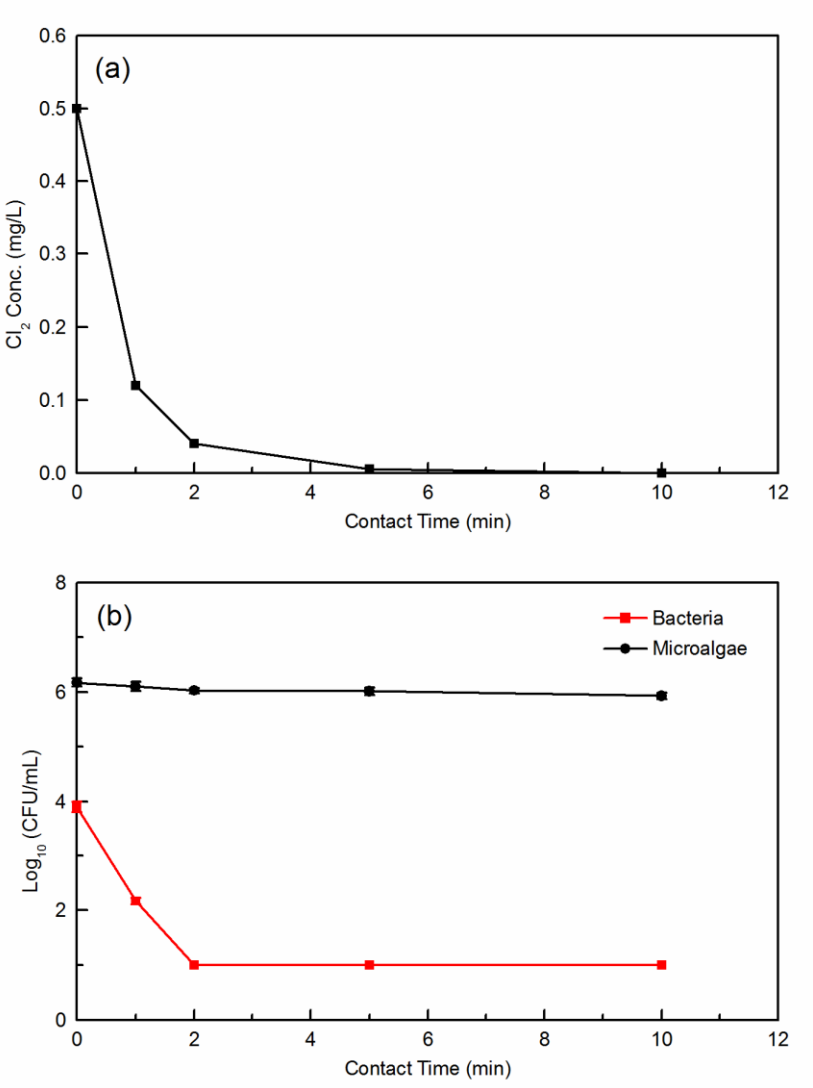

Fig. B5. (a) Chlorine decay curve, and (b) live concentration of bacteria and *Chorella sorokiniana* in authentic recycled water treated with 0.5 mg/L chlorine. Cell count data are shown as means ± S.D., n=3.

B.4. Conclusion

The main purpose of this work was to compare the effectiveness of different disinfectants on the removal of bacteria from recycled water of microalgae *Chlorella sorokiniana* DOE1412 cultures. Chlorination (0.5 mg/L) was proven to be most efficient and cost-effective on both artificial and authentic recycled water. The bacteria were deactivated by 5-log while at least 50% of the algae were able to grow on plates after treatment. Also, there was no chlorine residual detected. The variations in effectiveness of disinfectants are primarily because they react with different functional groups in algal and bacterial cell membranes. The target sites for chlorine are believed to be cell walls and the amino groups in proteins, where chlorine oxidizes thiol groups to different forms, and affects DNA synthesis by disrupting nucleotide regulation (Russell, 2003). Chlorine can damage all types of microorganisms, however in this study, an appropriate dose was determined that damaged the thin cell wall of gram negative bacteria while leaving the stronger *Chlorella sorokiniana* cell wall intact. This work shows the bacteria in the recycled medium can be deactivated by chlorine at a reasonably low cost, while the survival rate of algae is relatively high. In the future, chlorination may be investigated for deactivating *Vampirovibrio chlorellavorus*, the predatory bacteria of *Chlorella* sp. *V. chlorellavorus* attaches to the outside of algal cells, transfers hydrolytic enzymes to the algae, ingests algal lysates for replication, which results in algal cell death (Soo et al., 2015). This type of study would require quantitative measurements of *V. chlorellavorus* which is challenging since it is an obligatory parasite. Also, long term studies are required to conquer the challenges on a commercial scale. Once the optimized technique is scaled-up, it will reduce the replenished algal inoculum and nutrients for the next run of cultivation, more importantly, it will significantly lower the demand of fresh water to enhance the economical

and sustainable viability of algal biomass. This technique also has the potential to be implemented in using impaired water sources, such as wastewater, to cultivate algae.

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The authors declare no conflicts of interest. No conflicts, informed consent, human or animal rights applicable.

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APPENDIX B1: Supplementary Materials for APPENDIX B

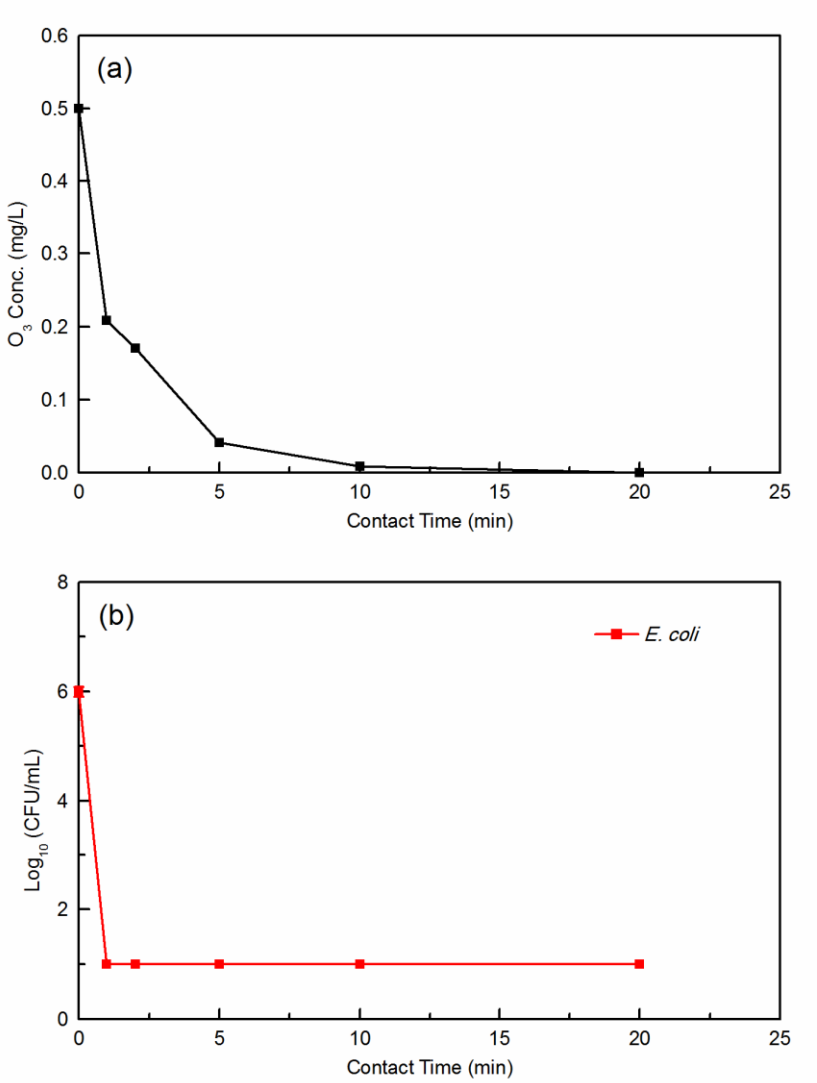

Fig. BS1. (a) Ozone decay curve, and (b) live concentration of *E. coli* in 250 mL water sample (~1×10^6 CFU/mL *E. coli* cells) treated with 0.5 mg/L ozone. Cell count data are shown as means ± S.D., n=3.

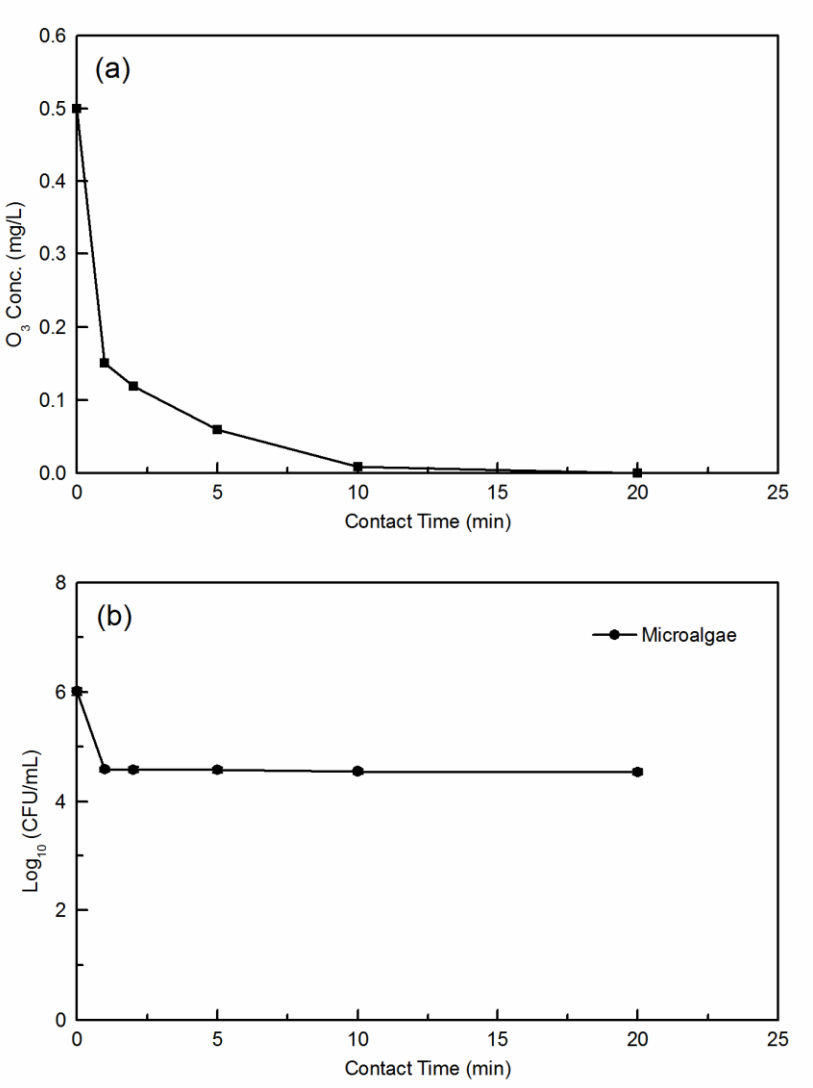

Fig. BS2. (a) Ozone decay curve, and (b) live concentration of *Chorella sorokiniana* in 250 mL water sample (~1×10^6 CFU/mL DOE1412 cells) treated with 0.5 mg/L ozone. Cell count data are shown as means ± S.D., n=3.

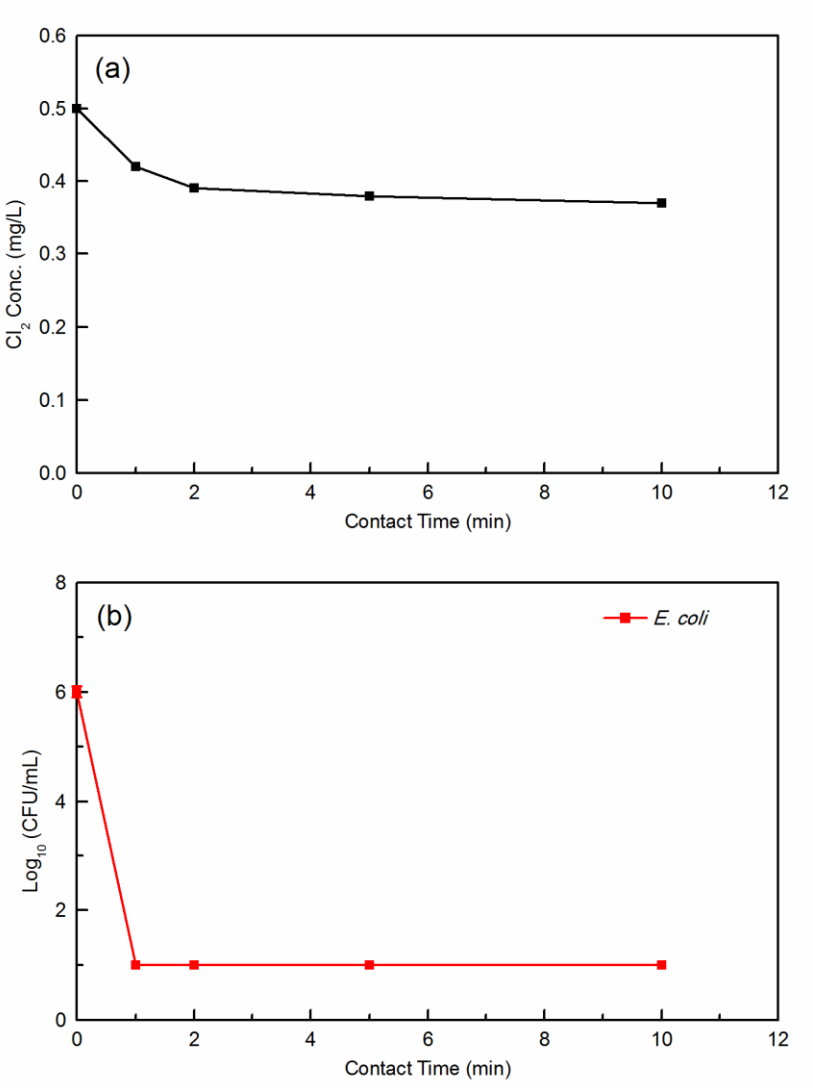

Fig. BS3. (a) Chlorine decay curve, and (b) live concentration of *E. coli* in 250 mL water sample (~1×10^6 CFU/mL *E. coli* cells) treated with 0.5 mg/L chlorine. Cell count data are shown as means ± S.D., n=3.

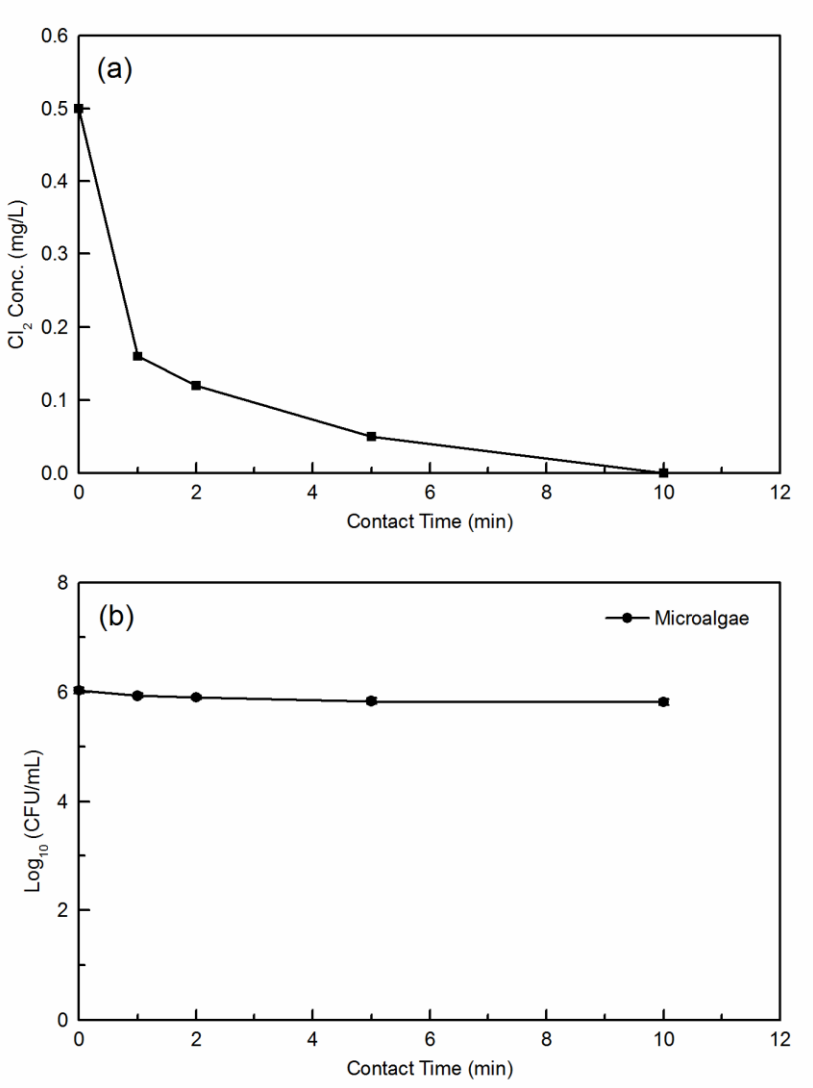

Fig. BS4. (a) Chlorine decay curve, and (b) live concentration of *Chorella sorokiniana* in 250 mL water sample (~1×10^6 CFU/mL DOE1412 cells) treated with 0.5 mg/L chlorine. Cell count data are shown as means ± S.D., n=3.

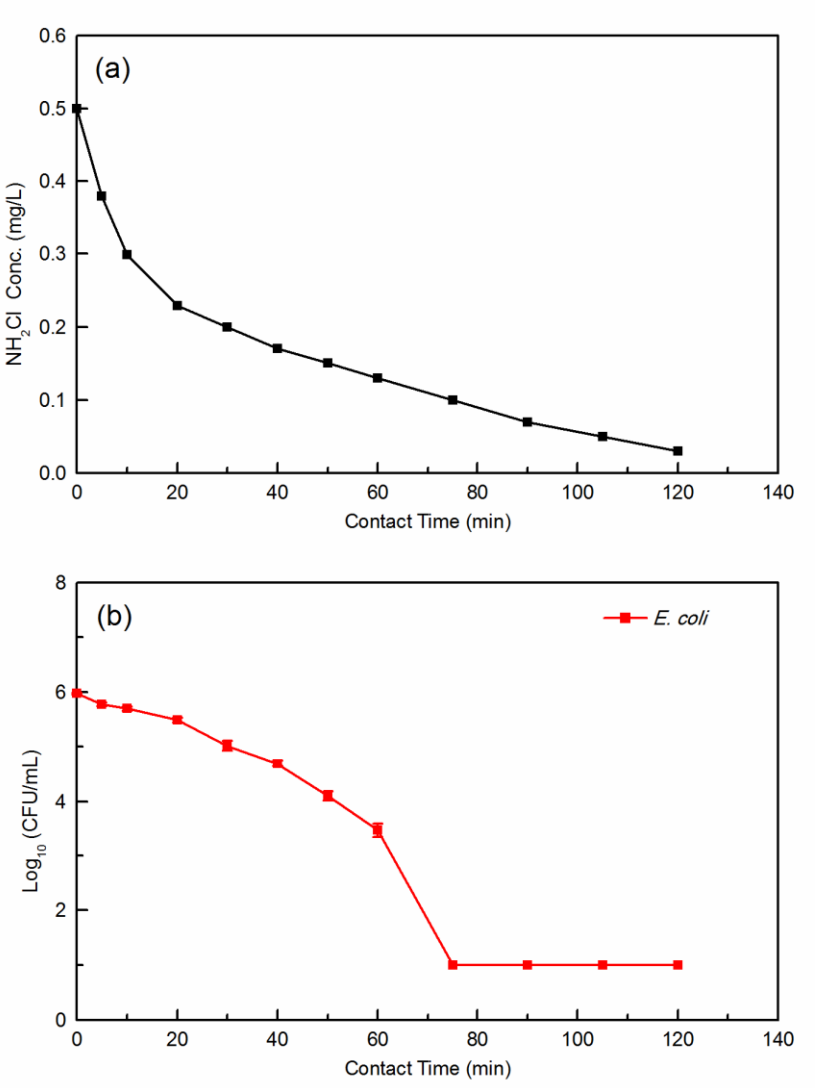

Fig. BS5. (a) Monochloramine decay curve, and (b) live concentration of *E. coli* in 250 mL water sample (~1 × 10^6 CFU/mL *E. coli* cells) treated with 0.5 mg/L monochloramine. Cell count data are shown as means ± S.D., n=3.

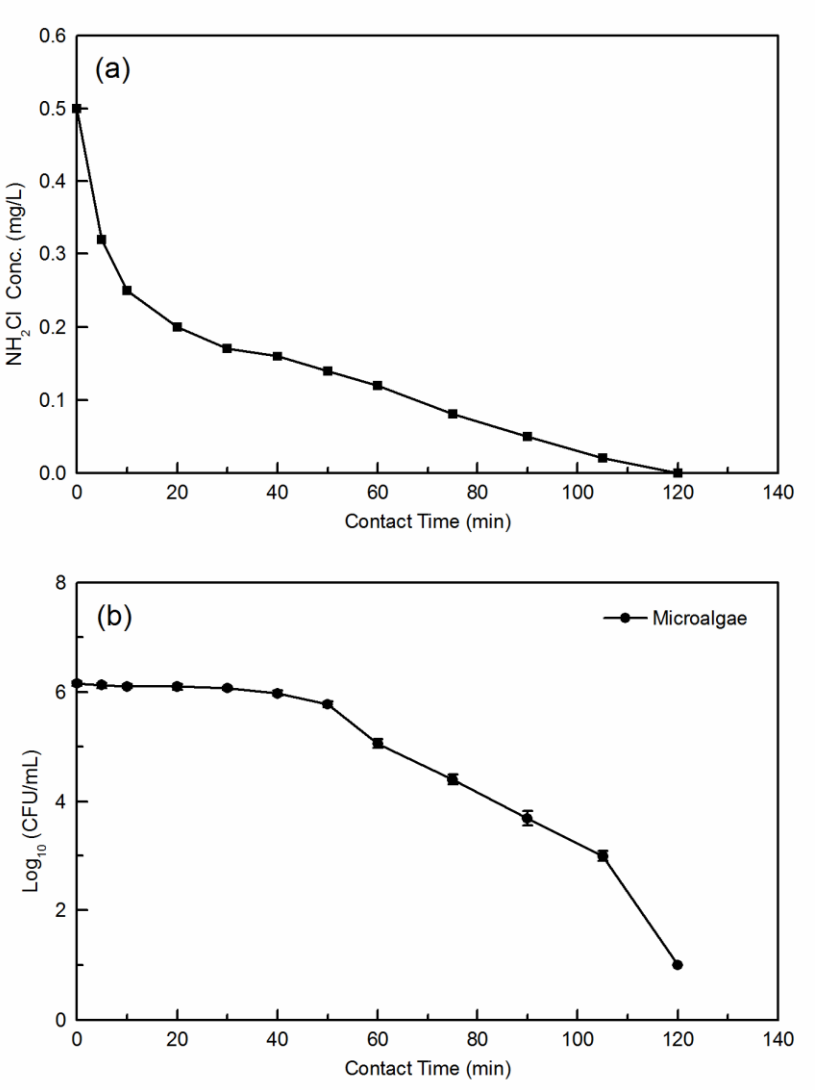

Fig. BS6. (a) Monochloramine decay curve, and (b) live concentration of *Chorella sorokiniana* in 250 mL water sample (~1×10^6 CFU/mL DOE1412 cells) treated with 0.5 mg/L monochloramine. Cell count data are shown as means ± S.D., n=3.

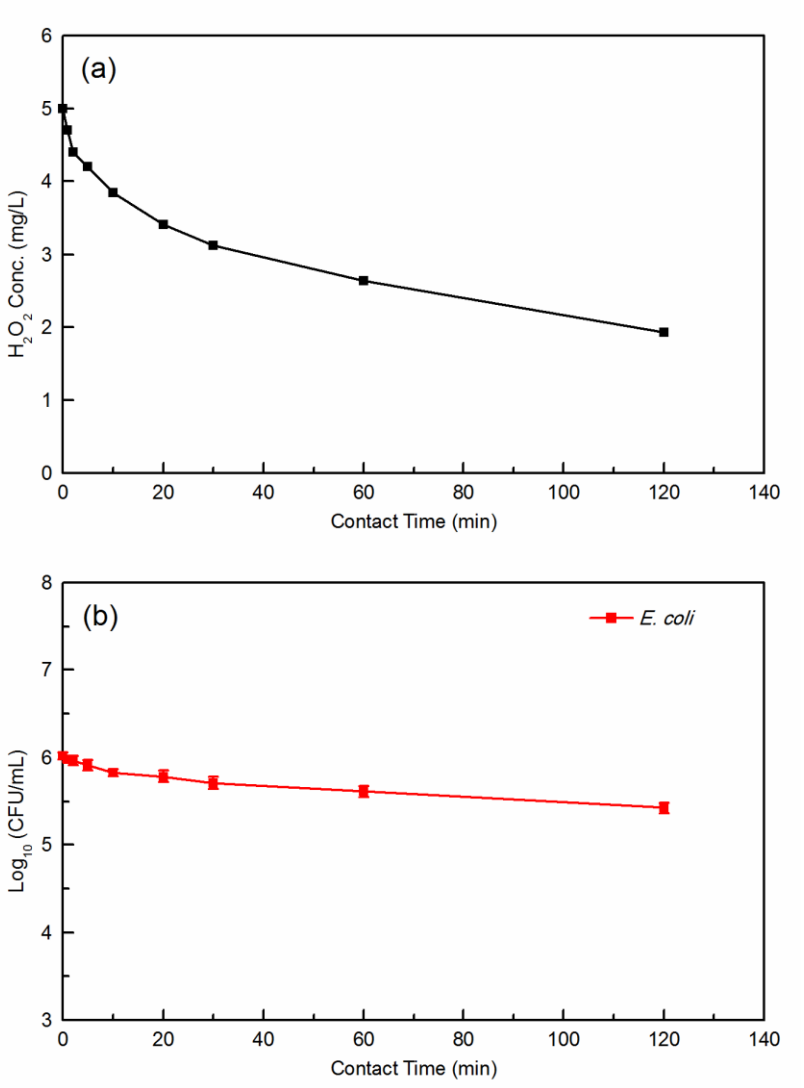

Fig. BS7. (a) H_2O_2 decay curve, and (b) live concentration of *E. coli* in 250 mL water sample (~1 × 10^6 CFU/mL *E. coli* cells) treated with 5 mg/L H_2O_2. Cell count data are shown as means ± S.D., n=3.

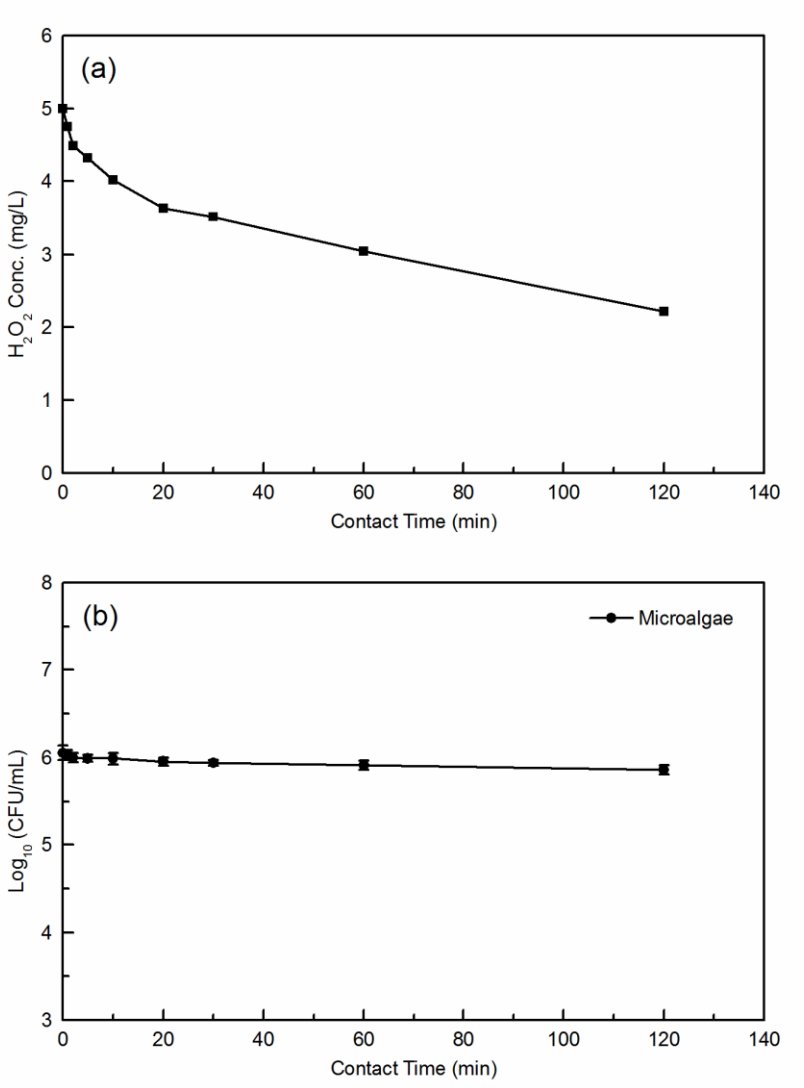

Fig. BS8. (a) H_2O_2 decay curve, and (b) live concentration of *Chorella sorokiniana* in 250 mL water sample (~1×10^6 CFU/mL DOE1412 cells) treated with 5 mg/L H_2O_2. Cell count data are shown as means ± S.D., n=3.