# Nitrogen Removal by Algae-Bacteria Consortia: Batch Tests and Photo Sequence Batch Reactor

by

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**Abstract**

Exceeding nitrogen discharge into natural water bodies can lead to eutrophication in natural aquatic environments, as well as the decline in shellfish habitat and aquatic plant life. Currently, bacterial biological treatment process is the most common process employed in wastewater treatment plants, which requires extensive oxygen. The large demand for oxygen provided by mechanical aeration is costly and can strip out volatile compounds. Microalgae are photosynthetic micro-organisms can be a good source of oxygen in the wastewater treatment process. The effect of using microalgae, either solo or in consortia systems along with other micro-organisms (mainly bacteria) have been studied by researchers to improve their contaminant removal efficiency. In a consortia system, microalgae generate oxygen through photosynthesis to satisfy the oxygen requirement of bacteria. Simultaneously, they also remove contaminating nutrients, nitrogen and phosphorus, throughout their growth cycle. Various factors affect the performance of the consortia systems such as lighting, pH, and species of microalgae and bacteria. Since microalgae are typically suspended and dispersed in the media, harvesting is crucial to achieving a high-quality effluent.

The effects of ammonium nitrogen concentration, algae biomass concentration, and light conditions (wavelength and intensity) on the ammonium removal efficiency of algae-bacteria consortia from wastewater were investigated in initial short term small scale experiments. The results indicated that ammonium concentration and light intensity had a significant impact on nitrification. It was found that the highest $NH_4^+$-N concentration (430 mg N/L) in the influent resulted in the highest ammonia removal rate of 108 ± 3.6 mg N/L/days, which was two times higher than for the influent with low $NH_4^+$-N concentration (40 mg N/L). At the lowest light intensity of 1000 Lux, algae biomass concentration, light wavelength, and light cycle did not show

a significant effect on the performance of algal–bacterial consortium. Furthermore, the $NH_4^+$-N removal rate was approximately 83 ± 1.0 mg N/L/days, which was up to 40% faster than at the light intensity of 2500 Lux. It was concluded that the algae-bacteria consortia can effectively remove nitrogen from wastewater and the removal performance can be stabilized and enhanced using the low light intensity of 1000 Lux that is also a cost-effective strategy.

To evaluate the effect of photoperiod on consortium performance in long-term low-light intensity, a photo batch sequence reactor was operated under 24 hours' illumination and 16h/8h light-dark cycle alternately. After the stabilization phase, a constant $NH_4^+$-N removal rate of 60 mgN/L/d was achieved for both photoperiods. Without loading organic carbon in the wastewater, maximum denitrification efficiency of 28% was achieved in photoperiod of 16h/8h, which was 36% higher compared to that achieved under continuous lighting condition. Less biomass was produced with photoperiod of 16h/8h L/D even though similar $NH_4^+$-N removal rate and higher denitrification rate were achieved. Biomass settled within half an hour of the start of the experiment with a sludge volume index (SVI) of 109 ml/g. The results indicate a sustainable way for reduced energy consumption and provide a way to reduce bio-waste production by treating wastewater with algae-bacteria consortia.

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## List of Abbreviations

| | |
|---|---|
| AOB | Ammonia-Oxidizing Bacteria |
| AOA | Ammonia oxidizing archaea |
| BBM | Bold's Basal Medium |
| BOD | Biochemical oxygen demand |
| COD | Chemical oxygen demand |
| DI water | Deionized water |
| DO | Dissolved oxygen |
| EPS | Extracellular polymeric substances |
| FA | Free ammonia |
| FNA | Free nitrous acid |
| HRAP | High rate algal ponds |
| HRT | Hydraulic retention time |
| L/D | Lighting to dark cycle |
| LED | Light-emitting diode |
| LI | Light intensity |
| NiR | Nitrite reductase |
| NOB | Nitrite-oxidizing bacteria |
| NR | Nitrate reductase |
| RAS | Return activated sludge |
| SRT | Solids retention time |
| SVI | Sludge volume index |

| | |
|---|---|
| TKN | Total Kieldahl nitrogen |
| TN | Total nitrogen |
| TOC | Total organic carbon |
| TSS | Total suspended solids |
| WAS | Waste-activated sludge |
| WSPs | Waste stabilization pond systems |
| WWTPs | Wastewater treatment plants |

# Chapter 1 INTRODUCTION AND OBJECTIVES

City, industry, and agriculture operations create a large volume of wastewater every year. Natural water bodies receive the major proportion of various wastewater streams. The water body is eutrophic when the nitrogen concentration is higher than 1.9 mg/L (Brown et al., 2001), which can result in algal blooms. Many microalgae grow on the surface of the water that blocks the sunlight and exerts oxygen from aquatic bodies. Habitats of aquatic life decrease due to the decline in oxygen thereof.

Biological (activated sludge) treatment is the most commonly used process for nitrogen removal in wastewater treatment plants (WWTPs). There are two steps for removing nitrogen in biological treatment: nitrification and denitrification. In this process, nitrifiers, including ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), convert total ammonia (free ammonia and unionized ammonia) to nitrate. Denitrification happens in an anoxic environment in which denitrifiers reduce nitrate and nitrite to nitrogen gas. It takes 4.57 grams of $O_2$ to oxidize per gram of total ammonia to nitrate. In WWTPs, mechanical aeration supplies a large amount of oxygen and costs 45–75% of total energy demand of the plant (Oilgae, 2010). The aeration has the risk of stripping out volatile compounds (Muñoz et al., 2004). In addition, waste-activated sludge (WAS) is the major by-product of biological treatment. The production of WAS is corresponding to the amount of wastewater treated (Metcalf and Eddy, 2003). To treat 1 million liters of wastewater, the activated sludge process generates about 70–100 kg dry WAS. With the continued increase in wastewater generation, the amount of WAS is also increasing (Athanasoulia et al., 2012). To treat and dispose of WAS, significant energy and a large land area are required.

Microalgae are primarily oxygen-releasing photosynthetic organisms with a simple cell having no roots, stems, or leaves. Microalgae grow fast, produce ubiquitously, and can double their biomass within 24 h. They can grow exponentially within 3.5 h (Bala et al., 2016). They are ubiquitous in the environment and can thrive in almost any habitat as long as the required nutrients are available. Some microalgae grow on rocks, in soils, and/or in symbiotic relationships with plants. Most commonly, however, microalgae grow in fresh and marine aquatic systems, as well as in wastewater streams from a variety of sources (Zhou, 2014). Microalgae can produce a wide range of valuable metabolites including fats, sugars, and bioactive compounds (Andersen, 2013). Generally, lipid content is in the range of 20 to 50% of their dry weight. Depending on the group of algae, such as *Botryococcus* (green algae), the content could be as high as 90% (Metting, 1996). After lipid extraction, the protein-rich residue is ideal for feeding animals and fertilizing crops (Cai et al., 2013). There have been many studies conducted on microalgae for applications in biotechnology. So far, microalgae have been implemented in industry for different purposes, such as synthesis of supplements, food additives, and other bioactive compounds for the cosmetic and pharmaceutical industries (Priyadarshani et al.,2012). In natural aquatic systems, microalgae act as tiny aeration devices that produce oxygen for other bacteria and serve as carbon dioxide sinks that fix $CO_2$. Other than oxygen–carbon dioxide exchange, the interactions between microalgae and bacteria also include other aspects (Figure 1-1), which indicates the potential of microalgae–bacteria consortia system on wastewater treatment.

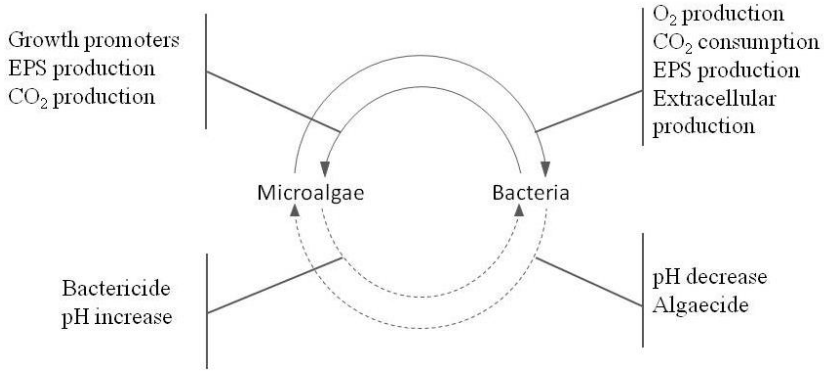



**Figure 1-1 Interactions between microalgae and bacteria (Plain line: negative interactions; Dashed line: positive interactions) (Riquelme, 2012; Muñoz et al., 2006)**

In terms of wastewater, studies on microalgae-based treatments have been conducted for more than a half-century. Waste stabilization pond systems (WSPs) and high rate algal ponds (HRAP) are two currently available technologies. A WSP is an open system where microalgae and heterotrophic bacteria form a symbiotic relationship as shown in Figure 1-1. In this system, microalgae assimilate nitrogen and phosphorus, and bacteria removes organic matter. An HRAP is a shallow, paddlewheel-mixed open raceway pond. In this system, microalgae grow rapidly and produce extensive oxygen, driving aerobic treatment and the assimilation of wastewater nutrients in algal biomass. The end products from the microalgae-based treatments can be used as animal feed or crop fertilizer (Oilgae, 2010). The advantages of microalgae-based wastewater treatment as stated by the U.S. Department of Energy (DOE) National Algal Biofuels Technology, included the "potential to treat agricultural drainage and eutrophic water bodies, wastewater treatment revenue that offsets microalgae production costs, lower capital and operation and maintenance costs than conventional wastewater treatment, and lower energy intensity than conventional wastewater treatment (a green-house gas benefit)"(US DOE, 2010).

The use of high-quality effluent is important for wastewater treatment. Harvesting of suspended growing microalgae or microalgae-bacteria consortia is expensive (Zhang et al., 2012). The efficiency of common industrial processes, such as filtration, centrifugation, and micro-straining, have worked poorly at removing microalgae. Adding chemicals such as lime and alum are efficient but costly. Biomass auto-flocculation have been observed in the laboratory condition, however, the mechanism is still unclear. Other methods including biomass immobilization and dosing edible chitosan are promising strategies for harvesting (Muñoz et al., 2006).

The objectives of batch tests are as follows:

- Explore the effect of ammonium concentration, algal biomass, and light on the performance of an algae–bacteria consortium on nitrogen removal from synthetic wastewater.
- Optimize the nitrogen removal process via algae–bacteria consortia thereby making it more feasible for implementation in the WWTPs.

The objectives of photo sequence batch reactor are as follows:

- Examine the effect of different photoperiods on the nitrification performance of the consortia.
- Estimate the low light intensity effect on a long-term PSBR.

# Chapter 2 LITERATURE REVIEW

## 2.1 The application of microalgal treatment in different wastewater streams

Profiles of wastewater streams are significantly different from each other, with no distinguishing chemical characteristics or physical properties. Wastewater pollution comes from three sources: municipal systems, agricultural systems, and industrial systems.

In general, there are three types of municipal wastewater in WWTPs: primary effluent, secondary effluent, and centrate generated from the dewatering of anaerobic digested activated sludge (Constantine, 2006). Compared to primary and secondary effluents, centrate contains much higher nitrogen concentrations which have become a treatment issue for the WWTP. Current centrate treatments include physical-chemical treatments and biological treatments (Constantine, 2006). Some researchers have used reject water as cultivation media for algal biomass and lipid production (Cai et al., 2013; Min et al., 2011; Rusten et al., 2011), which shows the potential of microalgae in the treatment of reject water.

Agricultural wastewaters, especially from dairy and swine production, have high turbidity and nutrient concentrations, and large insoluble organic compounds. The contaminants with high turbidity can block light and decrease photosynthetic efficiency, which obstructs algal treatment applications in agricultural wastewaters. Therefore, in related studies, streams from agriculture wastewater were commonly diluted before algal-based treatments to decrease the turbidity and nutrient concentration.(González et al., 2008; Woertz et al., 2009; Zhu et al., 2013).

Industrial wastewaters also have complex constituents and high levels of toxic compounds. Generally, industrial wastewaters are treated using a variety of hazardous chemicals for pH correction, sludge removal, as well as color and odor removal (Oilgae, 2010). Based on published

reports, industrial wastewaters are not suitable for microalgae growth. However, a few studies have demonstrated the feasibility of microalgae-based remediation of some specific industrial wastewaters (Chinnasamy et al., 2010; Zhou, 2014).

Many benefits support the potential of microalgae-based treatments, such as low operating costs, ability to reduce atmospheric $CO_2$ level and/or capture of $CO_2$ from industrial flue gases, and production of valuable end bio-products (Oilgae, 2010). Studies of microalgae-based treatments on nitrogen removal include the microalgae solo system and the consortia system.

## 2.2 Microalga treatment systems

### 2.2.1 Microalgal solo systems

In natural aquatic systems, microalgae assimilate large amounts of nutrients and trace metals during the growing season. Microalgae can also digest inorganic nitrogen sources, such as nitrate, nitrite, and ammonium. Among those, ammonium is preferred since microalgae can assimilate it by consuming less energy than the other two forms. Microalgae shows advantages in many applications: they can grow rapidly, produce many bio-valuable by-products, the residue of waste microalgae can be used as animal feed and crop fertilizer, and do not need a large amount of land for waste disposal (Cai et al., 2013). The aforementioned advantages make this system a great candidate for wastewater treatment. Microalgae solo systems here refer to the methods using only microalgae in wastewater treatment (nitrogen removal) without the assistance of other organisms. Many experiments have been conducted to investigate the performance of microalgae solo systems on wastewater streams and these are summarized in Table 2-1.

**Table 2-1 Experiments on microalgal solo systems**

| Medium source | Microalgal species | Experimental settings | References |
|---|---|---|---|
| Secondary wastewater effluent | *Chlorella vulgaris* | Batch tests (6 days);<br>LI[a] =6000 lux;<br>L/D[b]: 16/8 | (J. Kim et al., 2013) |
| Primary settled wastewater | *Chlorella vulgaris* | Batch tests (15 days); LI=14580-16740 lux;<br>L/D[a]: 8/16 | (Lee et al., 2015) |
| Secondary effluent | Four species of green microalgae | Batch tests (7 days);<br>LI=7000 lux;<br>L/D: 8/16 | (Su et al., 2012) |
| Secondary effluent | *Scenedesmus obliquus*<br><br><br><br>*Chlorella vulgaris* | Batch test (2.5 days), LI=7290 lux;<br>semi-continuous (35 hrs/ cycle);<br>LI= 10800 lux;<br>cycle was not mentioned | (Alejandro et al., 2010) |
| Primary settled wastewater | *Chlorella vulgaris* | Batch test (10 days);<br>LI= 4300 lux;<br>L/D: 16/8 | (Lau et al., 1995) |
| Secondary effluent | Mixed green algal culture | Batch test (14 days);<br>LI=7000 lux;<br>L/D: 16/18 | (Su et al., 2012) |
| Source-separated urine | *Chlorella sorokiniana* | Continuous (HRT of 1 day);<br>LI= 27000 -97200 lux;<br>illuminating continuously. | (Tuantet et al., 2014) |

[a] d: days
[b] LI: Light intensity
[c] L/D: lighting to dark cycle
[d] HRT: hydraulic retention time

### 2.2.2 Microalgal-bacteria consortia systems

In microalgae-bacteria consortia system(Figure 1-1), microalgae can produce various organic substances that bacteria can assimilate. However, the relationships between microalgae and bacteria are very complex. Some species of bacteria can release hormones to promote algal growth (Mouget et al., 1995). For instance, when co-immobilizing *C. vulgaris* with *Azospirillum brasilense* in alginate beads, populations of algae, pigments in algal cells, and size of micro-algal colonies were significantly improved (Gonzalez & Bashan, 2000). However, the experiment also showed that *Pseudomonas vesiculark* improved algal performance without producing any plant hormones (Mouget et al., 1995). Classification of consortia systems can be based on the effect of

microalgae in systems, which are microalgae-assistant systems and algae-dominant systems. Microalgae can also act as an oxygen producer for other organisms in the former system and removes nutrients in the latter system.

#### 2.2.2.1 Microalgae-assistant systems

WWTPs demand intensive oxygen for nitrification, which is accomplished with mechanical aeration. However, it is costly and can strip out volatile contaminants (Muñoz et al., 2005) . In algae-assistant systems, microalgae mainly supply dissolved oxygen for bacteria to remove nutrients and actively uptake nutrients as well (Karya et al., 2013). Rapid growth rate of microalgae corresponds to sufficient oxygen release. In conventional oxidation (stabilization) ponds or the developed suspended algal pond systems such as HRAP, microalgae are combined with heterotrophic aerobic bacteria, which provides sufficient oxygen for bacteria to remove organic and inorganic pollutants (Pittman et al., 2011). Karya et al. (2013) had successfully cultivated a bio-flocculent alga—activated sludge which could remove up to 100% of $NH_4^+$ (50 mg/L) in a semi-continuous reactor. According to experiments carried out by Wolfaardt et al. (1994), the degradative efficiency of bacteria increased by 37% in the presence of microalgae. They hypothesized that algal products enhanced the performance of bacteria. Table 2 presents a summary of the experimental results related to ammonia removal and oxygen production.

#### 2.2.2.2 Microalgae-dominant systems

In this type of system, microalgae play the key role in nutrient removal and are essentially required to produce adequate biomass for sufficient uptake. For this reason, strategies for improving microalgae density have been investigated. *Azospirillum brasilense* is known as plant-growth-promoting bacteria as it produces several phytohormones in vitro (Gonzalez & Bashan, 2000).

According to research (Mouget et al., 1995) , *Pseudomonas diminuta* and *Pseudomonas vesicularis* promoted the growth of green microalgae *Chlorella sp*. and *Scenedesmus bicellularis* without releasing any growth-promoting substance. Researchers assumed that stimulation happened because of photosynthetic oxygen tension reduction (air suction) by bacteria

**Table 2-2 Experiments on microalgae-bacteria consortia**

| Medium source | Consortia content | Experiment settings | DO mg/L | References |
|---|---|---|---|---|
| Artificial medium containing acetonitrile | *C. sorokiniana, C. vulgaris, Sc. Obliquus, Se. capricornutum* (separately tested)/ bacteria mixed culture | Continuous; HRT: 3.5d; Continuous lighting, LI= 18500 lux | 1.66 mg/L | (Raul Muñoz M. J., 2005) |
| Artificial wastewater | *Scenedesmus quadricauda*/ nitrifier enriched activated sludge | Semi-continuous; HRT:1d/2d; SRT:30d/15d; Continuous illumination, LI= 2300 lux | 12mg/L after 4 hours running. (0.46 kg/m3/day) | (Karya et al., 2013) |
| Artificial wastewater | Mixture of *Scenedesmus quadricauda, Anabaena variabilis, Chlorella sp., Chlorococcus sp., Spirulina sp.*/activated sludge | Continuous; HRT: 1d; SRT: 15d; | 6.5± 2.1 mg/l (0.156kg/m3/day) | (Vandaele. S., 2000) |
| Pre-treated swine manure | *Chlorella sorokiniana*/ mixture bacteria | Continuous; HRT: 10d; continuous illumination LI: 10,000 lux | 10mg/L | (C. González, 2008) |
| Pre-treated municipal wastewater | Wastewater born microalgae /activated sludge | Batch test; HRT: 10d; L/D=12/12; LI: 7000 lux | Stay below 0.33 mg/l at the first week, gradually increased to 5 mg/l | (Yanyan Su, 2012) |

[a] DO: dissolved oxygen
[b] d: days
[c] LI: lighting intensity
[d] HRT: hydraulic retention time
[e] SRT: solids retention time

### 2.3 Efficiency of solo algal systems and consortia systems

Nitrogen is essential for building up the algal cells' components, such as genetic material, enzymes, proteins, hormone, vitamins, alkaloids, amides, and energy transfer molecules. Nitrogen is the

second most abundant element making up 6–10% of dry weight of green algae *Chlorella*. Nitrogen content ranges from 1-10 % of the cell dry weight (Grobbelaar, 2013). Carbon is the predominant element of green algae *Chlorella* and accounts for approximate 50% of cell dry weight (Cho et al., 2011). Most species of microalgae can utilize both organic and inorganic nitrogen. For inorganic nitrogen, eukaryotic microalgae can only assimilate nitrite, nitrate, and ammonium/ammonia. Cyanobacteria (also called blue-green algae) are prokaryote that can convert atmospheric nitrogen to ammonia by fixation (Cai et al., 2013). To assimilate the inorganic nitrogen, nitrate and nitrite must be reduced to ammonium by nitrate reductase (NR) and nitrite reductase (NiR), respectively. Microalgae assimilate ammonium to glutamine and release the hydrogen ion. Since the assimilation of ammonium does not require the redox reaction, it consumes less energy than the assimilation of nitrite and nitrate. Besides, in most microalgae species, NR activity is fully repressed in the cell when sufficient ammonium is supplied. Therefore, ammonium is ones of the most preferred inorganic source of nitrogen. (Cai et al., 2013; Zhou, 2014).

In activated sludge, removing nitrogen requires two groups of microorganisms namely nitrifiers and denitrifiers. Nitrifiers are autotrophic bacteria, which do not need an organic carbon source but consume large amounts of oxygen. Inorganic nitrogen sources are electron donors in the nitrification process. Nitrification process has two steps: oxidation of ammonia to nitrite by AOB and oxidation of nitrite to nitrate by NOB. Through these two steps, both AOB and NOB obtain energy for assimilation. In the denitrification process, nitrate or nitrite are reduced by denitrifiers to accept electrons and provide energy for the assimilation of organic matter. The reduction involves four steps (eq. 2-1):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad \text{(eq. 2-1)}$$

Few studies have evaluated different parameters to increase the efficiency of solo microalgae and consortia systems for nitrogen removal. Table 2-3 lists the efficiency results of experiments carried on solo microalgae and consortia (algae-assistant and microalgae-dominant) systems, respectively.

**Table 2-3 Efficiency of algal treatments on nitrogen removal in different wastewater streams**

| | Medium source | Algal species | Initial nitrogen (mg/L) | Removal efficiency % | Reference |
|---|---|---|---|---|---|
| **Microalgae solo system** | Secondary effluent | *Chlorella vulgaris* | N-$NH_4^+$: 8.05±0.16; 18.31 ± 0.53 | 100 % within 2 days. | (Kim et al., 2013) |
| | Piggery wastewater | *Chlorella zofingiensis* | TN: 63.96-82.7 | 65 %- 80 % within 4 days. | (Zhu et al., 2013) |
| | Source-separated urine | *Chlorella sorokiniana* | TN: 4300-7100 | 20- 30 % per day | (Tuantet et al., 2014) |
| | Centrate | Microalgae consortium | TN: 220 | 65% per day | (Halfhide et al., 2015) |
| | Secondary effluent | *Phormidium sp., C. reinhardtii, C. vulgaris and S. rubescens* separately. | TKN: 26.4 ± 0.7 | 100% within 4 days for *Phormidium sp.*, & *C. reinhardtii;* within 6 days for the other two | (Su et al., 2012) |
| **Consortia (microalgae assistant)** | Artificial wastewater | *Scenedesmus quadricauda*/ nitrifier enriched activated sludge | N- $NH_4^+$: 50 | 100 % per day nitrification | (Karya et al., 2013) |
| | Artificial wastewater | Mixture of *S. quadricauda, A. variabilis, Chlorella sp., Chlorococcus sp., Spirulina sp.*/activated sludge | N- $NH_4^+$: 66 | 85 % per day | (Vandaele, 2000) |
| | Pre-treated swine manure | Chlorella sorokiniana/ mixture bacteria | N- $NH_4^+$: 250-120 | 99 % per day | (C. González, 2008) |
| | Pre-treated municipal wastewater | Wastewater born microalgae /activated sludge | N- $NH_4^+$: 39.4 ± 5.5 | 91.0 ± 7.0 % in 10 days | (Yanyan Su, 2012) |
| | Raw leachate | mixed culture of bacteria and algae | N- $NH_4^+$: 80 | 11% per day | (Sniffen et al., 2016) |
| **Consortia (microalgae dominant)** | Modified OECD medium | *Chlorella vulgaris and Bacillus licheniformis* | N- $NH_4^+$: 20 | 78% within 6 days | (Liang et al., 2013) |

[a] TKN: total Kieldahl nitrogen
[b] d: days
[c] TN: total nitrogen

## 2.4 Factors affecting the microalgal system

Both microalgae and bacteria are sensitive to their living environment. Temperature, pH, light intensity and pattern, nutrient concentrations, dissolved oxygen, and some other factors can influence their activity and performance in different ways. Therefore, it is necessary to take them into consideration and optimize the treatment environment. For microalgae solo systems, lighting intensity, pH value, biomass density, and different types of species should be considered. Since microalgae are providing oxygen and organic substance for bacteria, factors that affect microalgae performance are similar for consortia systems.

### 2.4.1 Light supply

Light is important for activity and growth of photosynthetic organisms. Microalgae can capture light very efficiently using chlorophyll. Microalgae use their pigments to capture light to produce oxygen and convert light energy into chemical energy (i.e. Adenosine triphosphate- ATP) and biochemical reductant, which reduces inorganic carbon to form organic molecules (Masojıdek, Koblızek, & Torzillo, 2004). Photosynthetic processes happening inside algal cells provide energy to build their cell structures and to reproduce. The production of more algal biomass results in more oxygen being produced. Adequate dissolved oxygen is required for efficient ammonia nitrogen removal. In another way, if algal density is too high, photons cannot penetrate deeply into the culture broth, causing inefficient light utilization (Park et al., 2001). To avoid such problems, photo-bioreactor should be properly designed with an ample surface to volume ratio for light capture, appropriate mixing for homogeneity to support sufficient mass and photon transfer, and adequate biomass density to prevent self-shading(Muñoz et al., 2006).

#### 2.4.1.1 Light wavelength and intensity

Pigments in microalgae cells including chlorophylls, carotenoids, and phycobilins are absorbed in a narrow spectrum range (Raaman, 2006). The pigments existing in microalgae are different depending on the species. Green microalgae have chlorophylls a and b, and most of them do not have accessory light-harvesting pigments, which could help to extend the absorption wavelength. Red microalgae contain chlorophyll a and phycobilisomes. Most cyanobacteria, also known as blue-green algae, possess chlorophyll a, phycocyanin, and phycoerythrin as light-harvesting molecules (Masojıdek et al., 2004). Studies reported that algal biomass production and nutrient removal are influenced by wavelength and light intensity (LI). Kim et al. (2013) reported a 50% higher biomass production of microalgae *Scenedesmus sp*. using red and blue lights than the culture cultivated under white light. In addition, the same study also showed better nutrient removal by mixed lights with specific ratios than white light. Another study conducted with different monochromatic lights and white light on *Chlorella vulgaris* presented a higher growth rate with blue light as compared to the wavelengths of white, red, and green light (Blair et al., 2014). Ho et al. (2012) reported that LI was related to algal growth, carbohydrate/lipid production, and $CO_2$ fixation efficiency. They tested LIs ranging from 140 μmol/$m^2$/s to 540 μmol/$m^2$/s, which exhibited that biomass productivity and $CO_2$ fixation rate increased with the increase in LI until the peaks reached an LI value of 420 μmol/$m^2$/s. Light inhibition happened after that stage, resulting in the decrease in both $CO_2$ fixation and biomass productivity. Liao et al. (2014) developed a novel tubular photo-bioreactor forming periodic light and dark regions. This study stated that algal specific biomass production increased with increasing LI values ranging from 120 to 240 μmol/$m^2$/s; however, a decrease in production was reported for LI values from 240 to 300 μmol/$m^2$/s at 100 Hz frequency of light/dark cycle.

Light wavelength and intensity also influence the performance of nitrifiers. Both ammonium and nitrite oxidizing activities can be inhibited by strong light in the ocean ecosystem. In these systems, the most efficient nitrification happens at the bottom of the euphotic zone where the light intensity is only 5-10% of that on the surface (Ward, 2011). Photoinhibition of nitrifiers is species dependent. According to the active zone, AOB work in more shallow area than the NOB. Therefore, it was proposed that NOBs are more sensitive to photoinhibition than the ammonia oxidizers (Olson, 1981). Guerrero et al (1996) studied light inhibition on marine AOB and NOB. The study revealed that photoinhibition is species-specific and dependent on dosage (light intensity and lighting period) and wavelength. The results showed that AOB were more sensitive to blue light than NOB. Cool-white fluorescent light inhibited AOB activity but did not influence NOB. Light inhibition on AOB and NOB were also reported for soil nitrifying bacteria (Guerrero et al., 1996) . According to Ward (2011), the possible reason for photoinhibition of nitrifiers is that cytochromes of both AOB and NOB that are involved in the energy transduction pathways of nitrification can be damaged by the light (Ward, 2011).

#### 2.4.1.2 Lighting period

In addition to wavelength and LI, the frequency of light also influences algal biomass production and nitrogen removal. It has been proved that dark periods between short flashes of light can increase photosynthesis efficiency, especially under high LI (Liao et al., 2014; Park et al., 2001). Liao et al. (2014) developed a novel tubular photo-bioreactor forming periodic light and dark regions. The average biomass productivity increased by 21.6± 2.1% at the artificial light/dark cycle frequency of 100 Hz. After photoinhibition, dark phase proved effective for recovering nitrifiers' activity (Guerrero et al., 1997; Yoshioka et al., 1984). A study carried out by Yoshioka et al. (1984) showed that the light activity damaged AOB and NOB; however, it was recovered in dark

condition in 10 days, which means that dark phase was necessary for proper functioning of nitrifiers.

### 2.4.2 pH of algal and bacterial growth media

The pH plays an important role in many cellular processes, which include energy metabolism, structure and function of organelles, enzymes, and proteins. For most algae, the pH range of culturing media is from 7 to 9. Extreme pH may cause the disruption of many cellular processes which could lead to culture collapse (Cautteau, 1996). Some components are required in algal cultivation media to maintain a stable pH by avoiding metal ion precipitation, retarding contaminants' growth (microbial inhibitor), or serving other functions (Brand et al., 2013). The pH tolerance ranges vary among different algal species. Some species could only live in a narrow range; however, others could thrive in a wide range(Moss, 1973). *Chlorella ellipsoidea* grows in a wide pH range between 4 and 10. The best growth performance of *Chlorella vulgaris* was observed at pH 10 (Gong et al., 2014). The pH also impacts nutrient uptake. Zhou et al. (2015) studied the optimal pH ranges for *Chlorella vulgaris* on nutrient removal and found the optimal pH range for ammonia and nitrogen removal was 7-8. Different results were established by Liang et al (2013). In a co-cultured system of *Bacillus licheniformis* and *Chlorella vulgaris*, it was shown that the optimal pH for $N\text{-}NH_4^+$ removal was 7, while for phosphorus removal pH did not have significant impact (Liang et al., 2013; Zhou et al., 2015). However, the concentration of nutrients could be affected by pH. A high pH value could result in an increase in free ammonia (FA) concentration and phosphorus precipitation in the form of calcium phosphate (Cai et al., 2013). Meanwhile, excessive FA concentration affects algal photosynthesis, depressing their growth (Abeliovich et al., 1976). On the other hand, the activities of algal cells could also induce pH variation, such as $CO_2$ consumption and $N\text{-}NH_4^+$ uptake. It is known that dissolved $CO_2$

consumption by photosynthetic process increases $OH^-$ concentration and $N\text{-}NH_4^+$ uptake by microalgae while releasing $H^+$ (Knud-Hansen et al., 1998). Except for algae, nitrification by nitrifying bacteria also contributes to pH decrease. Low pH inhibits both groups of AOB and AOA, but their activity can be restored by pH adjustment (Ward, 2011).

### 2.4.3 Microalgal and bacterial species

Selection of microalgae and bacteria species depends on the role they play in the system and their performance in different kinds of wastewaters.

In solo algal systems, *Chlorella sp*., is one of the most common species used in wastewater treatments. Alcantara et al. (2013) confirmed the presence of nitrate reductase (an enzyme involved in the bacterial denitrification pathway) in axenic cultures of *Chlorella vulgaris*. Moreover, *Scenedesmus sp.* is also widely used in studies. They are equipped with flotation spines making their colonies buoyant, which contribute to the efficient uptake of light and nutrients. Both of the species have similar performances on nutrient removal(Cai et al., 2013). They can also grow under autotrophic (without organic substrate) and heterotrophic conditions (with the organic substrate and without light), but these species were inhibited when they grew in the mixotrophic mode (with the organic substrate under light/dark cycle) (Combres, Laliberte, Reyssac, & Noue, 1994; Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011). Su et al. (2012) studied four species on the effectiveness of nutrient removal. The species were three green microalgae and one cyanobacterium. In this study, ammonia concentration was 25 mg/L and it was completely removed in 6 days by all four species. Among these species, *Phormidium sp.*, a cyanobacterium, and *Chlamydomonas reinhardtii* removed all ammonia on the fourth day. On the fourth day, a peak of $NO_3^-$, which was less than 6 mg/L was observed in the *Phormidium sp. tr*eatment. It took another 3 days for *Phormidium sp.* to remove the nitrate.

In marine ecosystems, except providing oxygen, microalgae can also provide organic substrates for bacteria. In this mutual relationship, microalgae can provide proteins, organic carbon, and some other carbohydrates for bacteria. In exchange, bacteria supply inorganic nutrients and other metabolic compounds including vitamins, hormones, and EPS (extracellular polymeric substances), which contributes to bio-flocculation. However, this mechanism is not universal among all microalgae and bacteria species. It is species-specific as the microenvironment of each alga is different (Ramanan, Kim, Cho, Oh, & Kim, 2016). In studies about wastewater treatment, shown in Table 2, green microalgae species are prevalent in experiments and bacteria are commonly collected from activated sludge or wastewater (Karya et al., 2013; Ruiz-Marin et al., 2010; Su et al., 2012a) .

In algal dominant systems, some bacteria (such as *Pseudomonas*) were found to be effective at promoting algal growth. The appropriate selection of strains of plant growth promoting bacteria (PGPB) is important. As mentioned before, *Pseudomonas diminuta* and *Pseudomonas vesicularis* effectively enhanced the algal production while Dakhama et al. (1993) found that *Pseudomonas aeruginosa* depressed the growth rate of various green microalgae and cyanobacteria by releasing anti-algae substance. According to Bashan et al. (2000) (Gonzalez-Bashan, Lebsky, Hernandez, Bustillos, & Bashan, 2000), when co-immobilized with nitrogen-fixing bacteria *Phyllobacterium myrsinacearum*, pigment production in algal cells increased significantly while nutrient removal by microalgae was reduced. *Azospirillum brasilense* is the most studied PGPB, which is effective for numerous crops (De-Bashan, Hernandez, Morey, & Bashan, 2004) including *Chlorella vulgaris* (De-Bashan, Antoun, & Bashan, 2008). Studies showed that the addition of phytohormones IAA produced by *A. brasilense* increased the algal population. Co-culturing with *A. brasilense*, *Chlorella vulgaris* can grow well under unfavorable aquatic conditions (high pH

and presence of toxic substances). Moreover, when co-cultivated with *Bacillus*, the nutrient removal efficiency of *Chlorella vulgaris* also improved significantly (Liang et al., 2013)

### 2.4.4 Dissolved oxygen in aquatic media

Since dissolved oxygen is required by AOB, AOA, and NOB, they can only be active in aerobic environments (Ward, 2013). To oxidize each gram of ammonia, nitrifying bacteria group needs 4.7 gram of oxygen. However, denitrification happens under anoxic conditions, which means the dissolved oxygen concentration should be less than 0.5 mg/L. The DO level is commonly considered as a key factor for determination of nitrification and denitrification rate. The nitrification process stops when DO value drops below 0.2 mg/L. Other studies stated that complete simultaneous nitrification and denitrification occurred at the DO value of 0.3 mg/L. Below 0.3 mg/L, denitrification will prevail over nitrification(Li et al., 2008; Pochana et al., 1999). Oxygen saturation coefficients of Monod kinetics is 0.3 mg/ L for nitrititation and 1.1 mg/ L for nitratation (Wang et al., 2004). According to the coefficients, nitrite accumulation can occur when the DO value is maintained below 1.1 mg/L, meaning that NOB could become a rate-limiting factor for nitrogen removal. A fully aerobic membrane bioreactor was operated to treat black water under three low dissolved oxygen (DO) levels below 0.5 mg/L. In this study, the optimal DO range for denitrification was 0.15–0.35 mg/L; however, ammonia could only be reduced to 40 mg N/L (Hocaoglu et al., 2011) .

### 2.4.5 Organic matter

Denitrifiers are a group of heterotrophic bacteria, which use nitrite or nitrate as the electron acceptor in the respiration process and obtain energy from organic substances. In WWTPs, dosing organic matter is necessary to provide sufficient electron donors for nitrate removal. In general, to

reduce each gram of nitrate, 4 grams of biochemical oxygen demand (BOD) is required. The amount of BOD can change due to the varying carbon source and operational conditions (Metcalf, 2003). For example, with the same amount of chemical oxygen demand (COD), denitrifiers consume more nitrate by using acetate than methanol; The amount of nitrate removed by denitrifers can vary even using the same source of COD due to the different operational processes or conditions (Ahn, 2006). Meng et al. (2008) explored effects of different COD/N ratio on nitrogen removal. The results indicated that high COD/N ratio of 15 limited nitrification performance whereas low COD/N ratio of 5 was preferred by AOB; however, it limited the denitrification process

Some microalgae can grow heterotrophically without light when organic substrates are available. In this way, microalgae grow without light and uptake organic carbon to obtain energy. Three growth ways, heterotrophic, autotrophic, and mixotrophic were tested on *Chlorella vulgaris* by Perez-Garcia et al. (2010). In this study, microalgae performance on biomass production and nutrient removal was superior under heterotrophic conditions than the other two. Zhu et al. (2013) performed 10-day batch tests with different COD concentrations in piggery wastewater under continuous illumination. The results showed that microalgae *C. zofingiensis* had better performance on total nitrogen (TN) removal in higher COD-concentrated wastewater. They removed 81.03% TN with an initial concentration of 148 mg/L in the wastewater of 3500 mg/L COD and removed 68.96% TN with an initial concentration of 17 mg/L TN in the wastewater of 400 mg/L COD.

## 2.5 Harvesting of biomass

Both solo microalgae system and microalgae-based consortia systems showed potential in treating wastewater, however, the cost of biomass harvesting might be a barrier for practical applications (Karya et al., 2013).

In solo algal systems, harvesting efficiency depends on aspects such as algal species, cell density, and culture conditions. Microalgae are typically small sized (1–30 μm), have a low concentration (0.3–5 g/l), are negatively charged, and have a specific density that is close to that of their culture medium. These characteristics make them dispersedly suspended in media (Molina Grima et al., 2003). To capture all of the algae, the harvesting process could be very complex, involving one or more steps through several physical, chemical, or biological processes. The available industrial methods for harvesting include filtration, centrifugation, gravity sedimentation, and micro-straining. However, none of them has been proven to be cost-effective and suitable for large-scale microalgae removal (Muñoz et al., 2006). For large-scale microalgae removal, membrane filtration is costly because of extensive maintenance and the high-energy requirement for pumping. Conventional filtration processes are not reliable for small-size microalgae. Centrifugation method requires intensive energy (Muylaert et al., 2015) and the efficiency of the process depends on the microalgal species and centrifugation speed (Molina Grima et al., 2003). Gravity settling is suitable only to harvest large-sized microalgae cells but the process can be enhanced if it is followed by flocculation.

Coagulation and flocculation harvest the biomass by adding chemical coagulants and flocculants or raising pH over 10. Although these processes are effective at harvesting; however, they are costly and can increase the effluent's salinity(Muñoz et al., 2006). The flocculant type and microalgae species are the factors that affect the harvesting efficiency. Abomohra et al. (2014)

studied four different flocculants, NaOH, $MgSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, and NaCl, with different dosing concentrations, 0, 50, 150, 250 mg/L, on green microalgae *Scenedesmus obliquus*. The results showed that NaOH with a dosing concentration of 250 mg/L performed the best as it removed 85% microalgae cells within 2 hours. Other flocculants including $MgSO_4 \cdot 7H_2O$ and NaCl did not show any flocculent activity(Abomohra et al., 2014). Beach et al. (2012) conducted research on other flocculants such as chitosan biopolymer, ferric sulfate, and alum at the concentrations of 50, 75, 100, 125, 150 mg/L. In this research, chitosan was found to be the optimal flocculant for green microalga *N. oleoabundans* at the concentration of 100 mg/L(Beach et al., 2012). Chitosan is polymer flocculant and it is regarded as edible and nontoxic. Polymer flocculants are attractive because they are easily manufactured and typically have good performance with less dosage producing large and stable flocs. However, the salinity can affect the harvest efficiency(Molina Grima et al., 2003) . In addition, for different species, microalgae require different chitosan dosages. Polymer flocculants are often less effective for harvesting marine microalgae (Muylaert et al., 2015). For *Tetraselmis chui, Thalassiosira pseudonana* and *Isochrysis sp.*, the optimal dosage was 40 mg/L; *Chaetoceros muellari*; however, required 150 mg/L to get the desired flocculation (Heasman et al., 2000). Chen et al. (2012) used aqueous ammonia as the flocculant and tested it on marine microalgae and freshwater microalgae. The results showed that marine microalgae were eliminated easier than freshwater algae. To reach the pH above 10, marine microalgae species takes less amount of aqueous ammonia. The initiating flocculation was achieved by dosing $Ca(OH)_2$ at the pH value of 7.97 for wild type *Chlamydomonas reinhardtii* and pH value of 10 for *Nannochloris*. Except the aforementioned flocculants, filamentous fungi (Xie et al., 2013; Zhang et al., 2012) were also studied at lab scale. Xie et al. (2013) co-cultured fungi *C. echinulate* with green microalgae *Chlorella vulgaris* and

formed the pellets with diameters ranging from 2 to 10 mm, which enabled the complete removal of microalgae by simple filtration. Without dosing flocculants, auto-flocculation can happen when pH rises due to the photosynthetic carbon dioxide depletion (Muylaert et al., 2015).

For consortia systems, the methods used for harvesting are same as microalgae solo systems. To obtain a simple sedimentation by gravity, bioflocculent algae-bacteria biomass was developed (Karya et al., 2013; Su et al., 2011; Gutzeit et al., 2005). The formation of bioflocculent biomass is commonly achieved by continuously mixing and illumination followed by 1 h sedimentation. After settling, the supernatant is discarded and the algae-bacteria biomass could be obtained after the settling period of 1 month. The formation process is expected to be influenced by cell surface properties of the algae, EPS (extracellular polymeric substances), and other factors (e.g. amount of calcium) (Gutzeit et al., 2005); however, the mechanism is still unknown.

Using an immobilization system is another possible solution for harvesting. Immobilization in a polymeric material such as carrageenan, chitosan, or alginate has been reported by various researchers (Eroglu et al., 2012; Eroglu et al., 2015; Gonzalez et al., 2000). Advantages of immobilization include protecting microalgae and/or bacteria that are resistant to harsh environments, recycling the biomass in an economical way, and reducing the damage to cells before recovering. However, there are some disadvantages, such as limited oxygen and nutrient transfer, weak and costly matrices, and difficulties for implementation of large-scale applications ((Eroglu et al., 2015; Muñoz et al., 2005).

A reasonable harvest process is supposed to be cost effective and can be applied to large volumes for treatment. It is complicated to develop a universal method for microalgae or algae-bacteria harvest due to the various affecting factors, like microalgae species, salinity, etc. This area is

actively researched, and it targets different biomass types to develop a specific and economical harvesting system that is important for practical applications.

**2.6 Perspective**

In conventional nitrification process in WWTPs, providing dissolved oxygen for nitrifiers typically required high energy and state-of-the-art equipment to create very fine bubbles. As a tiny photosynthetic cell, microalgae are combined together with bacteria to serve as an aeration device. It gives us a promising way to save energy and develop a sustainable treatment process. According to Tables 2-1 and 2-2, microalgae-bacteria consortia systems have been mostly studied on artificial wastewater and real wastewater with low ammonia concentrations. However, studies on wastewater with high ammonia concentrations, such as reject water, are rare. To treat reject water, high rate of oxygen production is necessary for the removal of high concentration of ammonia. Among the studies that have been reported, the best result was obtained using the bio-flocculent biomass that produced 0.48 $kgO_2/m^3/d$. Illumination is essential for microalgae photosynthetic activity. In the studies listed in the tables, a typical photoperiod for consortia systems was 24 hours a day and LI values were strong (above 4000 lux). However, continuous illumination allows microalgae to continuously produce oxygen, which can inhibit the denitrification process. Thus, further research on finding optimal conditions for simultaneous removal of ammonia and nitrate nitrogen is necessary.

# Chapter 3 MATERIAL AND METHODS

## 3.1 Growth medium and microorganisms

Bacteria samples were acquired from the activated sludge, which was obtained from the West End Water Pollution Control Centre in Winnipeg. Algae were a mixture of cyanobacteria and green algae taken from Lake Devonian, Manitoba. The 1-L enriched algae consortium and 0.25-L return activated sludge (RAS) was added together to 5-L of modified Bold's Basal Medium (BBM) in a continuously mixing tank. The tank was set in an incubator (at 25°C) and illuminated by Light-emitting diode (LED) strips with the light intensity of 4000 Lux. To see the effect of high ammonia content, 800 mgN/L of ammonium chloride was used as nitrogen source for consortium cultivation in the preliminary batch tests. Ammonium nitrogen concentration was reduced one month before running the PSBR to 200 mgN/L to compensate for the initial $NH_4^+$-N loading (150 mgN/L). The other components used in the wastewater treatment are: $CaCl_2$ (25 mg/L), NaCl (25 mg/L), $MgSO_4$ (75 mg/L), $KH_2PO_4$ (105 mg/L), $K_2HPO_4$ (75 mg/L), EDTA - $Na_4$ (50 mg/L), KOH (31 mg/L), $FeSO_4.7H_2O$ (5 mg/L), $H_2SO_4$ (18 mg/L), and $H_3BO_3$ (11 mg/L). Furthermore, 0.1 mL of each of the trace metal solutions were added to the 1000 mL of the following solutions: $ZnSO_4.7H_2O$ (14.1 g/L), $MnCl_2.4H_2O$ (2.3 g/L), $CuSO_4.5H_2O$ (2.5 g/L), $Co(NO_3)_2.6H_2O$ (0.8 g/L), and $Na_2MoO_4.2H_2O$ (1.9 g/L) (Reading, 2007). No additional organic carbon was loaded into the reactors. The pH was maintained at 7.5 by adding sodium bicarbonate. The cultivation reactor was aerated only during the first month, and the medium was renewed by replacing half of the medium containing biomass every two weeks. The consortium data was analyzed when significant ammonium nitrogen removal (60–100 mgN/L/days) was observed.

## 3.2 Experiment set-up and operation

### 3.2.1 Batch tests

Prior to inoculation, 200 ml of consortium culture was centrifuged at 3600 rpm for 10 min, washed twice with deionized (DI) water, and re-suspended in the 400 ml synthetic wastewater. LED strips were placed behind the reactors and controlled by an LED dimmer (Armacost, Baltimore, MD). Light intensities were measured by a light meter (MeterShack, Randburg, SA). The working volume of each Erlenmeyer flask was 400 mL. All flasks were continuously mixed via magnetic stirrers (Fisher Scientific, Hampton, NH). Except the batch test evaluating the effect of wavelength, the light cycle (light: dark) of other batches was kept at 16h:8h L/D. The tests were carried at room temperature. It should be noted that no sterilization step was performed for the whole experiment since these batch tests were short term assays with 3 replications.

### 3.2.2 Photo sequence batch reactor

Prior to inoculation, consortium culture was centrifuged at 3600 rpm for 10 mins, washed twice with DI water, and re-suspended in artificial wastewater. Ammonium nitrogen, with a concentration of 140 ± 30 mg/L, was the only nitrogen source in artificial wastewater. Same light intensity of 1000 ± 200 Lux was used for all stages of the experiment. LED strips were placed behind the reactors and controlled by an LED dimmer (Armacost, Baltimore, MD). Light intensities were measured by a light meter (MeterShack, Randburg, SA). The working volume of the reactors was 2 L. Reactors were mixed using magnetic stirrers (Fisher Scientific, Hampton, NH) at 220 rpm. The pH in the reactors was maintained at 7.5 by pH controller (Milwaukee Instruments Inc., MC122, Melrose, MA). Light cycle of 16h:8h L/D and 24 hours continuous illumination were separately tested at different stages. HRT was maintained at two days. SRT was not controlled but monitored. The experiments were carried out in a PSBR with 20 cm diameter at room temperature for 280 days. The reactor setup and PSBR cycles schedule are indicated in Fig. 3-1.

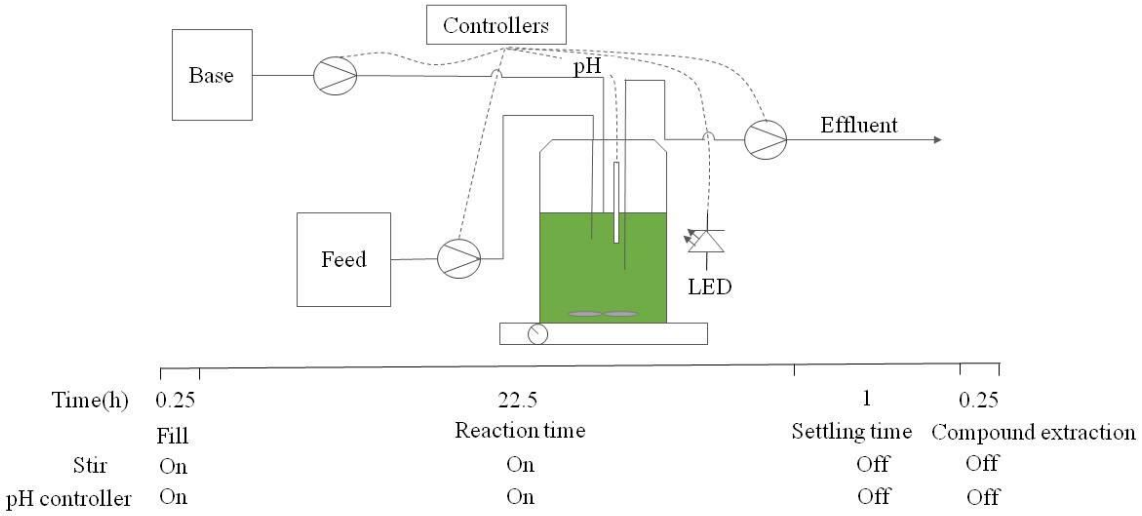



**Figure 3-1 PSBR setup and cycle schedule**

## 3.3. Sampling and analytical methods

### 3.3.1 Batch tests

Samples were taken daily from the evenly mixed flasks and filtered through a 0.45 µm hydrophilic filter paper. The parameters of nutrients such as ammonium, nitrite, nitrate, and ortho-phosphate were determined using the flow injection analyzer (Lachat Instruments, Quickchem 8500, Loveland, CO). Ammonium removal rate was calculated from the decrease in ammonium concentration with time observed during the batch tests. Prior to the absorbance measurement, samples were well mixed in three cycles on the vortex mixer (Fisher Scientific, Hampton, NH). Density of microorganisms was calculated by taking optical density (OD) measurements at 685 nm in three replicates using a spectrophotometer (Fisher Scientific, Ultrospec 2100pro UV/Visible, Hampton, NH). It should be noted that the OD results presented in this study are a proxy for the concentration of microorganisms; however, algae made up the majority of the biomass in the

consortium. Dissolved oxygen (DO) was determined by the DO meter (YSI Incorporated, ProODO, Yellow Springs, OH) and measured every day.

The data presented for each treatment are the means of three biological replicates. One-way analysis of variance (ANOVA) evaluated the significance of environmental factors including initial ammonium concentration, light intensity, dark/light cycle period, and light wavelength using the IBM SPSS Statistics software (SPSS 22.0; IBM Inc.).

### 3.3.2 Photo sequence batch reactor

Samples were taken from the effluent regularly. For kinetic tests, samples were taken from the reactor content every 2 to 4 hours. To determine $NH_4^+$-N, $NO_2^-$-N, $NO_3^-$NA and TOC content, samples were filtered through 0.45 µm hydrophilic filter paper and stored at 4$^o$C. The aforementioned nitrogen species were analyzed using the flow injection analyzer (Lachat, Quickchem 8500, Loveland, CO). Total suspended solids (TSS) were analysed according to standard methods for the examination of water and wastewater (APHA, 1995). Biomass samples was collected and sequenced and analysed in China using 16SV4. Dissolved oxygen was determined by the DO meter (YSI Incorporated, ProODO, Yellow Springs, OH). Total organic carbon (TOC) was measured by TOC Analyzer (Skalar, Formacs Series, Breda, NB). Results were taken from the mean value of two reactors.

Biomass sample was taken after the stabilization of the performance and sequenced by 16S (V4) for bacteria community composition analysis. DNA extraction procedure was performed using E.Z.N.A™ Soil DNA Kit (Omega Bio-tek, Inc.). DNA sample sequenced by NovoGene Corporation (Beijing, China). Variable region 4 of bacterial 16S rRNA genes were amplified and sequenced on an Illumina Hiseq 2000 pyrosequencer (Illumina, Inc.), producing 250 bp paired-end (PE) reads.

# Chapter 4 AMMONIUM REMOVAL USING ALGAE–BACTERIA CONSORTIA:THE EFFECT OF AMMONIUM CONCENTRATION, ALGAE BIOMASS, AND LIGHT

## 4.1 Introduction

Microalgae can capture light very efficiently using chlorophyll and convert light energy into chemical energy (i.e. Adenosine triphosphate-ATP). This process is also known as photosynthesis. During this process, oxygen and reducing agents that convert inorganic carbon (i.e. $CO_2$) to organic molecules are also produced (Masojıdek, et al., 2004). The production of more algal biomass results in the generation of more oxygen which is utilized in the form of DO for efficient ammonium nitrogen removal.

Biological (activated sludge) treatment is commonly used for nitrogen removal in WWTPs. In this process, nitrifiers, including AOB and NOB, convert total ammonia (free ammonia and unionized ammonia) to nitrate. Denitrification occurs in an anoxic environment in which denitrifiers reduce nitrate and nitrite to nitrogen gas. To oxidize a gram of ammonia to nitrate, nitrifying bacteria need 4.7 g of oxygen (Metcalf and Eddy 2003). In WWTPs, mechanical aeration supplies a large amount of oxygen and costs 45–75% of total energy demand of the plant (Oilgae 2010), which translates into a significant energy consumption and operational cost. In algae-bacteria consortia systems, algae mainly supply DO for bacteria to remove nutrients (Karya et al. 2013). It is expected that nutrient removal efficiency increase with the algal density. According to the study by Lee et al. (2015), nitrogen removal efficiency was positively related to algae biomass. The concentration of nitrogen present in water was considered one of the essential factors that directly affect algal growth kinetics. A study conducted by Uggetti et al. (2014) also showed that in the ammonia concentration range from 50 to 190 mg $NH_4^+$-N/L, a higher concentration was positively

associated with the algal growth rate. Light is important for activity and growth of photosynthetic organisms. Algal biomass production and nutrient removal are influenced by wavelength and light intensity (LI). Kim et al. (2013) reported 50% higher biomass production of microalgae *Scenedesmus sp*. using red and blue lights than the culture cultivated under white light. In addition, the authors also showed better nitrogen removal by using mixed lights with specific ratios than white light. Another study conducted with different monochromatic lights and white light on *C. vulgaris* presented a higher growth rate with blue light as compared to the wavelengths of white, red, and green light (Blair et al. 2014). Ho et al. (2012) reported that LI was related to algae growth. The biomass productivity peaked at 420 μmol/$m^2$/s from the range of LIs tested between 140 and 540 μmol/$m^2$/s. Photoinhibition occurred after the peak value and resulted in a decrease in biomass productivity.

Light wavelength and intensity also influence the performance of nitrifiers. Both ammonium and nitrite oxidizing activities can be inhibited by strong light. Merbt et al. (2011) stated that, photoinhibition of AOB and AOA were happened under continuously illumination of light intensity of 500 μE/$m^2$/s. NOB are supposed to be more sensitive to light than ammonium oxidizer, since they are active in lower depth than are the AOB and AOA. In ocean systems, the most efficient nitrification transpires at the bottom of the euphotic zone where the light intensity is only 5–10% of that on the surface (Ward 2011). Guerrero and Jones (1996) studied light inhibition of marine AOB and NOB to reveal that photoinhibition is species-specific and dependent on dosage (light intensity and lighting period) and wavelength. The results also showed that AOB were more sensitive to blue light than NOB. Moreover, cool-white fluorescent light inhibited AOB activity but did not influence NOB. Light inhibition on AOB and NOB were also reported for soil nitrifying bacteria (Guerrero and Jones 1997). According to Ward (2011), the possible reason for

photoinhibition of nitrifiers is that cytochromes of both AOB and NOB that are involved in the energy transduction pathways of nitrification can be damaged by strong light.

In algae–bacteria consortia systems, algae mainly supply DO for bacteria to remove nutrients and actively uptake nutrients as ammonia and phosphorus which can save energy and cost requirements in the wastewater treatment process. In the process, many factors, i.e. ammonium concentration, algal biomass, and light, could affect the activity of algae and nitrifying bacteria. Therefore, the primary objective of this study is to explore the effect of the aforementioned factors on the performance of an algae–bacteria consortium on nitrogen removal from synthetic wastewater. Another objective is to optimize the nitrogen removal process via algae–bacteria consortia thereby making it more feasible for implementation in the WWTPs.

## 4.2 Results and discussion

### 4.2.1 Pattern of oxygen concentration

All the batch tests followed a similar trend of change in DO. Figure 4-1 shows a sample batch test with initial algae biomass of 0.4 OD, ammonium nitrogen concentration 450 mg N/L, and L/D cycle of 2:1 h. The DO decreased to 1 mg/L after the first day and remained under 1 mg/L from day 2 to 7 until complete ammonium removal was achieved. After this stage, the DO produced by photosynthesis was no longer consumed since the ammonium oxidization process was stopped. This resulted in the increase of the DO. Similar results were obtained by Karya et al. (2013). The decreasing trend at the end is potentially due to the exhaustion of alkalinity.

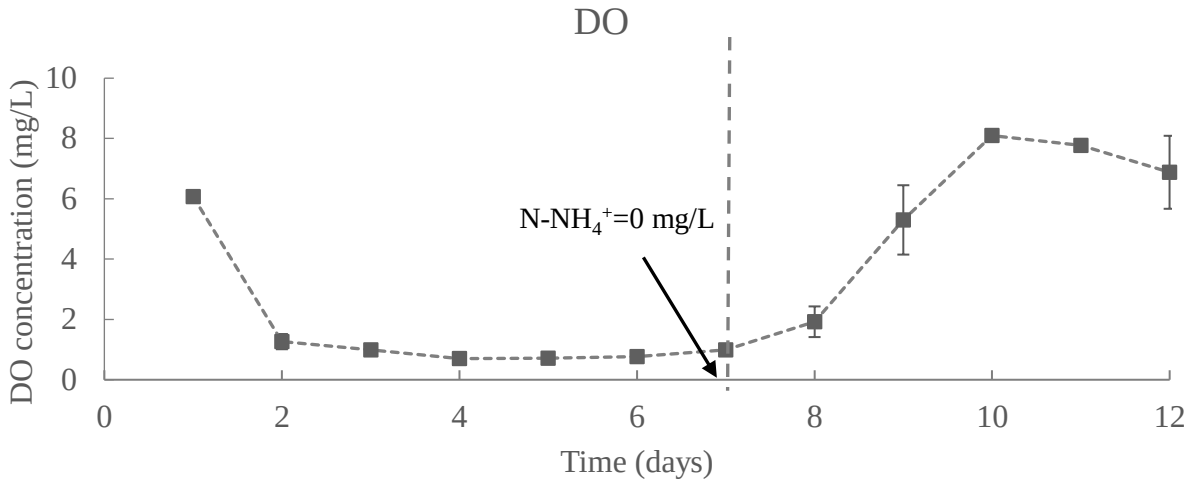



**Figure 4-1 Typical dissolved oxygen profile during the batch tests; error bars represent the standard error of the mean of three biogical replicates**

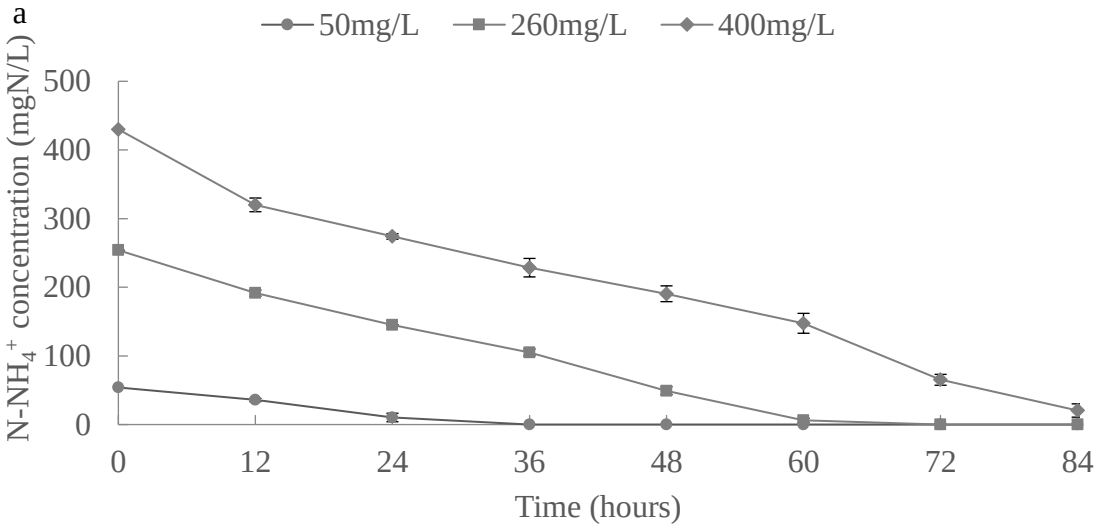
### 4.2.2 Effect of ammonium concentration and algae biomass density on ammonium removal

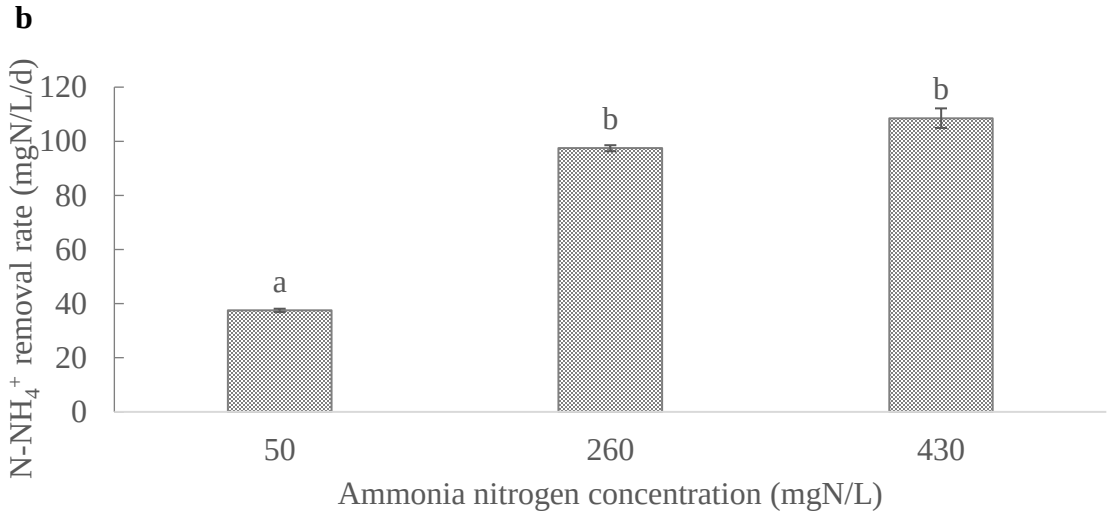
Initial ammonium concentrations of 50, 250 and 430 mg$NH_4^+$–N/L were evaluated for ammonium removal rate in a four-day batch test. After inoculation, all of the reactors were supplied 1000 Lux of nature white light.

The effect of ammonium nitrogen concentration on ammonium removal is shown in Fig 4-2a, b. In this batch-test, the influent with the concentration of 430 mg $NH_4^+$-N/L had the highest ammonium removal rate of 108 ± 3.6 mgN/L/day, which was two times faster than the influent with low ammonium concentration (40 mg N/L). This maybe due to the production of AOB (*Nitrosomas*), which was promoted by the high ammonium concentration (Chen et al. 2011). The ANOVA results also showed that initial ammonium concentration had a significant influence on the ammonium removal ($p = 0.001$). Similarly, Chen et al. (2011) reported a better sludge performance with the increasing ammonium concentration.

The optical density measurements at different initial concentrations (Fig. 4-2 c) showed a decrease

in biomass concentration over the course of the experiment. This is opposite to the findings of Uggetti et al. (2014) who reported that the increase in initial ammonium concentration enhanced biomass production. The low pH in the tests could be the reason for the decrease in biomass quantity. In the entire experiment, pH typically dropped to 5–6 after every pH adjustment. The slightly acidic condition may have affected the nitrification performance, as oxidation of each gram of ammonium nitrogen requires 7.15 g of alkalinity ($CaCO_3$) (Metcalf and Eddy 2003).



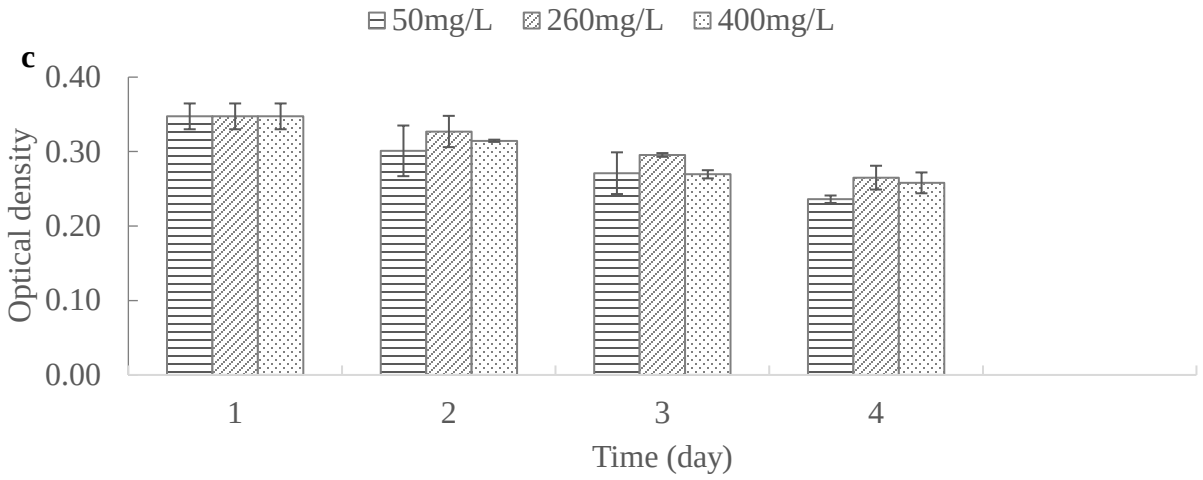


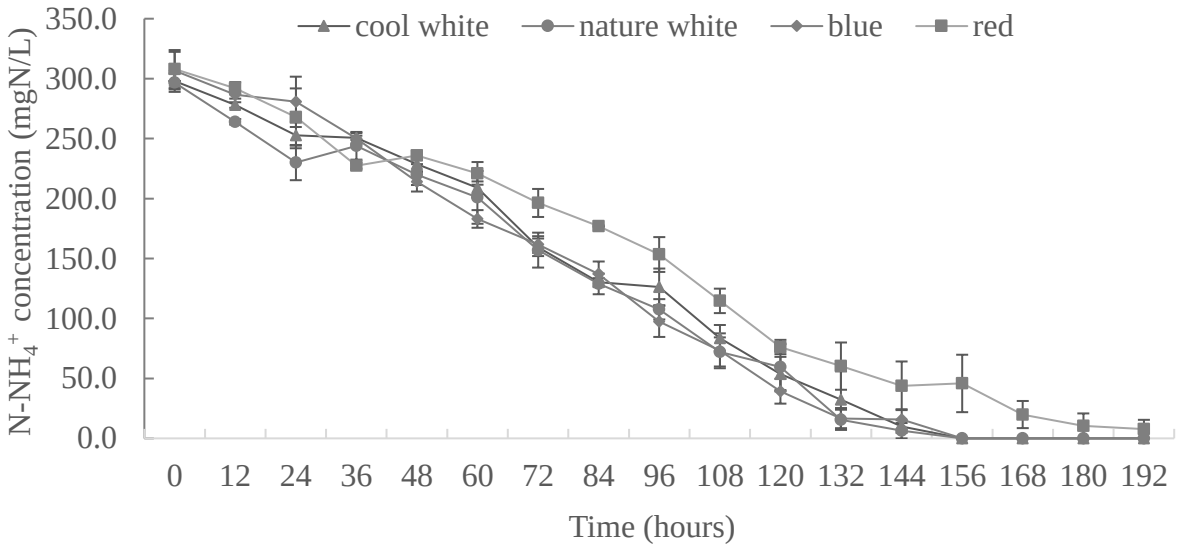

**Figure 4-2 Ammonium removal efficiency (a), ammonium removal rate (b), and optical density (c) for different initial ammonium concentrations over time; error bars represent the standard error of the mean of three biological replicates**

### 4.2.3 Effect of light wavelength on ammonium removal

To evaluate the effect of light wavelength on ammonium nitrogen removal efficiency, blue light (400–500 nm, with a peak at 450 nm), red light (600–700 nm, with a peak at 670 nm), cool white, and nature white light were used. The optical density of the initial algae–bacteria consortium was 0.640 ± 0.020 OD. Batch tests were performed for 8 days at 1000 Lux for each wavelength.

Previous studies have reported that algal biomass production and nutrient removal are influenced by the change in wavelength. Kim et al. (2013) reported a 50% higher biomass production of microalgae *Scenedesmus sp*. using red and blue lights than the culture cultivated under white light. The production of more algal biomass is expected to produce more oxygen. However, in the current study, light wavelength did not show a significant effect (ANOVA, $p > 0.05$) on algal biomass production (Fig.4-3). Complete ammonium removal was achieved after 6 days and the ammonium nitrogen removal rate was 50–53 mg N/L/day with no significant difference (ANOVA, $p>0.05$) between cool white, natural white, and blue light. The lowest rate (41 ± 3.71 mg $N\text{-}NH_4^+$/

L/day) was found under the exposure of red light source. According to literature (Guerrero and Jones 1996; Ward 2011), blue light and cool white light could inhibit AOB activity. However, in this batch test, no inhibition was observed. The reason could be the inability of the low LI (1000 Lux) to induce an inhibition. Secondly, the increased algae biomass prevented bacteria from excess light exposure.



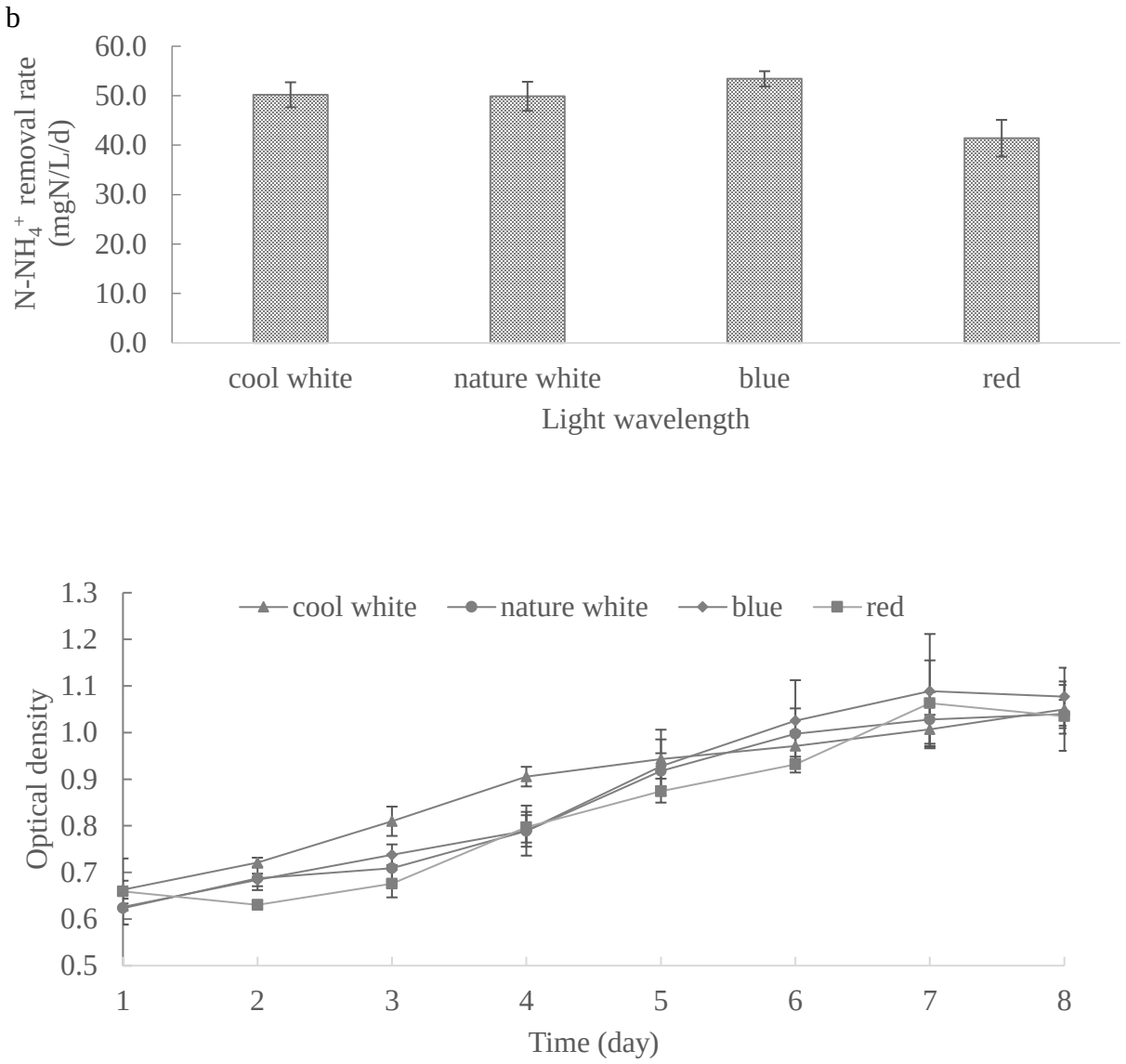


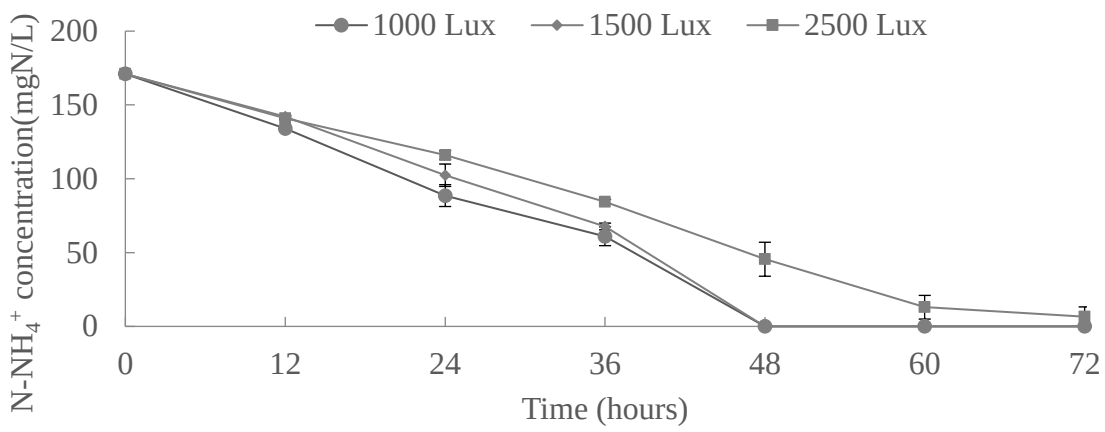

**Figure 4-3 Ammonium removal efficiency (a), ammonium removal rate (b), and optical density (c) for different light sources(wavelength); error bars represent the standard error of the mean of three biological replicates**

### 4.2.4 Effect of light intensity on ammonium removal

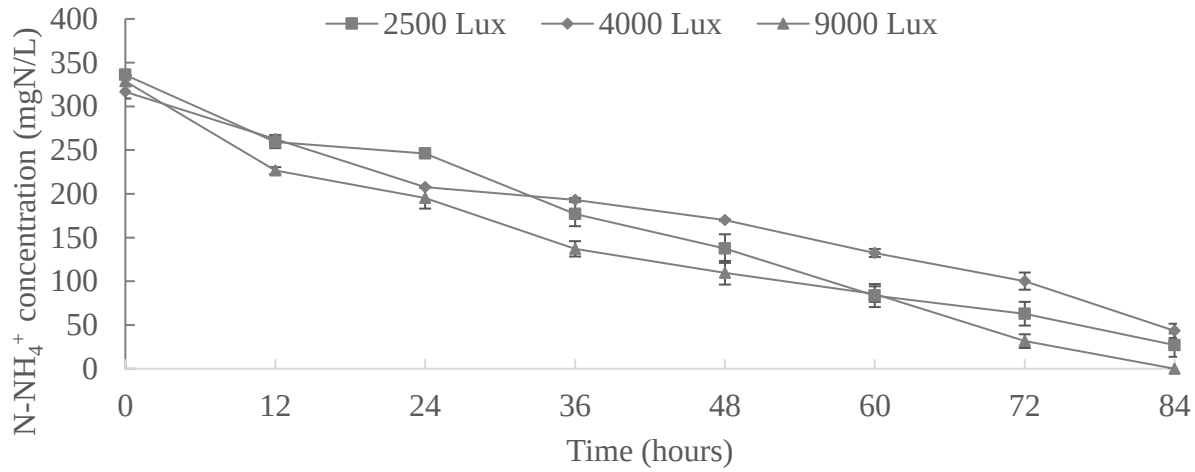
Ho et al. (2012) reported that LI was related to algal growth. They tested LIs ranging from 7560 to 30, 240 Lux, which exhibited that biomass productivity increased with the increase in LI until the peak value of 23,520 Lux. Light inhibition occurred after that stage, resulting in the decrease

in biomass productivity. The effect of light intensity is shown in Figs. 4-4 a–e. When ammonium concentration was 170 mg N/L, the lowest LI (1000 Lux) resulted in the ammonium removal rate of 83.0 ± 1.0 mg/L/day, which was up to 40% higher than the removal rate achieved at 2500 Lux. However, the difference in ammonium removal rate between the lowest LI and the highest LI was not significant (ANOVA, $p > 0.05$). The ANOVA results indicated that the light intensity had a significant effect ($p = 0.008$) on the nitrogen removal at the initial ammonium concentration of 340 mg N/L. When initial ammonium concentration was 340 mg N/L, LI of 4000 Lux obtained the lowest ammonium removal rate among all treatments; the fastest removal rate of 86.5 ± 2.2 mg N/L/day was found at the highest LI (9000 lux); however, it was not significantly different as compared to the removal rate at 2500 Lux. In the entire experiment, OD with the low LIs typically decreased or remained unchanged. The OD at the highest LI (9000 Lux) increased from 0.80 to 1.15 while the biomass at 4000 Lux was stable and close to that of a lower range from 0.78 to 1.02 was found at 4000 Lux. Before the complete ammonium removal, LI of 2500, while the biomass at 9000 Lux resulted in higher concentration. In addition, higher biomass did not promote the ammonium removal efficiency. The results might be due to the following reasons: (1) since the extensive amount of bacteria in the all treatments reactors limited the ammonium removal efficiency, the high algae biomass did not promote nitrogen removal; (2) high algal biomass concentration at 9000 Lux formed a self-shading mechanism that protected the bacteria from the strong LI while algal biomass at a lower LI (4000 Lux) did not adequately protect bacteria; (3) higher LI could inhibit the bacterial activity when OD value was around 0.8. Under the same LI of 2500 Lux, higher concentration of 340 mg N/L resulted in a better removal rate, which also confirmed that higher ammonium concentration promoted the removal efficiency of this consortium.

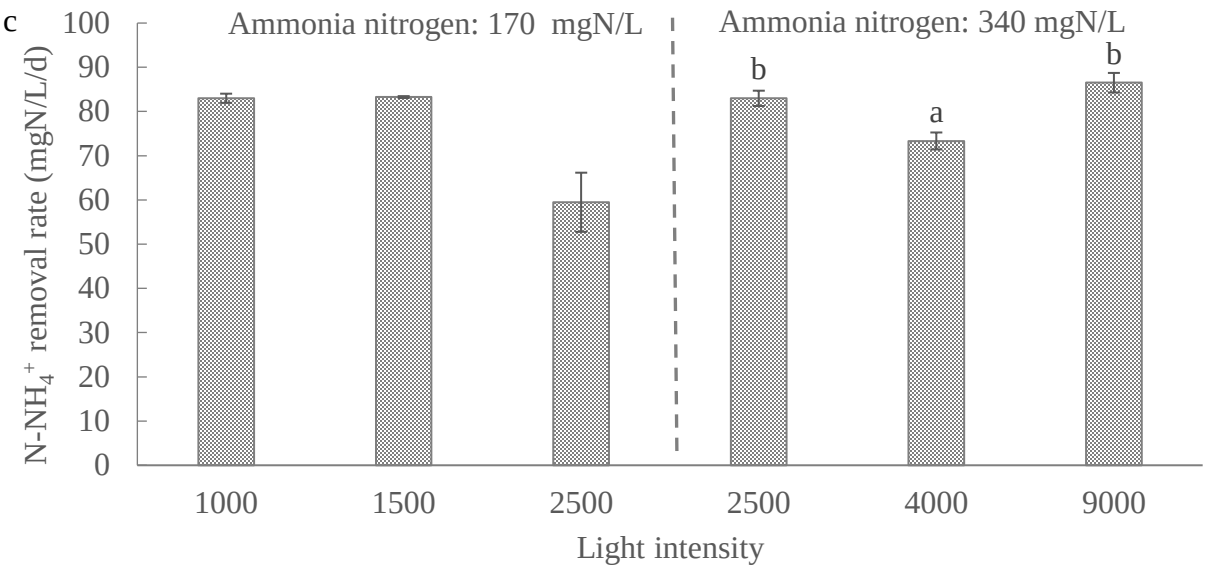
a

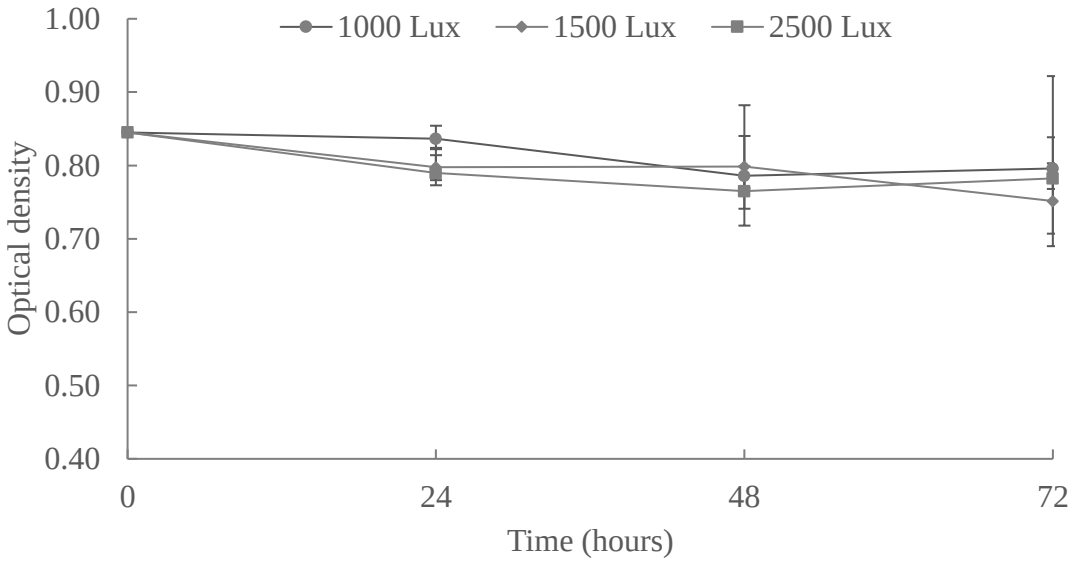

1000 Lux
1500 Lux
2500 Lux
N-NH4+ concentration(mgN/L)
200
150
100
50
0
0
12
24
36
48
60
72
Time (hours)

b


2500 Lux
4000 Lux
9000 Lux
N-NH4+ concentration (mgN/L)
400
350
300
250
200
150
100
50
0
0
12
24
36
48
60
72
84
Time (hours)

c


Ammonia nitrogen: 170 mgN/L
Ammonia nitrogen: 340 mgN/L
N-NH4+ removal rate (mgN/L/d)
100
90
80
70
60
50
40
30
20
10
0
b
a
b
1000
1500
2500
2500
4000
9000
Light intensity


1000 Lux
1500 Lux
2500 Lux
Optical density
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0
24
48
72
Time (hours)

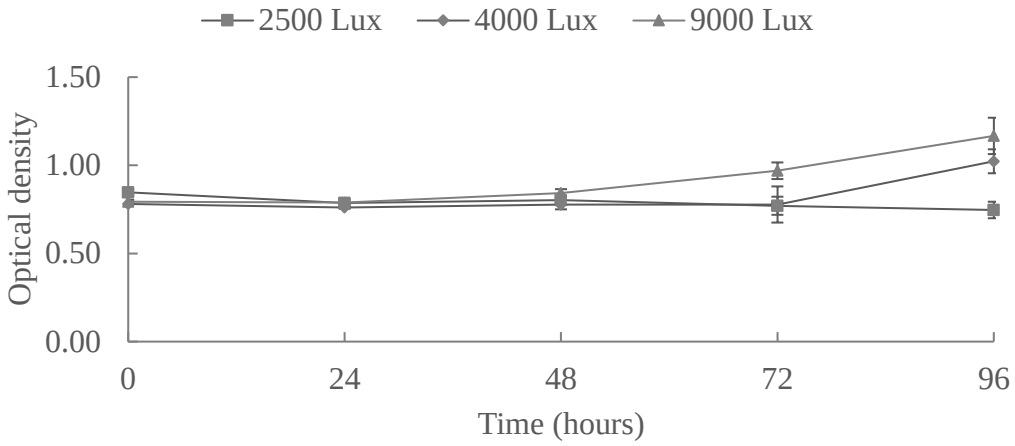



**Figure 4-4 Ammonium removal efficiency of low LI range (a) and high LI range (b), ammonium removal rate (c), and optical density of low LI range (d) and high LI range (e) for different light intensities; errors bars indicate the standard error of the mean of the three biological replicates**

### 4.2.5 Effect of light cycle on ammonium nitrogen removal

Effect of lighting cycle on ammonium removal was studied by establishing light to dark cycles (L/D) of 24:0 h, 16:8 h, and 2:1 h under 1000 Lux of natural white light, which lasted for 4 days. It has been proved that dark periods between short flashes of light can increase photosynthesis efficiency, especially under high LI (Liao et al. 2014; Park and Lee 2001). The effects of the light cycle are shown in Fig.4-5. Three different light cycles eliminated approximately 450 mg N/L of ammonium nitrogen in 8 days. However, the light cycles did not show a significant difference (ANOVA, $p>0.05$) on the consortium performance of ammonium nitrogen removal. This could be due to the low LI (1000 Lux) used in this test.

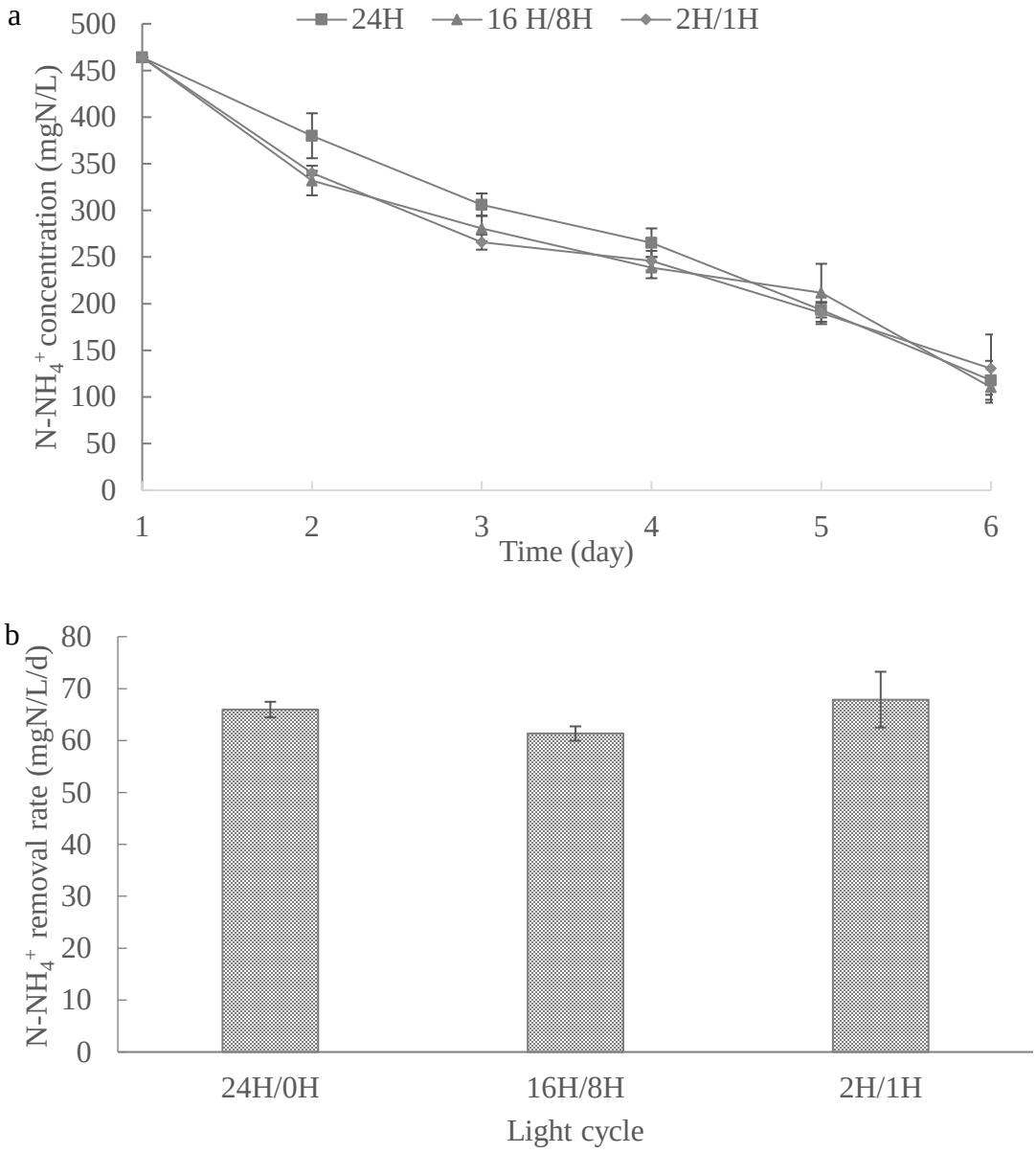


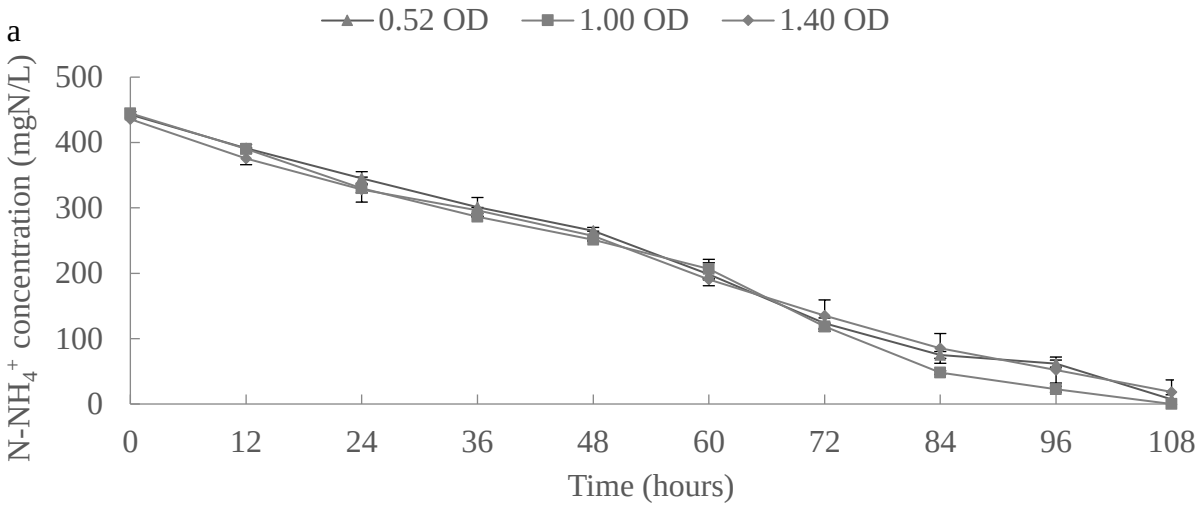

**Figure 4-5 Ammonium removal efficiency (a) and ammonium nitrogen removal rate (b) for different light cycles; errors bars indicate the standard error of the mean of the three replicates**

### 4.2.6 Effect of algal biomass on ammonium removal

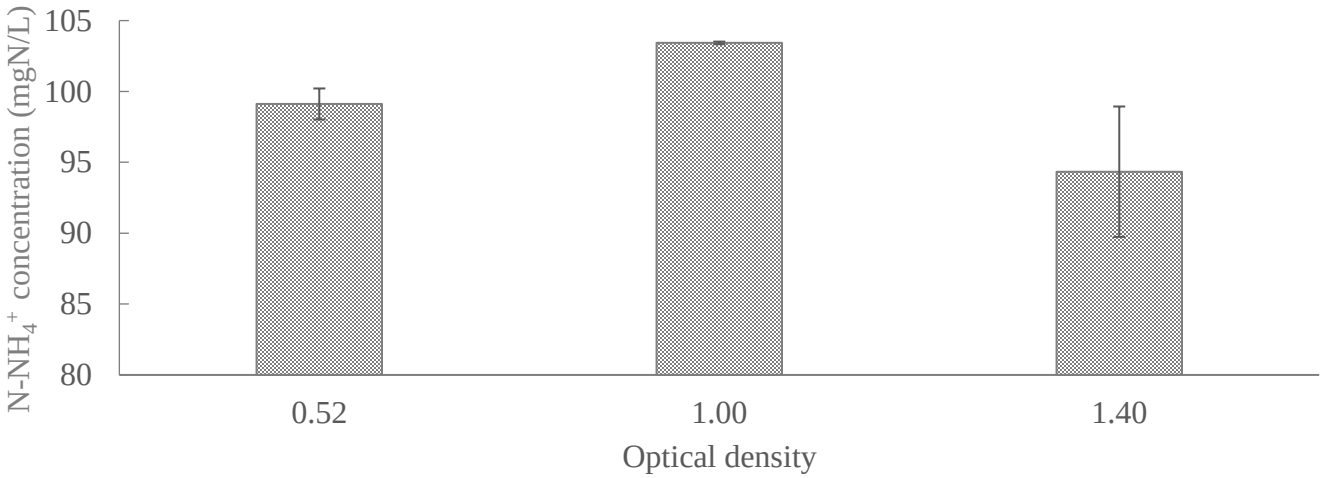
To determine the effect of initial algae biomass density, algae culture was added to the algae-bacteria consortium to get the optical densities of 0.500 ± 0.003, 1.000 ± 0.020, and 1.400 ± 0.020 OD. After inoculation, all of the reactors were supplied 1000 Lux of nature white light.

Dissolved oxygen is essential for bacteria to remove ammonium nitrogen. Without photoinhibition, it is expected that high algal density result in more oxygen production, which leads to higher nutrient removal efficiency. However, high algal density could result in self-shading that blocks the light and inhibits photosynthesis (Sforza et al. 2012). In this batch test, no significant (ANOVA, $p>0.05$) promotion or inhibition of nitrogen removal efficiency was observed between the initial OD of 0.520 and 1.000 (Fig. 4-6 b). The removal efficiencies at 0.520 and 1.000 OD were slightly higher than that of 1.400 and 1.000 OD had the best removal efficiency. This result corresponds to the batch test, which evaluated the LI having the highest biomass and did not promote ammonium nitrogen removal efficiency. This could be due to the same reason that high algal biomass density formed self–shading, which resulted in low photosynthesis efficiency and limited the ammonium removal rate. However, the ammonium removal efficiencies among three OD treatments did not show any significant difference (ANOVA, $p>0.05$). Fig. 4-6 c presents the ammonium removal rate by biomass. With the increase of the biomass, the ammonium removal rate was decrease. The least biomass of 0.5 OD achieved the highest ammonium removal rate of 157.3 $mgN/L \bullet OD^{-1} \bullet d^{-1}$ which was 70% higher than that of the 1.0 OD and 2.5 times higher than that of 1.4 OD. This result indicate that the increased cell density just increased the shading, under the flask configuration. Another reason could be that low LI of 1000 Lux limited the photosynthetic efficiency.

a


0.52 OD
1.00 OD
1.40 OD
N-NH4+ concentration (mgN/L)
500
400
300
200
100
0
0
12
24
36
48
60
72
84
96
108
Time (hours)

b


N-NH4+ concentration (mgN/L)
105
100
95
90
85
80
0.52
1.00
1.40
Optical density

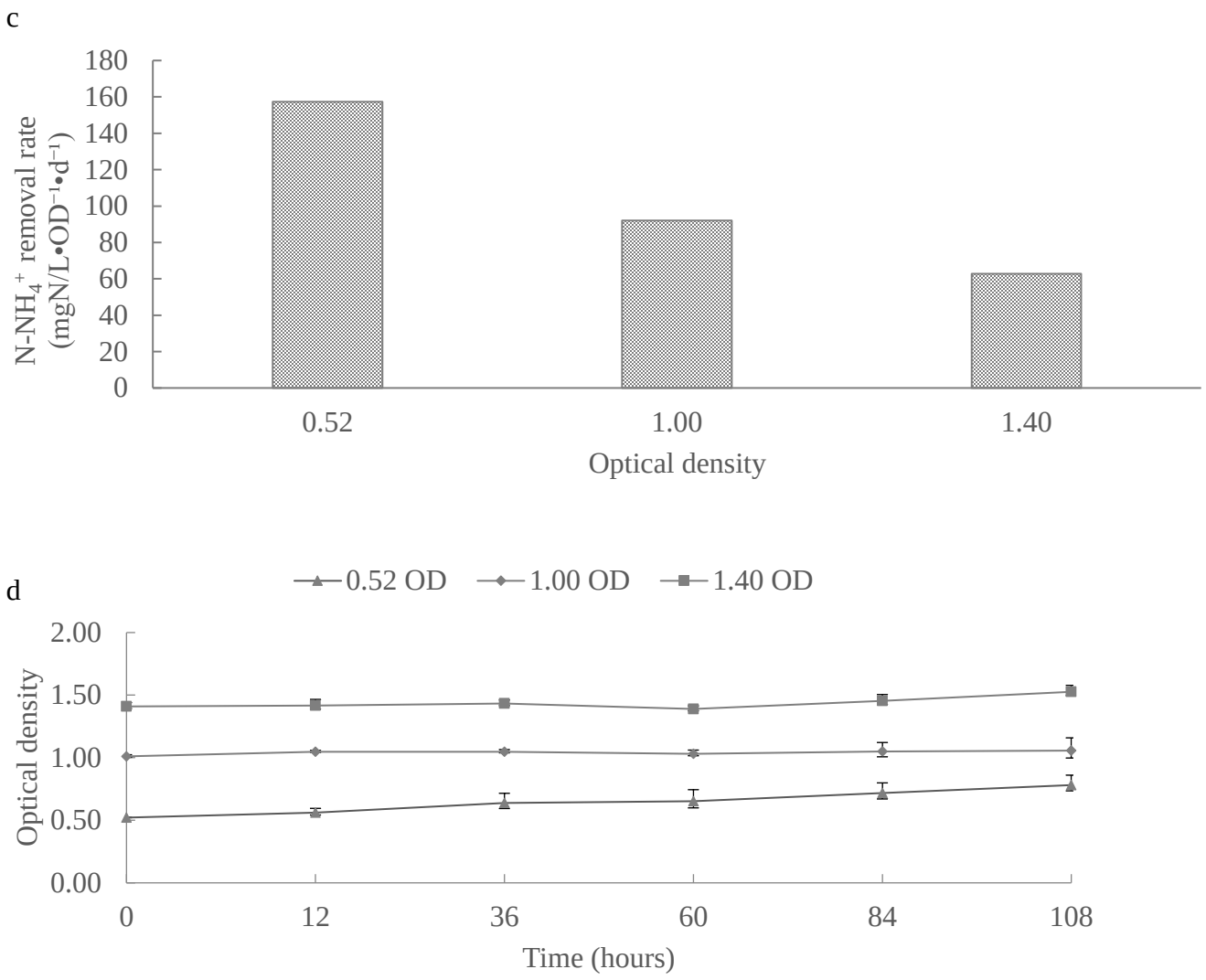



**Figure 4-6 The effect of algal biomass (different OD): ammonium removal efficiency (a), ammonium removal rate (b), and optical density (c); error bars indicate the standard of the mean of three replicates**

### 4.3 Conclusion

In this study, the impact of light, algal biomass concentration, and ammonium concentration on the performance of ammonium nitrogen removal via algae-bacteria consortia was investigated through batch tests. Results showed that the mixed culture of algae and nitrifying bacteria could achieve 100% ammonium removal without aeration. Higher ammonium concentration enhanced

the nitrogen removal rate. With the similar algal biomass quantity, the light intensity of 1000 and 9000 Lux resulted in similar ammonium removal efficiency. In this study, higher biomass did not show a superior ammonium removal rate than that of the lower biomass since the self-shading in the photo-bioreactor. This result presents that the higher cell biomass does not necessarily mean faster removal rates, especially when photosynthetic organisms are involved. In addition, at 1000 Lux, light wavelength, and light cycle did not present significant effects on the ammonium removal efficiency. This result suggests that the low light intensity of 1000 Lux could be used as an economic strategy to maintain a stable ammonium nitrogen removal performance of the algae–bacteria consortium, and high ammonia concentration could be used as a strategy to promote the efficiency of the algae–bacteria consortium. As the algae–bacteria consortium successfully removed ammonium nitrogen at low LI, it can be utilized in conjunction with other processes to treat the remaining nitrogen compounds (nitrite and nitrate) in the wastewater.

# Chapter 5 NITROGEN REMOVAL IN SEQUENCE BATCH PHOTO-BIOREACTOR USING ALGAE-BACTERIA CONSORTIUM

## 5.1 Introduction

Wastewater treatment is commonly accomplished through an activated sludge process. However, the high energy demand and the large waste sludge production of activated sludge has encouraged researchers to find a more sustainable and cost-effective way to treat wastewater.

As primarily oxygen-releasing photosynthetic organisms, microalgae function as "aeration devices" to replace the artificial aeration during wastewater treatment. In addition to oxygen, microalgae can produce a wide range of valuable metabolites including fats, sugars, and bioactive compounds (Andersen, 2013) to release simpler organic compound, which could easily be metabolized or consumed by heterotrophic bacteria (Sekaran et al., 2013). The exchange of inorganic and organic nutrients through photosynthesis and respiration support an advanced algae-bacteria interaction performance (Muñoz et al., 2006). Algae-bacteria consortia have been investigated for decades, with earlier research conducted on high rate algal ponds. Open systems usually need shallow liquid level, which allows the consortia to capture light and oxygen (Chang et al., 2015). Due to the large amount of wastewater and shallow environment requirement of open system, land demand becomes a limitation for the application. In addition, the construction of the open pond also makes the chemical stability becomes an issue due to the evaporation and precipitation (Tredici et al., 2009). Thus, the compact and enclosed system of photo sequence batch reactor (PSBR) using microalgae—bacteria consortia was developed recently. In PSBR, light is essential for biomass growth and photosynthesis. These parameters influence the production of oxygen and organic matter. Higher algal biomass production is expected to produce more oxygen but condensed algal biomass could block the penetration of photons and induce inefficient light utilization (Park et al.,

2001). Thus, algal biomass density will impact significantly on the design of photobioreactors. Dissolve oxygen (DO) concentration highly affects nitrogen metabolism. For instance, a study using continuous illumination produced DO mass of 0.46 kg/$m^3$/d, resulting in full nitrification without noticeable denitrification (Karya et al., (2013). DO range in the nitrification stage was 0.3-2.4 mg/L, which increased up to the oxygen saturation point after an hour. Another study explored the alternating light and dark periods to find that DO concentration lower than 0.5 during an entire cycle achieved rapid nitrification and removed over 90% total nitrogen and organic carbon (Wang et al. 2015). The aforementioned studies show the effect of different light conditions on DO concentration, which subsequently affects nitrification.

Poor effluent quality could limit the application of microalgae-bacteria consortia in wastewater treatment. Biomass harvest process is typically cost-effective and could be easily upgraded to large volumes. Currently, large-scale biomass harvest is performed using expensive methods such as filtration, centrifugation, gravity sedimentation, and micro-straining. The immobilization system developed at lab-scale could simply be used for harvesting and recycling but it is limited by the costly material and low oxygen and nutrient transfer efficiency. To obtain a simple sedimentation by gravity, a bioflocculent algae–bacteria consortium was developed. Bioflocculant produced by algae-bacteria consortia in PSBR could be settled by gravity to produce clear effluent (Wang et al. 2015, Karya et al. 2013), representing a relatively low-cost approach compared to the aforementioned methods.

Previous batch tests explored the effect of initial ammonium and algae biomass concentrations and light conditions on ammonium removal efficiency by algae-bacteria consortia during wastewater treatment (Chapter 4). It was found that low light intensity of 1000 Lux could be used as an economic strategy to maintain a stable ammonium nitrogen removal performance of the algae–

bacteria consortium. Therefore, low light intensity was used in the present experiment. The specific objectives of this study were to examine the effect of different photoperiods on the nitrification performance of the consortia, and to estimate the low light intensity effect on a long-term PSBR.

## 5.2 Results

### 5.2.1 Biomass growth of different photoperiods

Photoperiod of 24 hours illumination and 16h/8h L/D was applied alternately: the first 3 weeks was the starting phase and applied with the 16h/8h L/D photoperiod; the following phase from day 23 to day 120 was 24 hours illuminated; phase 3 was 16h/8h shifted light; the phase 4 began from day 215 was continuously illuminated. As shown in Fig. 5-1, total suspended solids (TSS) decreased in phase 1 and started to increase after the photoperiod shift to 24 hours illumination. Biomass reached 0.8 gTSS/L at the end of the phase 2. Decreased lighting period in phase 3 reduced the biomass to 0.57 gTSS/L from day 120 to day 139, but the stable level of 0.8 gTSS/L was achieved in 2 months. In phase 4, after a lag phase, biomass increased to 1.1 gTSS/L by the end of the experiment (280 days). Solid retention time (SRT) ranged from 5 to 13 days. Most of the biomass was settled within 20 minutes and the sludge volume index (SVI) as measured was 109 ml/mg.

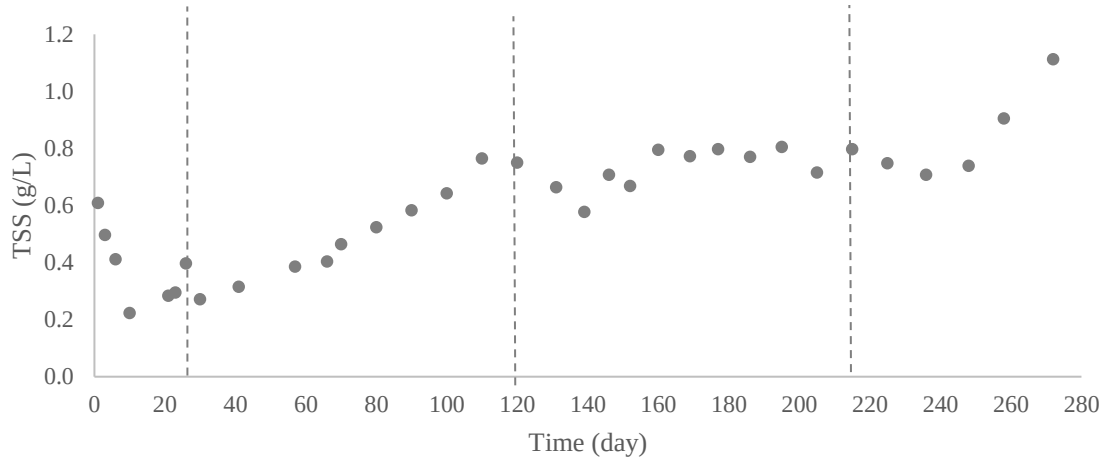



**Figure 5- 1 Variation of TSS over time**

### 5.2.2 Bacteria species in reactor

Since the interaction between algae and bacteria is very complex (Muñoz et al., 2006), it is especially important to know which kinds of nitrogen removal relate bacteria are active in the consortium system. A biomass sample taken at day 120 after the stabilization of the performance was sequenced using 16SV4 for bacteria community composition analysis (Table.5-1). In the reactor, both aerobic and anaerobic bacteria presented. It was found that Proteobacteria (34.7%), Bacteroidetes (22%), and Firmicutes (9.41%) were the three dominant bacteria phyla. Other than that, OP3 (7.5%), Cyanobacteria (5.3%), Chloroflexi (3.6%), Acidobacteria (3.4%), and Planctomycetes (2.3%) were also observed in the reactor. Chlorophyta (4.6%) as green algae was detected in the biomass as well. Bacteroidetes and Firmicutes are the dominant bacterial divisions in human gut and many of these are obligate anaerobes (Koliada et al., 2017, Guo et al., 2008). Proteobacteria is considered the most prominent phylum in wastewater treatment plants (Ibarbalz et al., 2013), including bacteria involved in nitrogen removal, such as ammonium oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), and denitrifiers. Under the phylum Proteobacteria, *Nitrosomonadaceae* is the most abundant bacteria, in the reactor and

*Nitrosomonadaceae* is the only reported bacteria under the family. This result explained why there was no nitrate detected in the effluent of the PSBR from day 120 to day 175. The bacteria associated with the anaerobic ammonium oxidation (Anammox) process are affiliated with the phylum Planctomycetes (Strous et al., 1999) and most of the identified Plactomycetes were present on aggregates (Fuchsman et al., 2012). Besides, Phylum Chloroflexi often exist during the anammox process (Sunja et al., 2010) as well. They survive by intake of organic substrates such as soluble microbial products and extracellular polymeric substances derived from anammox bacteria and AOBs (Chu et al., 2015, Tomonori et al.,2012). Furthermore, Chloroflexi reportedly play significant functions in microbial aggregates and granulation since they are mostly filamentous (Wang et al., 2017, Sunja et al., 2010).

**Table 5- 1 Dominant bacteria (relative abundance >0.02) in phylum level and minimum and analysed level**

| Dominant bacteria (phylum level) | Relative abundance | Dominant bacteria (minimum and analysed level) | Relative abundance |
|---|---|---|---|
| Alphaproteobacteria | 0.105431 | o__Rhizobiales | 0.021472 |
| Bacteroidetes | 0.220665 | f__Chitinophagaceae | 0.049173 |
| Betaproteobacteria | 0.15513 | f__Saprospiraceae | 0.049743 |
| Gammaproteobacteria | 0.045774 | g__Lactobacillus | 0.024345 |
| Firmicutes | 0.094135 | o__BD7-3 | 0.033798 |
| OP3 | 0.075339 | f__Nitrosomonadaceae | 0.111791 |
| Cyanobacteria | 0.053296 | c__PBS-25 | 0.075339 |
| Unassigned;Other | 0.043536 | o__Chlorophyta | 0.045774 |
| Deltaproteobacteria | 0.037264 | Unassigned;Other | 0.043536 |
| Chloroflexi | 0.036825 | o__Sphingobacteriales | 0.040663 |
| Acidobacteria | 0.03393 | o__Myxococcales | 0.03268 |
| Planctomycetes | 0.023227 | f__Ellin6075 | 0.031671 |
| | | f__A4b | 0.021713 |
| Amount | 12 | Amount | 13 |

### 5.2.3 DO concentration in different phases

DO was rapidly consumed over the course of filling stage until it reached a stable value between 0.4 and 0.7 mg/L. For the remaining part of the cycle, DO was remained constant in that range

until additional wastewater was fed to the reactor. The low concentration of DO could create both aerobic and anoxic region in reactors (Wang et al., 2015). Figure 5-2 shows an example with a stable DO value of 0.4 mg/L, which represents day 90 in phase 2. In phase 3 (day 167), the DO concentration was 0.6-0.7 mg/L in the lighting phase, which was comparable to previous phase, and decreased to 0.4-0.5 mg/L during dark phase. The higher DO in phase 3 is potentially a consequence of higher biomass concentration. However, DO value did not increase with the increase in lighting time and biomass in phase 4, results were in between 0.5 to 0.7 mg/L. This is due to self-shading caused by higher biomass density that blocks the light and inhibits photosynthesis (Chapter 4).

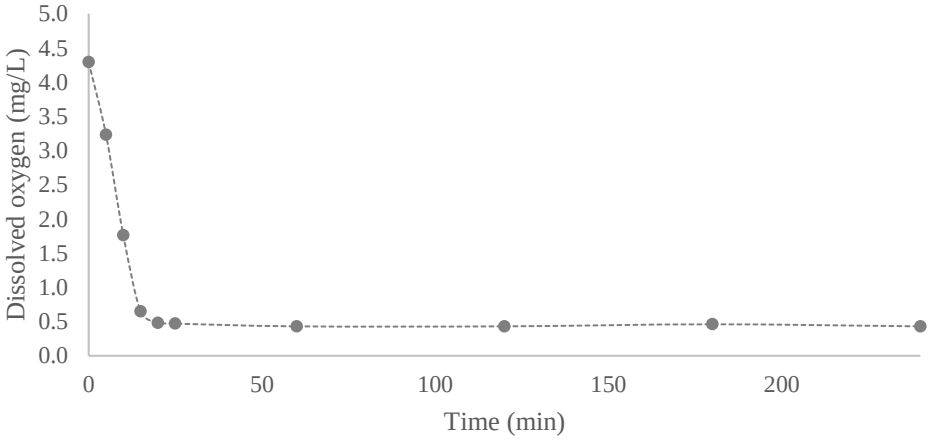



**Figure 5- 2 DO profile in SPBR cycle (phase 2)**

### 5.2.4 Effect of nitrification inhibitor on consortium performance

To estimate the nitrogen assimilation by algae and free ammonia (FA) volatilization, nitrification inhibitor was dosed 10 drops per liter in reactor during filling stage at the end of the experiment. Each cycle removed 8 mgN/L via biomass uptake and volatilization (Fig. 5-3). Since the biomass quantity reached the highest amount of 1.1 gTSS/L at the end of the experiment, the nitrogen assimilation and volatilization did not exceed 8 mg/L in any other phase. The inhibition of

nitrification activity resulted the increase of DO in the liquid bulk, that is why the denitrification process depressed and values of $N\text{-}NO_3^-$ did not decrease. The suppression of denitrification resulted in the increase of TOC, but it stopped after 8 hours. The organic matter produced in the reactor could be utilized by the other heterotrophic bacteria since the higher DO in the reactor could enliven them.

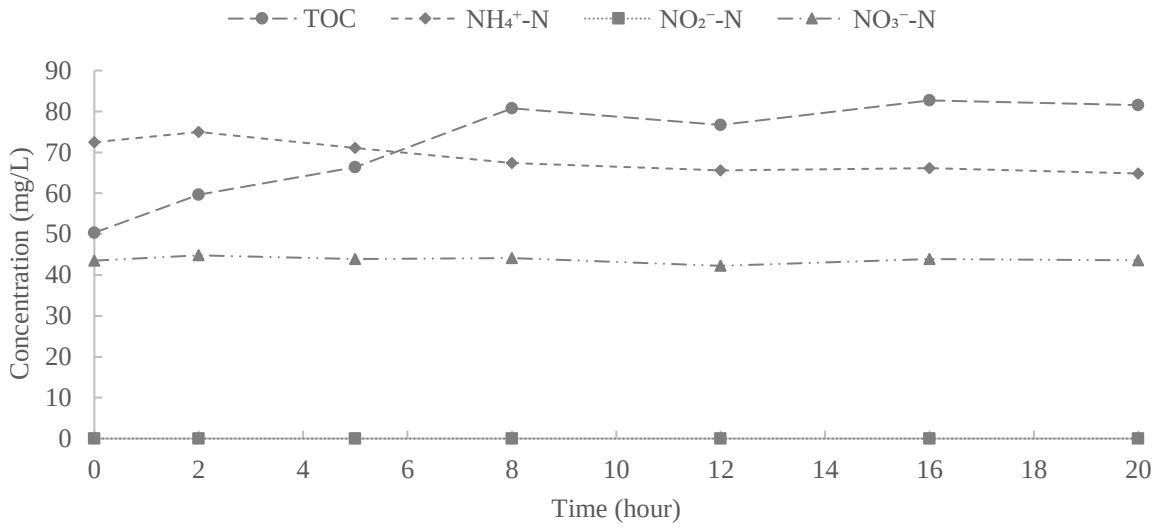



**Figure 5- 3 Kinetic tests with dosage of denitrification inhibitor**

### 5.2.5 Nitrogen removal performance of PSBR under different photoperiods

The SBRs were run for 280 days. Two types of photoperiod were tested interchangeably. The phases 1 and 3 were operated in 16 h/ 8 h L/D mode whereas phases 2, 4 were operated in 24 hours continuous illumination.

The $NH_4^+$-N removal efficiency on the first day was 117 mgN/L/d (Fig 5-4), which was similar to the best results of 108 ± 3.6 mg N/L/d achieved in previous batch test (Chapter 4). However, after 4 weeks of cultivation in PSBR, the value decreased to 43 mgN/L/d. At this point, the biomass was washed out and reduced to 0.3 gTSS/L from its initial value of 0.6 gTSS/L. The decrease in nitrogen removal efficiency could be due to the presence of less amount of biomass. Additionally,

the concentration of $NO_2^-$-N in effluent increased gradually to 47 mgN/L while the concentration of $NO_3^-$-N was maintained below 10 mgN/L. To ensure that proper functioning of reactors, biomass in the effluent was settled and recirculated after 10 days.

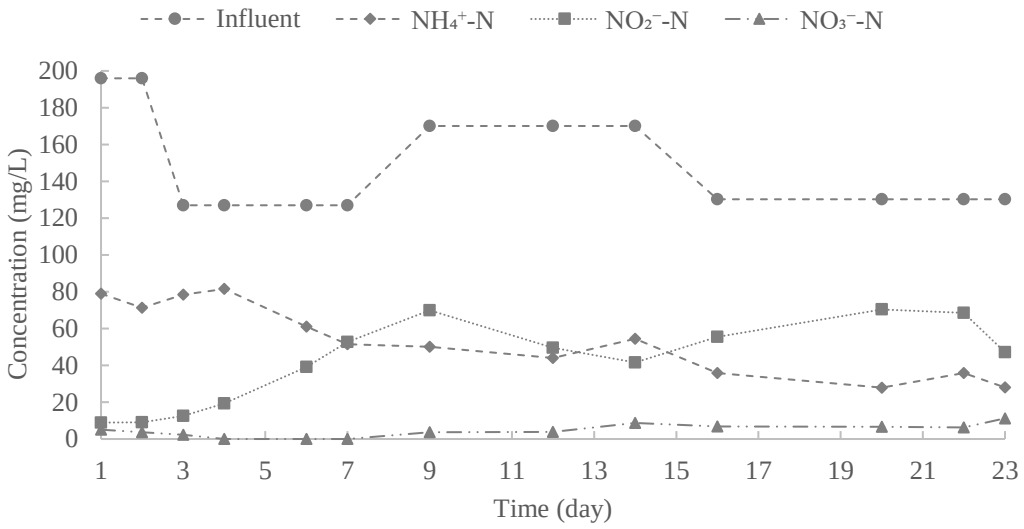



**Figure 5- 4 Nitrogen removal performance during phase 1 (16 h/8 h light to dark photoperiod)**

#### 5.2.5.1 Nitrogen removal performance in phase 2

In phase 2 (Fig 5-5 a-b), the photoperiod was changed to 24 hours illumination. $NH_4^+$-N removal efficiency decreased slightly to 30 mgN/L/d during the first week. However, it steadily increased to achieve a stable efficiency of 62 ± 3 mgN/L/d after 4 weeks. Moreover, the biomass also increased to 0.76 gTSS/L. The higher biomass quantity did not lead to a higher $NH_4^+$-N removal efficiency. Similar results were achieved from previous batch tests (Chapter 4). The $NO_2^-$-N concentration in effluent stayed around 60 mgN/L while the $NO_3^-$-N increased to 55 mgN/L at day 56 and gradually decreased to 15 mgN/L at the end of this phase, indicating NOB inhibition.

Kinetic tests were also carried out for better understanding the nitrogen removal process. Figure 6c shows the data collected in a cycle at day 90. During the course of this cycle, $NH_4^+$-N concentration decreased from 71.7 to 5.1 mgN/L with an overall rate of 2.68 mgN/L/h, $NO_2^-$-N increased steadily to 58.6 mgN/L while $NO_3^-$-N remained at a concentration lower than 14 mgN/L. TOC remained stable around 41.1 ±1.5 mg/L during the cycle.

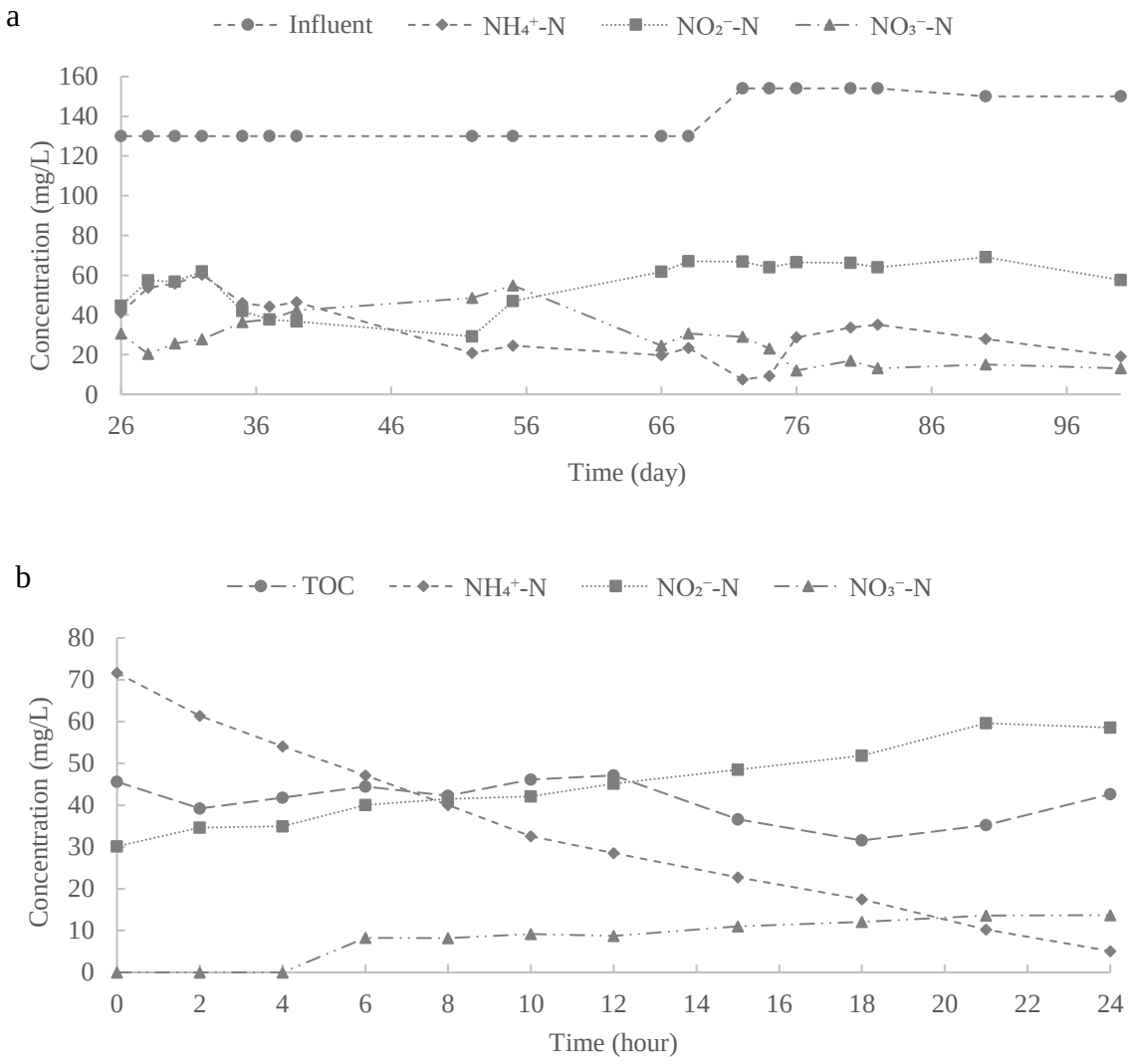



**Figure 5- 5 Nitrogen removal performance during phase 2 (24-hour continuous lighting) (a), Kinetic test of phase 2 (b).**

#### 5.2.5.2 Nitrogen removal performance in phase 3

In phase 3 (Fig. 5-6 a), photoperiod was reverted to 16/8 L/D. Within the dark phase, $NH_4^+$-N removal efficiency was 57.9 ± 1.9 mgN/L, which was similar to phase 2. The biomass was stabilized after a month in this period and remained around 0.78 ± 0.01 gTSS/L. Concentration of $NO_2^-$-N was 51.4 ± 1.7 mgN/L while the $NO_3^-$-N dropped below the detection limit. However, a reduction in the salt concentration in wastewater from 0.56 % to 0.24% was observed after two months under the same light condition. The lower salinity wastewater was loaded to the reactors for a month. During this course, $NH_4^+$-N removal efficiency decreased to 33 mgN/L/d at first, and increased thereafter. After stabilization, almost 100% of $NH_4^+$-N was removed. This could be due to the low concentration of salts that stimulated AOB growth (Li et al., 2018). The $NO_2^-$-N concentration dropped below the detection limit within 2 weeks while $NO_3^-$-N increased to 70 mgN/L. Ye et al. (2009) reported that NOB can completely oxidize $NO_2^-$-N to $NO_3^-$-N without additional salinity while the increase in salinity led to inhibition of NOB and partial nitrification. Moreover, adjusting the wastewater salinity back to 0.56 % did not increase $NO_2^-$-N concentration within two weeks. The reason could be a change in microbial community due to the low salinity (Li et al., 2018). Furthermore, NOB group favors growth in low salinity condition.

During the phase 3 cycles (Fig. 5-6 b), dark phase started at 10 pm and ended at 6 am, which indicated 7.5 to 15.5 hours of the cycle. The data in Fig. 6e were collected on day 167. Removal of $NH_4^+$-N took place throughout the cycle, but the rate was evidently slower during the dark phase compared to light phase. dark This result could be due to the nitrogen uptake by algae during dark phase when the algae grow heterotrophically. According to Perez-Garcia et al. (2010), nitrogen removal by *Chlorella vulgaris* was doubled in heterotrophic condition compared to that achieved in autotrophic condition. The removal efficiency was 1.71 mgN/L/h during dark phase. During the

first lighting phase, removal efficiency was 3.3 mgN/L/h, which was higher than the rate of 24-hour continuous illumination. That value decreased to 2.05 mgN/ L/h at the end of lighting phase. $NO_2^-$-N progressively increased but at a slower rate than phase 2. Similar to the nitrification efficiency, denitrification rate was reduced in the dark phase compared to the light phase. 6.8 mgN/L of inorganic nitrogen was removed in 8 hours dark period while the first 7.5 hours and the last 8.5 hours removed 22 and 9.3 mgN/L, respectively. $NO_3^-$-N concentration was below the detection limit, indicating that rapid nitrification was achieved in this photoperiod mode. The lower TOC concentration of phase 3 could be the result of shorter lighting period.

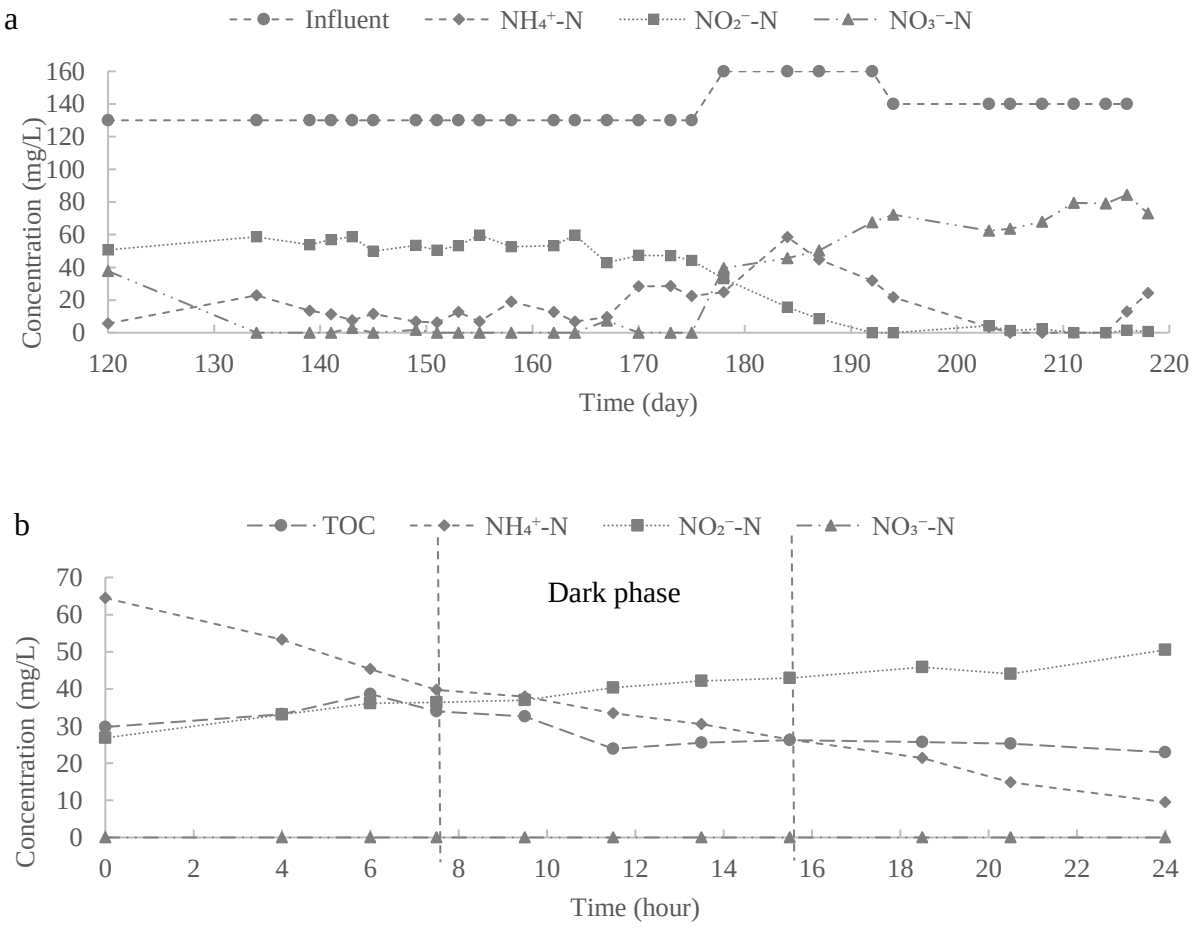
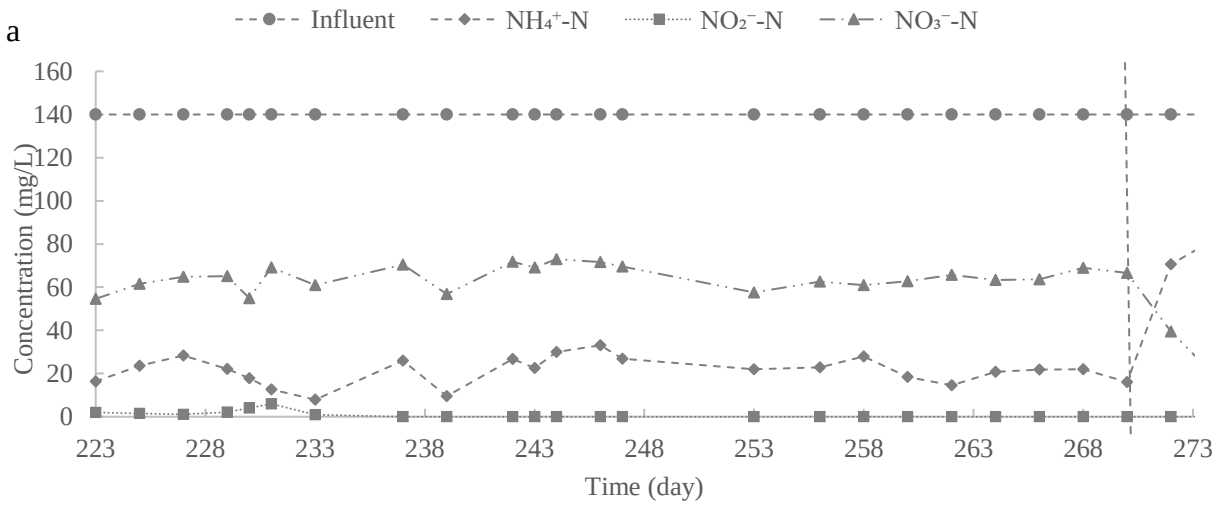



**Figure 5- 6 Nitrogen removal performance during phase 3 (16 h/8 h light to dark photoperiod) (a), Kinetic test of phase 3 (b)**

### 5.2.5.3 Nitrogen removal performance in phase 4

The salinity was recovered to 0.56% on day 208. After 2 weeks, the photoperiod was changed to continuous illumination. The initial purpose of changing illumination back to 24 hours was to verify if rapid nitrification could be achieved in this photoperiod as well. However, rapid nitrification was not observed after changing the salinity at the end of phase 3, which is due to the failure in recovering microbial community. Biomass increased in this phase but did not improve the nitrification efficiency. The $NH_4^+$-N removal efficiency was 58.7 ± 1.4 mgN/L/d, $NO_3^-$-N in the effluent was 64.6 ± 1.2 mgN/L, and $NO_2^-$-N was under the detection limit.

The kinetic test was conducted on day 262 (Fig. 5-7 b). $NH_4^+$-N decreased steadily with the a removal rate of 2.37 mgN/h, $NO_3^-$-N increased constantly from 36.7 mgN/L to 65.7 mgN/L, and TOC decreased from 65.4 to 42.2 mg/L. Even though rapid nitrification did not occur, denitrification was observed in this phase.



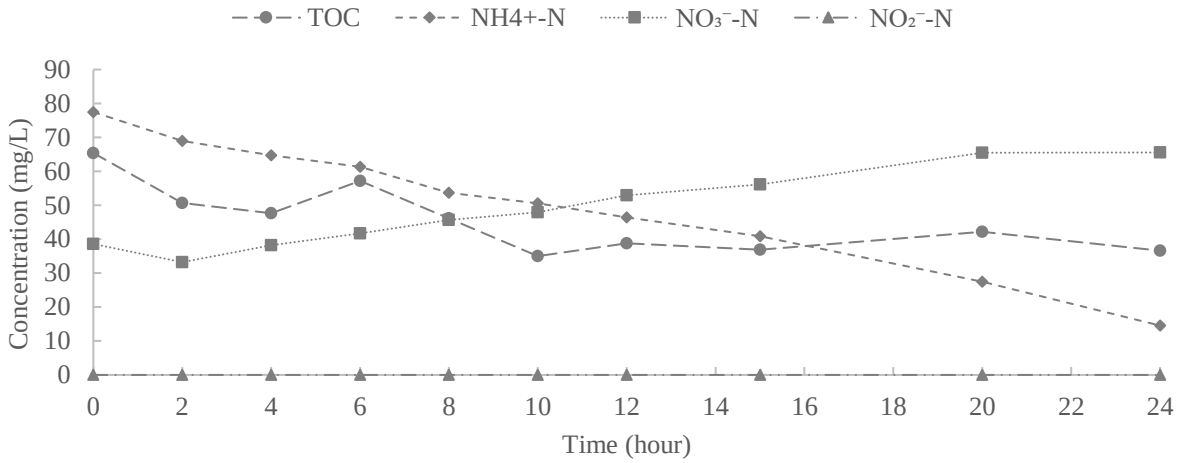



**Figure 5- 7 Nitrogen removal performance during phase 4 (24-hour continuous lighting) (a), Kinetic test of phase 4 (b).**

## 5.3. Discussion

### 5.3.1 Nitrogen metabolism with different photoperiods

**Table 5- 2 Nitrogen removal in different phases**

| Phase | Initial N | Effluent-$NH_4^+$-N | Effluent-$NO_2$-N | Effluent-$NO_3$-N | A&V | Denitrification |
|---|---|---|---|---|---|---|
| **2** | 102 | 5% | 57% | 13% | 8% | 18% |
| **3** | 91 | 10% | 55% | 0 | 7% | 28% |
| **4** | 114 | 12% | 0 | 60% | 7% | 21% |

A&V: Biomass assimilation and volatilization

The percentage of the nitrogen profile in three kinetic tests is summarized in table 5-2. The FA volatilization and biomass uptake of nitrogen was estimated by adding nitrification inhibitor at the end of the experiment, which maximized the $NH_4^+$-N at 8 mgN/L in a cycle. Thus, $NH_4^+$-N was primarily removed by nitrification and nitritation. In phase 3, the existence of dark phase in a cycle limited the photosynthesis process and produced less oxygen. Within this phase, nitrate became the unique product due to the less DO in the reactor (Ruiz et al., 2003, Wang et al., 2015). Even

though the phase 3 cycle was characterized by lower organic carbon in the liquid bulk compared to phases 2 and 4, nitrogen removal was higher compared to the 24-hour illumination photoperiod. This result could be explained considering the hypothesis that a medium with low DO concentration favors denitrification. However, as a result of the low organic carbon available in the reactor, both denitrification took place at a low efficiency (Wang et al., 2015). $NH_4^+$-N was entirely converted into $NO_3^-$, which could be the result of microbial community change during the load of low salinity wastewater of 0.24 % (Li et al., 2018) and an increase of DO concentration from 0.6 to 0.9 mg/L induced by the longer lighting period and higher biomass. For the removal of $NH_4^+$-N (Table 5-3), same as chapter 4, the lower biomass the higher efficiency. It confirmed that the higher biomass only increased the shading and is unnecessarily for a high removal rate, especially when the photosynthetic organisms are involved. Before phase 4, the accumulation of $NO_2^-$ indicated the inhibition of NOB activity. According to Villaverde et al. (1997), FA higher than 1.5 mg/L inhibits NOB. The maximum FA concentration of 1.84 mgN/L was produced during the start-up days while it decreased below 1.5 mg/L after one week. It was also found that free nitrous acid (FNA) higher than 0.011 mg/L inhibited NOB. According to the equation below FNA in the reactor ranged from 0.012 to 0.015 mg/L before phase 4, which was higher than the reported concentration of 0.011 mg/L that inhibited NOB (Vadivelu et al., 2006).

$$HNO_2\,(mg/L) = \frac{46}{14} \times \frac{{NO_2}^- - N(mg/L)}{Ka \times 10^{pH}}$$ (Anthonisen et al., 1976)

Moreover, the condition of low DO (Pollice et al., 2002) and short SRT (Hellinga et al., 1998, Wang et al., 2015) were also reported to favor the nitritation. The combination of alternating high $NO_2^-$ concentrations and low DO could be the reason for $NO_2^-$ accumulation. However, the decrease in salinity to 0.24% promoted NOB activity. As a consequence, $NO_2^-$ concentration

decreased below the detection limit for the remaining period, thereby elimination the inhibition of FNA on NOB.

**Table 5- 3 Overall $NH_4^+$-N removal rate with different biomass concentrations**

| Kinetic | Biomass (g/L) | Initial TOC (mg/L) | Overall removal rate of $NH_4^+$-N ($mgN/L \cdot gTSS^{-1} \cdot h^{-1}$) |
|---|---|---|---|
| **24/0** | 0.583 | 45.6 | 4.62 |
| **16/8** | 0.77 | 29.7 | 2.84 |
| **24/0** | 0.905 | 65.4 | 2.62 |

### 5.3.2 Effect of photoperiod on biomass and TOC concentration

Due to the higher biomass achieved in phase 4, the TOC content was 43% higher than that of phase 2 (Table 5-3). However, higher biomass in phase 3 did not stimulate TOC production. It could be due to less photosynthesis activity during the dark phase and the heterotrophic growth of some algae species during the dark phase that could uptake the organic matter in the reactor (Perez-Garcia et al., 2010). In addition, higher TOC produced in phase 4 did not achieve effective denitrification. This result is in accordance to an *ex situ* denitrification test carried by Wang et al. (2015). It was reported that without additional biodegradable organic carbon, the denitrification could not be accomplished in a sample of centrate containing 209 mg/L BOD.

DO is essential for nitrification. Without photoinhibition, it is reasonable to expect that high algal density results in higher oxygen production, which leads to better nitrification efficiency. In the current experiment, biomass increased in phases 2 and 4, and was stable in phase 3 with less lighting period. Chang et al. (2015) reported that a higher lighting period percentage in a cycle resulted in higher algal and bacterial growth with a longer dark phase period. However, as shown in table 2, the presence of higher biomass did not positively affect nitrification, which showed similar rates to those previously reported (Jia et al., 2018). The reason could be due to the self-

shading formed by the increased biomass. Biomass in phase 3 remained stable and did not lead to reduced nitrification or denitrification rate. The lower biomass production in phase 3 was accomplished with the SRT around 10 days, indicating a less bio-waste production compared to 24-hour illumination.

### 5.3.3 Settleability of the biomass

The cost due to biomass harvesting influences the feasibility of consortia application. It is a challenge to capture the dispersedly suspended microalgae for a large-scale treatment. The harvesting process typically involves physical, chemical or biological methods. None of the available industrial methods for harvesting microalgae is cost effective (Muñoz et al., 2006). In this study, floc size in the consortium ranged from 500 to 3000 μm. The bioflocculant algae-bacteria biomass was commonly established in PSBR (Karya et al., 2013, Su et al., 2011, Wang et al., 2015), since the interactions of algae with other microorganisms could favor the formation of bioflocculant consortia (González-Fernández et al., 2013). EPS excreted by bacteria bioflocculant enhances the aggregation of microalgae and bacteria (Gutzeit et al., 2005, Lee et al., 2009). Microorganism species also contributes to the flocculation of biomass. The result of 16sRNA demonstrates that the bacteria community composition of the consortium was very abundant. The presence of phyla Chloroflexi and Planctomycetes contributed to the formation of microbial aggregates (Wang et al., 2017, Fuchsman et al., 2012, Sunja et al., 2010). Filamentous microorganisms were also observed in the cultivation reactors by microscope, it could be represented by some species of algae or Chloroflexi, which typically mediate with other organisms and cause aggregation formation (González-Fernández et al., 2013). The achieved consortium

resulted in an effluent with low TSS concentration (average 33 ±3.4 mg/L), indicating a good settleability.

## 5. Conclusion

The current study used low light intensity of 1000 Lux, HRT of 2 days, and SRT ranging from 5-13 days to treat wastewater loading with 130 mgN/L using PSBR. A daily ammonium removal rate of 60 mgN/L/d was achieved with both photoperiods of 24-hour continuous illumination and 16h/8h L/D. Different with the result in other literature, the nitrification process did not stop in the dark phase of the photoperiod of 16h/8h L/D. Longer lighting period favored the growth of biomass and higher biomass produced 43% more TOC in the same photoperiod. However, the higher biomass results in a lower nitrification rate. In addition, the existing TOC was not sufficient to achieve effective denitrification/dinitritation rates. External organic carbon dosing or wastewater with moderate BOD could be used to enhance nitrogen removal efficiency. The photoperiod of 16h/8h produced less biomass but exhibited same nitrification rate and a higher denitrification/denitritation efficiency. Therefore, this study demonstrates that the use of algae-bacteria consortia to treat $NH_4^+$-N-loaded wastewater could be a sustainable and cost-effective method that requires less energy and it is worth continuing to optimize.

# Chapter 6 ENGINEERING SIGNIFICANCE

The presented research was 1) to estimate the effects of different factors on the performance of algae bacteria consortium on nitrogen removal and 2) to evaluate the feasibility of the PSBR working in low light intensity with two types of photoperiod. This chapter focuses on the engineering and economic significance of the key findings.

## 6.1 Major operation costs in wastewater treatment plant

The worlds' population in 2015 was 7.4 billion and it will keep grow up to 9.2 billion at 2024 as expected (United Nations, 2016). The increasing density of population in cities result in billions of tons of waste a year, including wastewater and WAS. The most widespread treatment in WWTP is biological (activated sludge) treatment which carried out by aerobic and anaerobic stage. In conventional treatment process, the costs for energy usually amount up to 10 – 30 % of the total operation costs. While for aerobic stage, mechanical aeration cost 45–75% of total energy demand in WWTPs (Oilgae, 2010). In addition, fouling issue of the fine bubble diffuser also demonstrate major energy operating cost efficiencies and maintenance cost. Besides, to treat and dispose of WAS, significant energy and a large land area are required. Disposal costs of WAS for a WWTP can differ between 15 and 50 % of the total operation costs. Other operations include personnel and maintenance amount up to 30-65 % of the total operation costs (Wendland, 2016).

## 6.2 Cost saving in electrical requirements

The presented research showed that no external aeration is needed for nitrification in the algae-bacteria consortium system, which means the use of algae-bacteria consortia could reduce the mechanical aeration cost in WWTPs. The nitrification performance with photoperiod of 16h/8h L/D was 60mgN/L in PSBR and similar to that of 24 hours' continuous illumination, while the denitrification/denitritation efficiency was 36% higher with 16h/8h L/D than that of the continuous

illumination. The result indicates that the nature day/ night period is feasible for the treatment which could eliminate the electrical cost for lighting.

### 6.3 Cost saving in bio-waste treatment

WAS is the major by-product of biological treatment. To treat and dispose of WAS, significant energy and a large land area are required. In conventional process. to treat 1 million liters of wastewater, the activated sludge process generates about 70–100 kg dry WAS (Metcalf and Eddy, 2003). In this experiment, Similar $NH_4^+$-N removal rate and higher denitrification efficiency were achieved with phase 3 compared to continuous illumination, but less biomass was produced. Consortium biomass was not wasted and the good settleability resulted in TSS concentration of 33 ± 3.4 mg/L in effluent. The bio-waste production is 1/3 to 2/3 of the WAS production in conventional treatment. This result demonstrate that the natural photoperiod is also applicable for cutting back the treat and dispose cost for bio-waste.

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