Development of advanced light-tolerant microalgae-nitrifying bacteria consortia for ammonia removal under strong light irradiation using light-shielding hydrogel

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Kento Nishi

Contents

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ABSTRACT

Normally, wastewater containing nitrogen is treated through microbial nitrification using aeration, and denitrification. However, in wastewater treatment plants, aeration is an most energy-intensive and costly process and may account for 45–75% of energy consumption. From the viewpoint of cost reduction and energy saving, photosynthetic aeration can be a potential candidate for replacing mechanical aeration. Therefore, it has been attracting great interest to develop economical nitrogen removal process using microalgae-nitrifying bacteria consortia without mechanical aeration recently and many studies have been tried. However, since it is well known that nitrifying bacteria suffer from photoinhibition when light irradiation exceeds a certain limit, this will limit possible light irradiation strength and total nitrification performance. To overcome this issue, a new technique of "light-shielding hydrogel" to immobilize only nitrifying bacteria and mitigate photoinhibition was developed and a highly sophisticated advanced light-tolerant microalgae-nitrifying bacteria consortia (nitrifying system combined with microalgae) is proposed.

In this study, first, "light-shielding hydrogel" in which bacteria were immobilized in hydrogel with light-shielding particles (carbon black) was developed and evaluated its effectiveness to mitigate photoinhibition for bacteria under strong light irradiation. Then, to establish consortia of *Chlorella sorokiniana* and nitrifying bacteria immobilized in light-shielding hydrogel, their nitrification performance under strong light irradiation was evaluated. In these experiments, three nitrifying bacteria conditions were used: light-shielding hydrogel, hydrogel containing only nitrifying bacteria without carbon black ("hydrogel"), and dispersed nitrifier without immobilization ("dispersion") as a control. Furthermore, the effect of as inoculum biomass ratio (microalgae to nitrifying bacteria) on the performance of consortia under strong light irradiation was conducted in a batch experiment using different inoculum biomass ratios.

In the light exposure experiment of only nitrifying bacteria at 1600 μmol photons m^{-2} s^{-1},

the nitrification performance markedly decreased to 15.1 and 48.0% compared to the dark condition in the dispersion and the hydrogel, respectively. Meanwhile, it was successfully maintained for the light-shielding hydrogel. The obtained result indicates that the effectiveness of light-shielding hydrogel to mitigate photoinhibition on nitrifying bacteria even under strong light irradiation.

Next, the developed light-shielding hydrogel was combined with microalgae under strong light irradiation. At 1600 μmol photons m^{-2} s^{-1}, the dispersion afforded a significant decrease in nitrification activity and subsequent process breakdown. In contrast, light-shielding hydrogel achieved complete nitrification without nitrite accumulation and had nitrification rates of approximately nine and two times higher than those for the dispersion and hydrogel conditions, respectively. Based on the overall evaluation, a possible sequence of process breakdown under strong light was also proposed. To take advantage of the easy separation of immobilized nitrifying bacteria and microalgae in this consortium, then, the effect of different biomass ratios on nitrogen removal performance of the consortia was evaluated. The results show the microalgal specific growth rate of 1.3 d^{-1} and the ammonia removal efficiency of 100% was obtained for the biomass ratio of 1:9 (microalgae: nitrifying bacteria) for the condition using the light-shielding hydrogel. Finally, based on the results in this experiment, a new method of controlling the appropriate biomass ratio based on the unique feature of the proposed system of using the light-shielding hydrogel for continuous operation was proposed.

In summary, this study demonstrated for the first time that the light-shielding hydrogel/consortia combination had potential for applications of nitrogen containing wastewater treatment, which require mitigation of photoinhibition under strong light irradiation. Further, it is expected that the proposed method will contribute to realize the practical application of microalgae–nitrifying bacteria consortia in various countries that experience high sunlight intensity due to their location in the sunbelt areas.

Chapter 1

General introduction

1.1 Nitrogen containing wastewater treatment process

With the expansion and diversification of human activities, massive discharge of nutrients causing eutrophication in aquatic environments has been identified worldwide. (McDowell et al., 2020). Notably, the number of sewer users is estimated to increase from 2 to 4 billion between 2010 and 2050 reported by van Puijenbroek et al. (2019). Therefore, it is essential to install more effective wastewater treatment plants and build infrastructure to connect them in order to prevent pollution of the water environment. However, many of the current wastewater treatment plants with conventional biological processes require a lot of energy and disposal of excess sludge. In the nitrogen removal process, nitrogen-containing wastewater such as rejected water after anaerobic digestion, and landfill leachate (Monballiu et al., 2020) are commonly treated with biological nitrification-denitrification processes. This process needs two types of reaction in an aerobic tank for nitrification and an anoxic tank for denitrification. An aerobic tank needs mechanical aeration to oxidize from ammonia to nitrate. In an anoxic tank, oxidized nitrate and/or nitrite were reduced by adding an organic substance, and finally, nitrogen gas was released into the air. (Figure 1-1(a); Ruiz et al., 2006). Throughout the wastewater treatment plant, the mechanical aeration for nitrification accounts for a large proportion of the energy consumption with a percentage of 75% (Rosso et al., 2008). This high operating cost is an impediment to the installation of new treatment plants, especially in developing countries. Therefore, there is a need to improve the configuration of conventional wastewater treatment plants with cost- and energy-saving approaches toward environmental and economic sustainability.

To reduce the high cost of aeration, anaerobic ammonium oxidation (ANAMMOX) process and partial nitrification/ANAMMOX (PN/A) process have been investigated. ANAMMOX bacteria are microorganisms that can directly reduce NH_4^+ and NO_2^- to N_2 and can maintain high nitrogen

removal activity under anaerobic conditions of 0.1 mg-O_2 L^{-1} (Wu et al., 2018). The PN/A process requires low DO concentrations using ammonia oxidizing bacteria (AOB) for partial oxidation of NH_4^+ to NO_2^- in addition to ANAMMOX bacteria, allowing 42% reduction of aeration costs compared to the conventional processes (Bae et al., 2015). Further, a more advanced treatment methods utilizing biofilm formation, granulation, or gel immobilization of microorganisms have attracted attention for its advantages in reducing bacterial stress and realizing high nitrogen removal performance and bacterial growth. The use of attached bacteria is an effective means of maintaining biomass and preventing washout of slow-growing nitrifying bacteria and ANAMMOX. However, although the ANAMMOX process helps to reduce oxygen requirements, the necessity of more advanced and sophisticated operation control and the production of excess sludge are the remaining challenges.

1.2 Recent studies with microalgae-bacterial consortia for wastewater treatment

Whilst, using the microalgae-bacterial consortium for nitrogen containing wastewater treatment has attracted attention owing to its advantages such as its energy- and cost-efficiency (Figure 1-1(b)). The wastewater treatment using microalgae can efficiently treat various wastewater containing abundant nutrients including not only nitrogen but also phosphorus due to microalgae uptake (Li et al., 2019). In addition, microalgae-bacterial consortia have the following attractive advantages; (i) they can efficiently treat a variety of wastewater with wider C/N/P ratios (Chiu et al., 2015); (ii) highly tolerant to harsh wastewater environments and successfully treats complex wastewater. (He et al., 2013); (iii) the grown microalgae biomass can subsequently be used for other applications such as biofuel production and/or fertilizer. (Salama et al., 2017).

Microalgae and nitrifying bacteria have different roles in the ammonia removal process (Gonçalves et al., 2017). As shown in Figure 1-2, ammonia in wastewater is removed by two pathways that is uptake by microalgae and nitrification by nitrifying bacteria. Nitrifying bacteria use

produced O_2 by microalgal photosynthesis for nitrification, thus eliminating mechanical aeration-associated costs, and thereby significantly reducing the total operation cost. The interrelationship of increasing and decreasing pH effects derived from microalgae photosynthesis and nitrification processes typically helps the consortia maintain a stable pH environment in the reactor (Jia and Yuan, 2016). Furthermore, the competitive interactions between microalgal uptake and nitrification by nitrifiers allows rapid ammonia removal as compared to microalgae monoculture (Rada-Ariza et al., 2017).

In recent years, the effects of various factors such as wastewater type (Wirth et al., 2020), process conditions (Zhang et al., 2020), and light exposure (Kang et al., 2018) have been investigated in microalgae-bacteria consortium (Delgadillo-Mirquez et al., 2016; González-Camejo et al., 2019). However, the effects of light intensity, particularly intense light intensity comparable to sunlight, on the consortium have rarely been examined, even though it is an important parameter for successful process toward practical application. In outdoor environment, the high-rate algal pond (HRAP) reactor is typically used for wastewater treatment and cultivation by microalgae as it needs a large surface area and low depth (0.2–0.4 m) to obtain light efficiently and can be operated with low energy of 1.5–8.4 W m^{-3} (Mendoza et al., 2013). However, it has been known that the sunlight intensity varies depending on the region, time, and season. Hence, the high photosynthetically active radiation (PAR) reaches approximately over 2250 μmol photons m^{-2} s^{-1} in some places (Arthurs et al., 2013), and this may negatively affect the microalgae-nitrifying bacteria consortium in HRAP reactors with shallow depths.

1.3 Issue of microalgae-nitrifying bacteria consortia under strong light irradiation

In spite of its importance, the reason for the limited number of research on the consortia under strong light could be the difficulty of stable process operation, especially owing to the photoinhibitory effect on nitrifying bacteria. Nitrifying bacteria are generally more sensitive to light

than microalgae. The specific growth rate of microalgae *Chlorella sorokiniana* increases up to 250 μmol photons m^{-2} s^{-1}, however, decreases gradually at higher light intensities (Kumar et al., 2014). Conversely, Merbt et al. (2012) reported that the growth inhibition of nitrifying bacteria, such as ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB), begin at 60 μmol photons m^{-2} s^{-1}. They also found that nitrite-oxidizing bacteria (NOB) are more sensitive to light than AOB.

Recently, Arun et al. (2021) investigated the effect of intense light up to 2000 μmol photons m^{-2} s^{-1} on microalgae-bacterial consortia and reported that a significant decrease in ammonia removal efficiency to 6.2% at light intensity of 1500 μmol photons m^{-2} s^{-1}. It was also reported that nitrifying bacteria photoinhibition reduced nitrification activity by 70% at 1600 μmol photons m^{-2} s^{-1} as compared to the dark condition, although the presence of sufficient oxygen in dispersed microalgae–nitrifying bacteria consortia (Akizuki et al., 2020a). These studies clearly exhibited the need for developing new technique to protect nitrifying bacteria from strong light irradiation.

Therefore, to mitigate the photoinhibition, new approach of immobilizing and shading only the nitrifying bacteria in the consortia is proposed. As mentioned above, immobilization for bacteria has the advantage of protecting microorganisms from external stresses. In addition to immobilization, it is expected that the addition of black powder inside hydrogel as light-shielding material provides more protection of nitrifying bacteria from strong light exposure, as shown in Figure 1-3. Hereinafter, this hydrogel is referred to as “Light-shielding hydrogel” in which nitrifying bacteria is immobilized and protected from light exposure. If the light-shielding hydrogel effectively mitigate photoinhibition, it is expected that combining the light-shielding hydrogel with the microalgae–nitrifying bacteria consortia could establish a process capable of outdoor operation, even under intense light irradiation (Figure 1-1(c)).

1.4 Objective of this study

The objective of this study is to develop an advanced light-tolerant microalgae-nitrifying bacteria consortia for stable ammonia removal under strong light irradiation using light-shielding hydrogel, and to examine the effectiveness of the proposed system to establish a new low-cost and energy efficient nitrogen containing wastewater treatment process in countries that experiences high sunlight intensity due to their location in the sunbelt areas.

To achieve these goals, first, the preparation method of light-shielding hydrogel was established and its effectiveness to mitigate photoinhibition of nitrifying bacteria under strong light irradiation was examined prior to testing it in the microalgae–nitrifying bacteria consortium system (Chapter 2). Subsequently, the effects of the light-shielding hydrogel on nitrification and ammonia removal performance were evaluated in microalgae–nitrifying bacteria consortia under light irradiation between 0 and 1600 μmol photons m^{-2} s^{-1} to establish as new light-tolerant microalgae-nitrifying bacteria consortia (Chapter 3). Based on the obtained results, the secondary effects caused by photoinhibition were clarified, and a mechanism of consortia breakdown under intense light irradiation was also proposed in the chapter. To improve the ammonia removal performance of the proposed process, the effect of inoculum biomass ratio between microalgae and nitrifying bacteria on the nitrification performance was investigated (Chapter 4). Finally, the appropriate operation method for stable and continuous treatment of nitrogen containing wastewater by using the proposed light tolerant microalgae-nitrifying bacteria consortia was proposed and its cost analysis compared with the conventional process was conducted (Chapter 5).

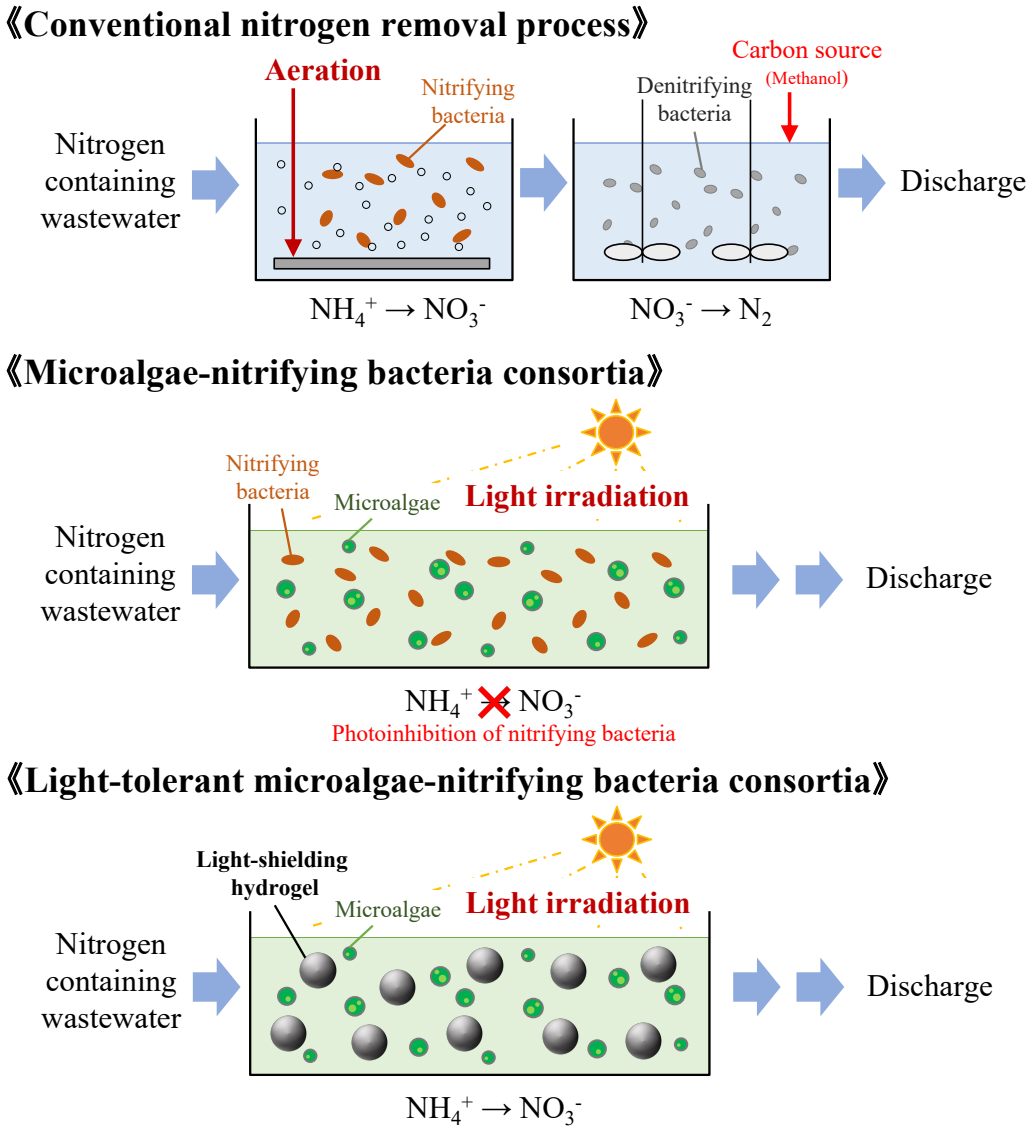

Figure 1-1 (a) (top) Conventional costly biological nitrification-denitrification process;

(b) (middle) Microalgae-nitrifying bacteria consortia under strong light;

(c) (bottom) Proposed light-tolerant microalgae-nitrifying bacteria consortia.

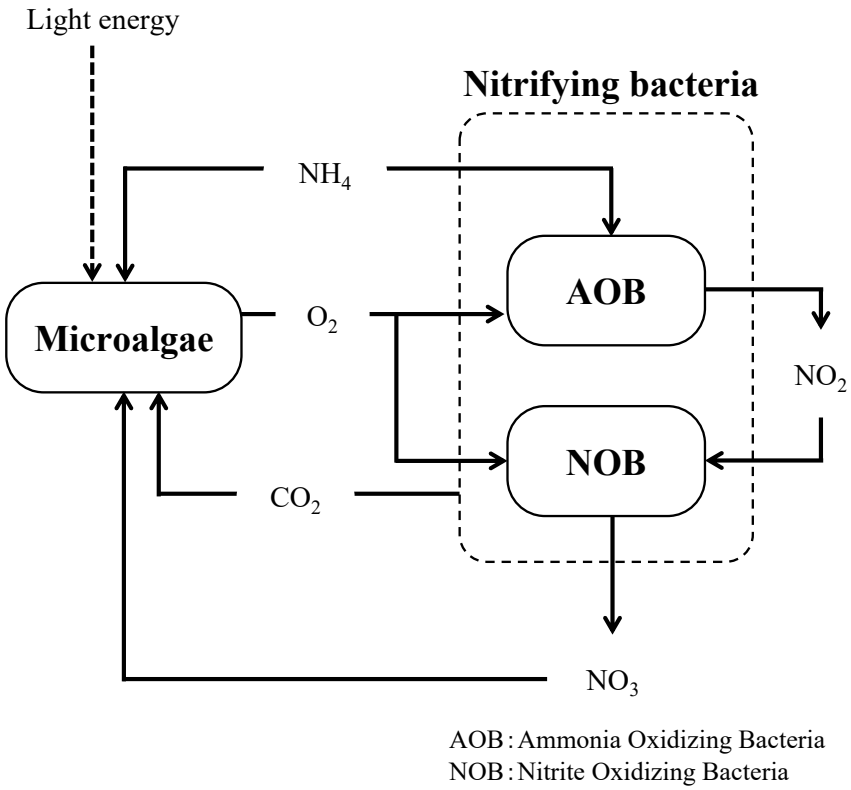

Figure 1-2 Nitrogen cycle by microalgae and nitrifying bacteria in microalgae-nitrifying bacteria consortia.

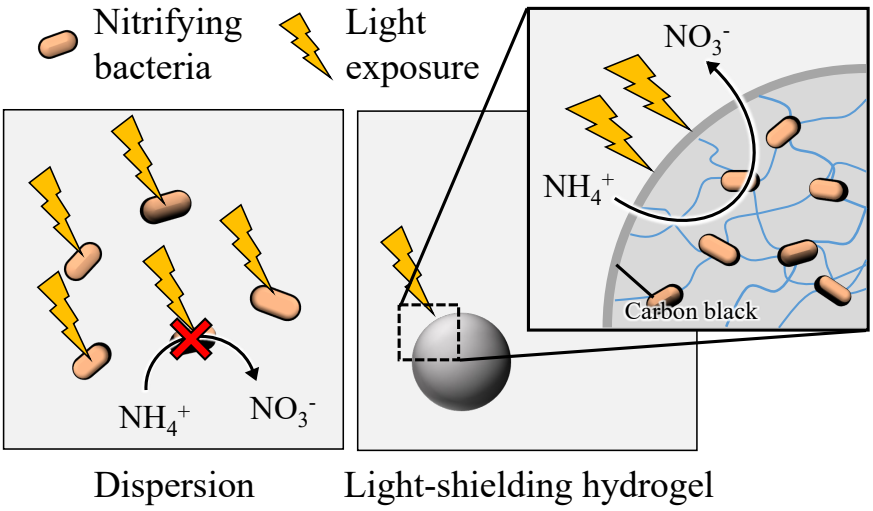

Figure 1-3 Dispersed nitrifying bacteria and

immobilized nitrifying bacteria in light-shielding hydrogel.

Chapter 2
Development of light-shielding hydrogel

2.1 Introduction

Recently, the wastewater treatment process with microalgae-bacterial consortium has gained attention because photooxygenation by microalgae can supply oxygen to bacteria, no need for costly mechanical aeration operations (Solimeno et al., 2017). It has been reported that microalgal growth increase under intense light irradiation up to 2000 μmol photons m^{-2} s^{-1} (Zhao et al., 2013), while nitrifying bacteria easily suffer from photoinhibition because of inactivation of ammonia monooxygenase (AMO) activities (Merbt et al., 2012). Wang et al. (2015) reported that the nitrification process in microalgae-nitrifying bacteria consortium was completely inhibited at 300 μmol photons m^{-2} s^{-1}, and the relatively low light intensity of 74 ± 5 μmol photons m^{-2} s^{-1} was recommended as the optimum intensity. In another study, the photoinhibition of nitrifying bacteria was confirmed above 60 μmol photons m^{-2} s^{-1} (Merbt et al., 2012). In order to provide a low-cost microalgae-bacteria consortia system for wastewater treatment, open and shallow reactors such as high-rate algal ponds (HRAP) are commonly used (Passos et al., 2014). However, sunlight irradiance in developing countries such as the Sunbelt region often exceeds 2000 μmol photons m^{-2} s^{-1} as mentioned above, making light inhibition of nitrifying bacteria activity likely to occur when this reactor is used under outdoor environment. Therefore, the supplying light-tolerant ability to microalgae-nitrifying bacteria consortium is needed. Key point to achieving such a consortium is the coexistence of microalgae and nitrifying bacteria in the same reactor, while the former needs to receive light and the latter must avoid light.

It has been reported that the granule form could mitigate photoinhibition on nitrifiers (Arcila et al., 2017; Akizuki et al., 2020b) and the use of nitrifier granules as an inoculum would be available for mitigating light inhibition. However, aggregation of dispersed nitrifiers normally requires more

than a few months (Kim and Seo, 2006). In addition, granule size is an important parameter that varies light penetration into the granules, and it should be controlled with an appropriate size. However, the granule size fluctuates easily depending on ambient conditions, wastewater types, and inoculum sources (Pishgar et al., 2019). To solve those problems, the concept of "Light-shielding hydrogel", in which only nitrifying bacteria are encapsuled in a light-shielding material added hydrogel is proposed in this study to mitigate photoinhibition of nitrifying bacteria. In this method, preparation of light-shielding hydrogel can be achieved quickly, and the hydrogel size can also be controlled easily. Since it is already known that by immobilizing bacteria in the hydrogel, bacteria can be maintained at a high concentration without leakage, the resistance of bacteria to pH and toxic substances can also be improved (Bouabidi et al., 2019).

Prior to testing the effectiveness of light-shielding hydrogel in the microalgae-nitrifying bacteria consortia, its effectiveness needs to be proved by using only bacteria under strong light irradiation. Although microbial immobilization was used with various polymers, (Sumino et al., 1992; Xu et al., 2017; Li-sheng et al., 2007), sodium alginate was selected as one of the most simple and easy ways to prepare. As a light-shielding material, carbon black was chosen because of its excellent light absorption property (Khajeh Mehrizi et al., 2012).

This chapter aims to develop light-shielding hydrogel, in which nitrifying bacteria were immobilized, by using sodium alginate as a polymer and carbon black as a shielding material. Then, the nitrification performance of the resulting samples was evaluated under a wide range of light irradiation in a batch test.

2.2 Materials and methods

2.2.1 Preparation of light-shielding hydrogel

Nitrifying sludge was obtained from the full-scale anaerobic-anoxic-oxic (A2O) treatment plant of Yokohama Hokubu Sludge Treatment Center in Kanagawa, Japan. The concentrated

nitrogen-containing synthetic wastewater composed of 2.4 g L^{-1} $(NH_4)_2SO_4$, 1.9 g L^{-1} NH_4Cl, 2.8 g L^{-1} KH_2PO_4, 2.0 g L^{-1} $MgSO_4$, 2.0 g L^{-1} NaCl, 17.5 g L^{-1} $NaHCO_3$, and 1.28 g L^{-1} $CaCl_2 \cdot 2H_2O$.

Light-shielding hydrogel in which immobilize with alginate hydrogel was prepared by following procedure: (1) Concentrated nitrifying sludge was obtained at 7.9 g-suspended solids (SS) L^{-1} of concentration by centrifuge at 3000 rpm for 10 min. (2) 2.0 wt% sodium alginate (Wako, Japan) was dissolved in deionized water, and concentrated nitrifying sludge was added in sodium alginate solution. (3) Then, carbon black powder (Yoneyama Yakuhin Kogyo, Japan) of 0.1 wt% was added to the mixture solution. (4) The resulting mixture solution was dripped into 1.0 wt% calcium chloride solution. (5) The resulting hydrogel beads were stored in calcium chloride solution at 25 ºC for 6 h. The resulting sample was referred to as "Light-shielding hydrogel". To evaluate the effect of immobilization and light-shielding, another type of immobilized nitrifying sludge excluding light-shielding materials (Carbon Black) was prepared by skipping step (3). The resulting sample was referred to as "Hydrogel". In addition to the light-shielding hydrogel and the hydrogel, the suspended sludge without immobilization in the hydrogel was used as a control, and was referred to as "Dispersion".

2.2.2 Light irradiation experimental setup

The effect of different light intensities on the nitrification performance of the resulting three sludge samples was examined by a batch test. Concentrated synthetic wastewater was diluted and adjusted to 50 mg-N L^{-1} of NH_4^+ concentration. The light-shielding hydrogel or hydrogel or dispersion were added to each serum bottle with an effective volume of 100 mL, respectively. The suspended solids (SS) concentration of nitrifying sludge in each bottle was set to 0.5 g L^{-1} for all the conditions. Pure oxygen gas was supplied to the bottles for 5 min to sufficiently saturate oxygen in the water. The samples were irradiated using a customized LED light irradiation device (Iida Lightning Co., Ltd., Japan). The Color Rendering Index (CRI) of the LED device presented 85%

similarity to CRI of daylight. The spectrum of the LED device and daylight are shown in Figure 2-1. The incident light intensities into each bottle were adjusted to 0 (as a control), 100, 450 and 1600 μmol photons m^{-2} s^{-1}. The three different light intensities were controlled by shading the bottles in three ways as shown in Figure 2-2; 100% of LED light without shading net (corresponding to 1600 μmol photons m^{-2} s^{-1}), about 30% of LED light with two layers of shading net (corresponding to 450 μmol photons m^{-2} s^{-1}), and around 6% of LED light with four layers of shading net (corresponding to 100 μmol photons m^{-2} s^{-1}). The bottle as a control (0 μmol photons m^{-2} s^{-1}) was sealed with aluminum foil to prevent light exposure. The samples were incubated at 25 ± 1 ºC for 12 h and shaken at 180 rpm. The experiments at each condition were conducted in triplicate.

2.2.3 Analytical methods

The suspended solids concentration of nitrifying sludge was measured according to a method from Japan Sewage Works Association. NH_4^+-N, NO_3^--N and NO_2^--N concentrations were analyzed by high-performance liquid chromatography at 40 ˚C (HPLC, Shodex Co. Ltd., electrical conductivity detector: CD-5). The cation column (IC YS-50, Shodex, Japan) and anion column (IC I-524A, Shodex, Japan) for HPLC was used.

The NO_3^- concentration under dark condition after 12 h was regarded as 100%, and the standardized nitrification performance was calculated based on the NO_3^- concentration at each light intensity after 12 h and at the dark condition using the following Eqn. 2-1;

$$\text{Standardized nitrification performance (\%)} = \frac{\text{Amount of } NO_3^- \text{ at } [LI] \text{ condition}}{\text{Amount of } NO_3^- \text{ at dark condition}} \times 100 \qquad (2\text{-}1)$$

where [*LI*] is each light intensity (μmol photons m^{-2} s^{-1}).

2.3 Results and Discussion

2.3.1 Ammonia removal and nitrification performance

The change of time of NH_4^+-N and NO_x^--N (NO_2^--N plus NO_3^--N) concentrations for all the batch conditions are shown in Figure 2-3. The dissolved oxygen (DO) was sufficient for nitrification under all conditions. In the dispersion, a gradual decrease in NH_4^+ concentration was observed at 0, 100 and 450 μmol photons m^{-2} s^{-1} throughout the experiment, and the decrease rate was almost the same (Figure 2-3A). Meanwhile, the significantly slower decrease of NH_4^+ was observed at the 1600 μmol photons m^{-2} s^{-1}. In the hydrogel, the NH_4^+ concentration decreased much faster than the dispersion for all the light intensity conditions (Figure 2-3B). Among the different light intensities in the hydrogel, a similar trend in NH_4^+ removal was observed during the first 6 h. However, thereafter the NH_4^+ removal slowed down considerably only at 1600 μmol photons m^{-2} s^{-1}. It is likely that these results in strong light intensity were due to photoinhibition. In the light-shielding hydrogel (Figure 2-3C), the NH_4^+ concentration also decreased faster than the dispersion, which was a similar trend for the hydrogel. Moreover, a very effective NH_4^+ removal comparable to the dark condition was achieved even at 1600 μmol photons m^{-2} s^{-1}. In the dispersion, NO_x^- production clearly decreased with the increase of light intensity (Figure 2-3A′). This tendency is different from that of NH_4^+ removal in the case of the dispersion. This result indicates that within the two steps of the nitrification process of "NH_4^+ to NO_2^- by ammonia-oxidizing bacteria (AOB)" and "NO_2^- to NO_3^- by nitrite-oxidizing bacteria (NOB)", strong light irradiation would inhibit the latter step. It means NOB is more sensitive to the strong light, which agrees with the results of previous study (Merbt et al., 2012). In the hydrogel, although an effective NO_x^- production was shown below 450 μmol photons m^{-2} s^{-1}, a remarkable decrease in the production occurred at 1600 μmol photons m^{-2} s^{-1} (Figure 2-3B′). This implies that the immobilization into the hydrogel has a stronger tolerance by light stress in AOB and NOB than the dispersion. Since the hydrogel is transparent without carbon black, the hydrogel does not have light-shielding effect. Therefore, this higher light tolerance is probably due to a higher

concentration of nitrifying bacteria in the hydrogel than in the dispersion. On the other hand, stable NO_x^- production was observed in the light-shielding hydrogel for all the light intensities up to 1600 μmol photons m^{-2} s^{-1} (Figure 2-3C′).

2.3.2 NO_2 accumulation by photoinhibition

Figure 2-4 shows the proportion of NO_2^- in NO_x^- at different types of sludge conditions after 12 h. In the dispersion, the proportion of NO_2^- increased with increasing light intensity. This result is different from the NH_4^+ removal (Figure 2-3A), in which there was no essential difference in the concentration decrease in the light intensity up to 450 μmol photons m^{-2} s^{-1}. The difference means that the NO_2^- accumulation started to occur already at 450 μmol photons m^{-2} s^{-1} despite the NH_4^+ consumption. As mentioned in the previous section, this finding implies that NOB are more sensitive to photoinhibition than AOB, and the photoinhibition of NOB occurs above 450 μmol photons m^{-2} s^{-1} at least. However, at 1600 μmol photons m^{-2} s^{-1}, both AOB and NOB seem to suffer from photoinhibition in the dispersion. Yang et al. (2022) reported that in their experiments where nitrifying bacteria were exposed to light at a combination of 200–2000 μmol photons m^{-2} s^{-1} light intensity, 1–6 h irradiation time, and in 2000–5000 mg-MLVSS L^{-1}, high specific light energy density (*Es*) affected photoinhibition of AOB and NOB. The results showed that electron transport system activity (ETSA) of AOB and NOB decreased by 6.6% and 23.9%, respectively, resulting in significant damage to the electron transport system in both bacteria. Normally, nitrifying sludge has three absorption peaks at 280 nm (proteins and nucleic acids), 408 nm (cytochrome-c), and 603 nm (cytochrome aa_3). Guerrero (1997) reported a positive correlation between cytochrome-c and photoinhibition at 408 nm among these wavelengths. Yang et al. (2022) clarified that the increase in absorption at 603 nm as well as at 408 nm found for the photo-inhibited nitrifying bacteria was correlated to the oxidation of cytochrome-c and cytochrome aa_3 and concluded that the damage of these cytochrome-c and cytochrome aa_3 could be the mechanism of photoinhibition of nitrifying

bacteria.

In the hydrogel, the proportion of NO_2^- was very small up to 450 μmol photons m^{-2} s^{-1} and then increased to about 15% at 1600 μmol photons m^{-2} s^{-1}. Therefore, photoinhibition was mitigated up to 450 μmol photons m^{-2} s^{-1} in the case of the hydrogel. On the other hand, NO_2^- was almost negligible at all the light intensities in the light-shielding hydrogel. This result demonstrates that both AOB and NOB were successfully protected from intense light even up to 1600 μmol photons m^{-2} s^{-1} by immobilizing bacteria in hydrogel and incorporating light-shielding particles.

2.3.3 Standardized nitrification performance under different light intensities

Figure 2-5 shows the relationship between light intensity and standardized nitrification performance. The standardized nitrification performance in the dispersion decreased monotonously as the light intensity increased. The same tendency was reported in previous research reported by Merbt et al. (2012) and Vergara et al. (2016) as shown in the same Figure. The standardized nitrification performance in the hydrogel was higher than that in the dispersion at all light intensities. The standardized nitrification performance in the hydrogel and the light-shielding hydrogel did not decrease at 450 μmol photons m^{-2} s^{-1}. However, at 1600 μmol photons m^{-2} s^{-1}, the standardized nitrification performance in only the hydrogel decreased by up to 48.0%. The result of the hydrogel is coincident with the previous study that granulated nitrifiers represented high tolerant to light irradiation especially below 450 μmol photons m^{-2} s^{-1} (Akizuki et al., 2020). It is worth noted that the standardized nitrification performance was hardly affected by strong light irradiation up to 1600 μmol photons m^{-2} s^{-1} in the case of the light-shielding hydrogel.

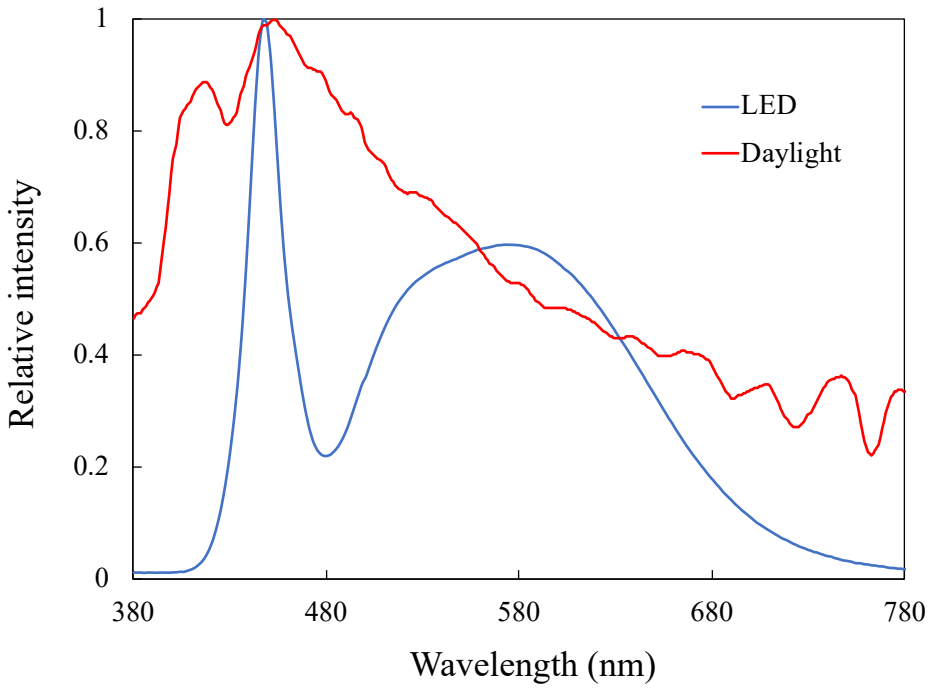

Figure 2-1 Spectrum of LED emission and daylight emission

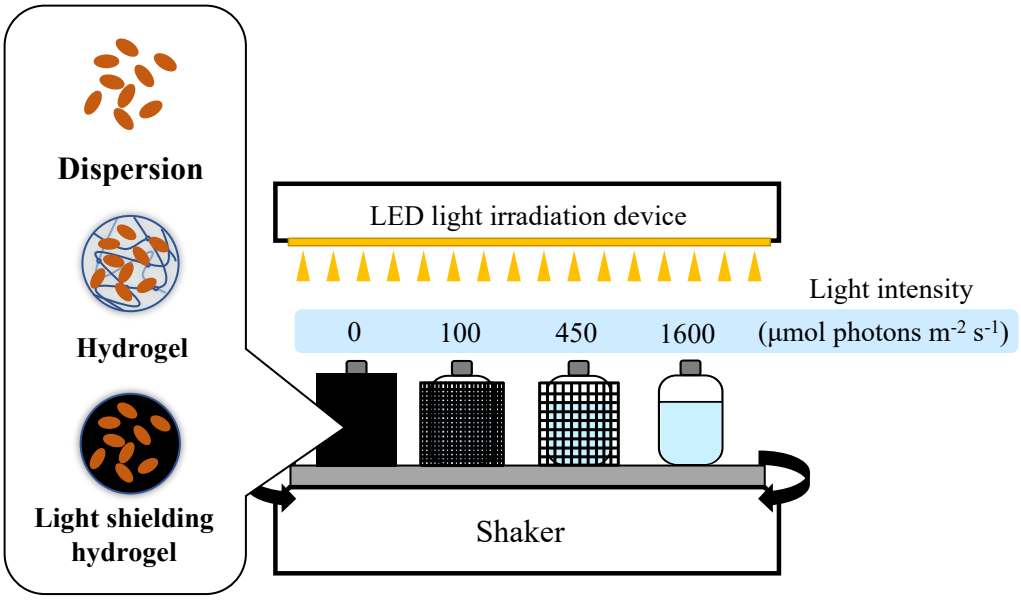

Figure 2-2 Experimental set up for light irradiation test with different light intensities on three nitrifying sludge samples of dispersion, hydrogel and light-shielding hydrogel.

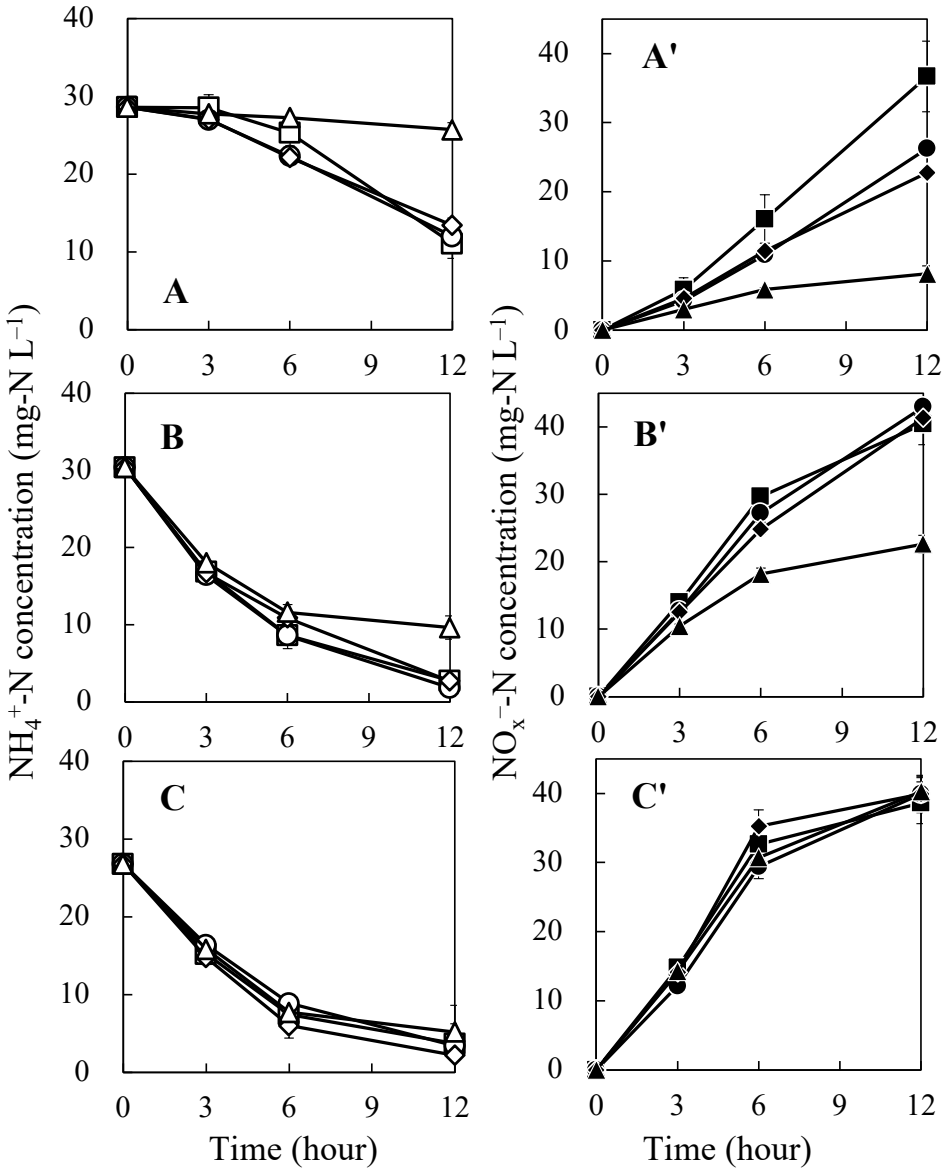

Figure 2-3 Time course change of NH_4^+-N and NO_x^--N concentration under different light intensity for various nitrifying sludge conditions.

A: Dispersion; **B**: Hydrogel; **C**: Light-shielding hydrogel

Light intensity (μmol photons m^{-2} s^{-1}): 0(□), 100(◯), 450(◇), 1600(△)

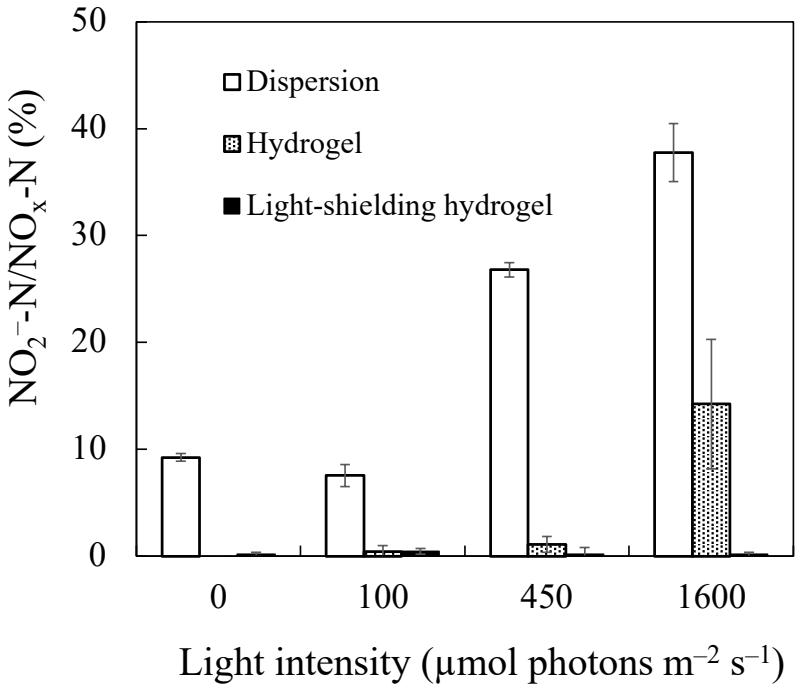

Figure 2-4 Proportion of NO_2^--N in NO_x-N at the end of experiment.

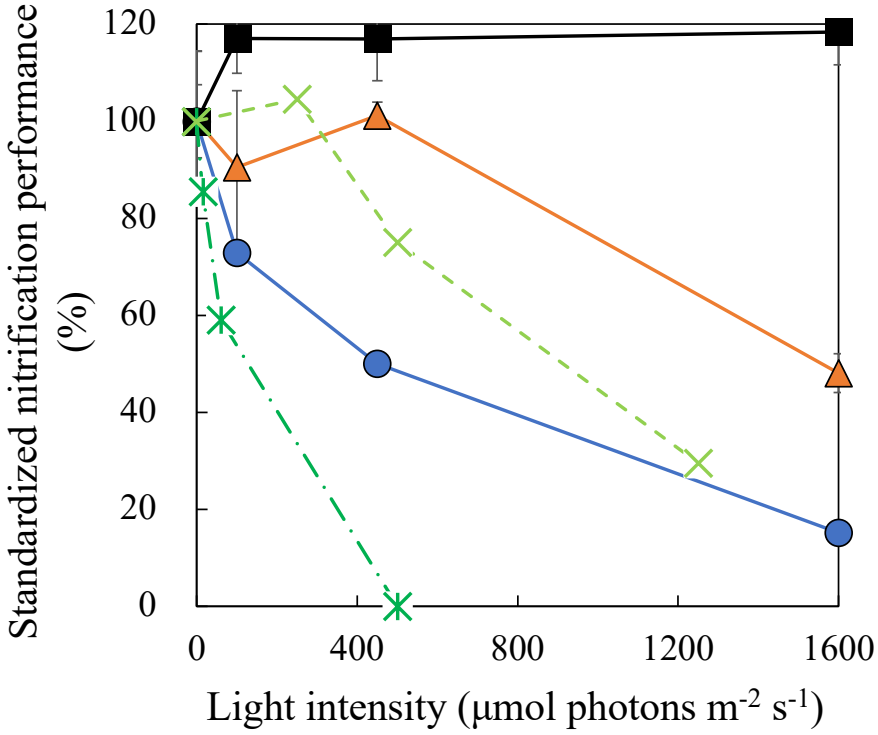

Figure 2-5 Standardized nitrification performance (regards as 100% at 0 μmol photons m^{-2} s^{-1}).

Dispersion (◯); Hydrogel (△); Light-shielding hydrogel (□);

nitrifying bacteria and *C. sorokiniana* (×) reproduced from Vergara et al. (2016);

N. maritimus (＊) reproduced from Merbt et al. (2012)

Chapter 3

Establishment of light-tolerant microalgae-nitrifying bacteria consortia

3.1 Introduction

To mitigate the photoinhibition of nitrifying bacteria, a new “Light-shielding hydrogel” was developed and its effectiveness was confirmed under intense light irradiation of up to 1600 μmol photons m^{-2} s^{-1} in the chapter 2. Therefore, it is hypothesized that combining then nitrifying bacteria immobilized in light-shielding hydrogel with the microalgae could establish the consortium process capable of outdoor operation even under intense light irradiation.

In this chapter, the microalgae-nitrifying bacteria using light-shielding hydrogel was tried to establish and evaluated its effect on nitrification and ammonia removal performance under light intensity range of 0–1600 μmol photons m^{-2} s^{-1}. Although the sunlight intensity can reach more than 2000 μmol photons m^{-2} s^{-1}, photoinhibition starts to appear above 250 μmol photons m^{-2} s^{-1} for microalgae and above 60 μmol photons m^{-2} s^{-1} for nitrifying bacteria. Therefore, the light intensity of 1600 μmol photons m^{-2} s^{-1} is strong enough to evaluate the effect of strong exposure on the proposed system. In addition, light attenuation inside the light-shielding hydrogel was experimentally examined by measuring light transmission through a mixture of comparable materials and concentrations. Finally, based on the obtained results, the secondary effects by photoinhibition for nitrifying bacteria were clarified, and a mechanism of consortium breakdown under strong light irradiation was proposed.

3.2 Materials and methods

3.2.1 Inoculates and cultivation media

Nitrifying sludge was obtained from Yokohama Hokubu Sludge Treatment Center in

Kanagawa, Japan. The sludge was collected from a full-scale wastewater treatment plant which used the A2O process. The light-shielding hydrogel was prepared with 0.1 wt% carbon black added as a light-shielding material following the procedure described in Chapter 2. The freshwater microalgae *Chlorella sorokiniana* (NIES-2173) was selected from the National Institute for Environmental Studies (NIES), Tsukuba, Japan. The concentrated ammonium-containing synthetic wastewater comprised of 2.4 g L^{-1} $(NH_4)_2SO_4$, 1.9 g L^{-1} NH_4Cl, 2.8 g L^{-1} KH_2PO_4, 2.0 g L^{-1} $MgSO_4$, 2.0 g L^{-1} NaCl, 17.5 g L^{-1} $NaHCO_3$, 1.28 g L^{-1} $CaCl_2 \cdot 2H_2O$, and trace metal solution (3 mL). The components of the trace metal solution were as follows; 1.0 g L^{-1} $Na_2EDTA \cdot 2H_2O$, 200 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 36 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 10.4 mg L^{-1} $ZnCl_2$, 4.0 mg L^{-1} $CoCl_2 \cdot 6H_2O$, and 2.5 mg L^{-1} $NaMoO_4 \cdot 2H_2O$.

3.2.2 Experimental conditions for batch test

The light-irradiation batch test is illustrated in Figure 3-1. The nitrification performance of the three different types of sludge, i.e., “light-shielding hydrogel”, “hydrogel”, and dispersed sludge without immobilization (“dispersion”), was examined under various light intensities. The experiment was conducted using serum bottles with 100 mL of working volume capped with a rubber stopper and covered with an aluminum seal. The initial ammonium concentration was set to 50 mg-N L^{-1} by diluting the concentrated synthetic wastewater. The nitrifying sludge and microalgae were added to achieve 0.5 and 0.3 g-SS L^{-1} in each bottle, respectively (biomass ratio of nitrifying bacteria to microalgae was approximately 5:3). Nitrogen gas was supplied for 5 min to expel dissolved oxygen from the bottle before sealing. The initial pH was set to 8.0 with 1 M HCl solution. A customized light-emitting diode (Iida Lightning Co., Ltd., Japan) was used as a light source. The incident light intensity was adjusted to 0, 100, 450, and 1600 μmol photons m^{-2} s^{-1} using a shading net as in Chapter 2. To achieve 0 μmol photons m^{-2} s^{-1} for the control, the bottles were wrapped with aluminum foil to block light exposure. All the serum bottles were maintained at 25 ± 2 °C and shaken at 180 rpm for 24 h. Each experimental condition was conducted in triplicate.

3.2.3 Analysis

The pH and dissolved oxygen (DO) concentrations were measured using a pH meter (9625-10D, Horiba, Japan) and a DO meter (9520-10D, Horiba, Japan). Following the sewage analysis method of the Japan Sewage Works Association (1997), the SS concentration was measured after filtering and drying well in a dryer at 105°C. After a sample was filtered through a 0.45 μm filter (GC-50, Advantec, Taiwan), the nutrients such as ammonium (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) was measured using high-performance liquid chromatography (Shimazu, Japan) with a Shodex column (IC YS-50, IC NI-424). The operation was set at 5.6 MPa for pressure and 40°C of the column oven temperature.

3.2.4 Estimation of light transmission within the light-shielding hydrogel

Since the light-shielding hydrogel was spherical (3.0 mm) and small, the transmitted light inside the hydrogel bead was difficult to measure. Therefore, a mixed solution of alginate (1.0%) and carbon black (0.1%) at the same concentrations as in the light-shielding hydrogel was prepared. The light attenuation inside the hydrogel was estimated by measuring its transmitted light intensity. The mixture solutions with thicknesses of 1.0, 1.5, and 2.0 mm towards the light path were irradiated using the LED light source at 1600 μmol photons m^{-2} s^{-1}. The transmitted light intensity through the mixture solution was measured using a light analyzer (LA-105, NK system, Japan).

3.2.5 Calculations

The amount of nitrogen uptake by microalgae, ΔN_{algae}, was calculated based on the mass balance of nitrogen using the measured concentrations of the three types of nitrogen. Due to its lower energy requirement for the cell, microalgae preferentially take up ammonium rather than nitrite and nitrate (Glibert et al., 2016), hence the nitrogen uptake by microalgae was calculated using the

following equation:

$$\Delta N_{algae} = \Delta C_{NH_4} - (\Delta C_{NO_2} + \Delta C_{NO_3}) \quad (3\text{-}1)$$

Where ΔN_{algae} is the amount of nitrogen uptake by the microalgae (mg L^{-1}), ΔC_{NH_4} is the decremental change in NH_4^+-N concentration (mg-N L^{-1}), and ΔC_{NO_2} and ΔC_{NO_3} are incremental changes in NO_2^--N and NO_3^--N concentrations (mg-N L^{-1}) in the batch experiments, respectively. However, since nitrate uptake by microalgae is initiated upon ammonium depletion, Eqn. (3-1) may not be applicable under low ammonia concentrations.

The nitrification rates were calculated as follows:

$$\text{Nitrification rate (mg-N } L^{-1} \text{ } h^{-1}) = \frac{(C_t - C_0)}{t} \quad (3\text{-}2)$$

where t is time (h), C_t is the NO_3^--N concentration at t (mg-N L^{-1}), and C_0 is the initial NO_3^--N concentration (mg-N L^{-1}).

Free ammonia (FA) was calculated as follows (Anthonisen et al., 1976):

$$\text{FA (mg } L^{-1}) = \frac{17}{14} \times \frac{[NH_4^+\text{-N}] \times 10^{pH}}{\exp\left(\frac{6344}{273+T}\right) + 10^{pH}} \quad (3\text{-}3)$$

where [NH_4^+-N] is the NH_4^+-N concentration (mg-N L^{-1}), and pH and T are the pH value and temperature (°C) in the bottles at the experimental endpoint, respectively.

The amount of volatilized ammonia in the bottles was calculated according to the following equation (modified after Zimmo et al. (2004)):

$$\text{Amount of volatilized ammonia (g)} = \sum_{i=1}^{n} \{ SA \times (3.3 \times FA + 4.90) \} \quad (3\text{-}4)$$

where SA is the surface area in each bottle (2.12×10^{-3} m^2, in this study), FA is the free ammonia concentration (mg L^{-1}), and n is the experimental time (day).

The amount of nitrogen assimilated into nitrifiers ($N_{nitrifier}$) during the nitrification reaction was calculated stoichiometrically using the following nitrification reaction (Mara, 2004):

$$NH_4^+ + 1.32\ O_2 + 1.98\ HCO_3^- + 0.98\ H_2O \rightarrow 0.021\ C_5H_7NO_2 + 0.98\ NO_3^- + 2.02\ H_2O + 1.88\ H_2CO_3 \quad (3\text{-}5)$$

The amount of ammonium uptake by microalgae and others at the experimental endpoint was calculated as follows:

$$\text{Amount of ammonia uptake by microalgae and others (mg L}^{-1}) = [NH_4^+\text{-}N]_{initial} - ([NH_4^+\text{-}N]_{end} + [NO_2^-\text{-}N]_{end} + [NO_3^-\text{-}N]_{end} + \text{Volatilized ammonia} + N_{nitrifiers}) \quad (3\text{-}6)$$

where $[NH_4^+\text{-}N]_{initial}$ is the initial NH_4^+-N concentration (mg-N L^{-1}); $[NO_2^-\text{-}N]_{end}$, $[NO_2^-\text{-}N]_{end}$, and $[NO_3^-\text{-}N]_{end}$ are the NH_4^+N, NO_2^--N, and NO_3^--N concentrations at the experimental endpoint, respectively (mg-N L^{-1}). Volatilized ammonia is the amount of volatilized ammonia in bottles (g). $N_{nitrifiers}$ is the amount of ammonia uptake by nitrifying bacteria (mg L^{-1}).

The ammonia removal efficiency was calculated at the endpoint as follows:

$$\text{Ammonia removal efficiency (\%)} = \left(1 - \frac{[NH_4^+\text{-}N]_{end}}{[NH_4^+\text{-}N]_{initial}}\right) \times 100 \quad (3\text{-}7)$$

where $[NH_4^+\text{-}N]_{end}$ is the NH_4^+-N concentration at the experimental endpoint (mg-N L^{-1}) and $[NH_4^+\text{-}N]_{initial}$ is the initial NH_4^+-N concentration (mg-N L^{-1}).

To estimate the exposed light intensity for nitrifying bacteria in the dispersion condition, the light intensity I_x in the serum bottle at depth x (cm) was calculated according to the following equation of the modified Lambert–Beer law (Akizuki et al., 2021):

$$I_x\ (\mu\text{mol photons m}^{-2}\ \text{s}^{-1}) = I_0 \exp\{-(C_{algae} \times \alpha_{algae} + C_{nitrifiers} \times \alpha_{nitrifiers}) \times depth\} \quad (3\text{-}8)$$

where I_x and I_0 are the estimated transmitted light intensity at depth x (cm) and incident light intensity (μmol photons m^{-2} s^{-1}), respectively. In the present study, to estimate the lowest possible light exposure intensity for the nitrifying bacteria in the dispersion case, the distance from the liquid surface to the bottom of the serum bottle (8.0 cm) was used as the depth x (cm) in the equation. C_{algae} and $C_{nitrifiers}$ are the initial concentrations of microalgae and nitrifiers (mg-SS L^{-1}), respectively. a_{algae} and $a_{nitrifires}$ are the light attenuation coefficients of microalgae (1.045 cm^2 mg^{-1}) and nitrifiers (0.003

cm^2 mg^{-1}), respectively, obtained from Vergara et al. (2016).

3.3 Results and Discussion

3.3.1 Environmental conditions (pH and DO concentration)

Table 3-1 shows the pH values for all conditions at the end of the experiment. The pH values increased above 8.0 for all the light-irradiated samples. However, the pH for the control (0 μmol photons m^{-2} s^{-1}) were maintained or decreased. In microalgae–nitrifying bacterial consortia, pH values varied depending on the balance between photosynthesis by microalgae and nitrification by nitrifying bacteria: increasing owing to CO_2 capture and decreasing due to H^+ production during ammonia oxidization. The appropriate pH value for ammonia oxidization of both *Nitrosomonas*-like AOB and *Nitrobacter*-like NOB is 7.9–8.1. Beyond that value, their activity is reduced by approximately 40–80% at pH 9.0 (Grunditz and Dalhammar, 2001). Moreover, it has been reported that the biomass productivity of the microalgae *Chlorella sorokiniana* is significantly reduced at pH 9.0 as compared to pH 8.0 (Qiu et al., 2017). In the dispersion, higher pH values caused by light irradiation may have inhibited both microalgae and nitrifying bacteria. In the hydrogel and light-shielding hydrogel, lower pH values (< 10) than that for the dispersion was observed. This indicates that the inhibition for the nitrification activity and microalgal biomass productivity by light exposure was severe in the dispersion, however, it was reduced in both the immobilized conditions.

In a consortium, the DO concentration also varied depending on the balance between photosynthesis and nitrification. The DO concentration increased with the increase in microalgal O_2 production and decreased with bacterial O_2 consumption. Table 3-1 shows the DO concentrations at the end of the experiment. it represents indirectly the relationship between O_2 supply and consumption. Regardless of sludge conditions and light intensity, DO concentrations were above 2.0 mg L^{-1} for the light-irradiated condition. Therefore, sufficient O_2 supply by photosynthesis could be achieved with a light intensity of at least 100 μmol photons m^{-2} s^{-1}. Moreover, under light irradiation,

DO concentration was 3.84–4.31 mg L^{-1} for the dispersion, 2.63–3.30 mg L^{-1} for the hydrogel, and 2.18–2.99 mg L^{-1} for the light-shielding hydrogel. As shown, the DO concentration for dispersion was higher than that for immobilized conditions. Relatively high residual DO concentration implies that O_2 consumption by the nitrification reaction was more greatly prevented under dispersion condition. This is likely due to the photoinhibition of nitrifying bacteria. These results are consistent with the findings in the chapter 2 and other reports that dispersed nitrifying bacteria are susceptible to light exposure. Conversely, light-stress tolerance was shown under aggregated or light-shielded bacteria (Arcila and Buitrón, 2017; Nishi et al., 2020). The higher pH values (i.e. lower nitrification activity) observed in the dispersion further support this assumption (Table 3-1). Immobilization of bacteria can also mitigate the negative effects such as pH changes (Bouabidi et al., 2019). Overall, the light-shielding and external stimuli tolerant properties of the proposed method may be why nitrification enhanced with a lower DO even under intense light irradiation.

DO concentrations requirement to completely nitrify depend on the dispersed bacteria or immobilized bacteria. The DO concentrations required for complete nitrification in dispersed bacteria was above 1.4 mg L^{-1} (Ruiz et al., 2006), whereas complete nitrification was achieved under the DO range of 2.0–4.0 mg L^{-1} when using immobilized activated sludge (Xu et al., 2017). The high diffusion resistance of immobilized hydrogel requires higher DO concentrations for nitrification than dispersion. (Benyahia and Polomarkaki, 2005). Although relatively low DO concentrations were confirmed in the hydrogel and light-shielding hydrogel conditions (2.18–3.30 mg L^{-1}) as shown in Table 3-1, the DO concentration still remained above 2.0 mg L^{-1}. This indicated the supply of sufficient oxygen for all conditions.

3.3.2 Nitrification and ammonia removal performance

Figure 3-2 shows the changes in nitrogen compounds with time for all conditions. Nitrogen uptake by microalgae, ΔN_{algae}, was calculated from the nitrogen compound and is also shown in the

same figure. Ammonium concentrations decreased in all sludge types under dark conditions. Microalgal uptake was the primary pathway of ammonium removal (Figure 3-2A–A″). Microalgae can uptake nutrients heterotrophically under dark conditions (Mooij et al., 2015). In this experiment, although carbonate was included in substrate, heterotrophic uptake by microalgae might be observed under dark condition. There are two possible reasons for this phenomenon. One is the contamination by organic matter contained in the used original nitrifying sludge which used to treat the reject water after anaerobic digestion. The other is the increase in organic matter by self-degradation of bacteria under anaerobic conditions. It is likely that microalgae uptake ammonia heterotrophically using organic matter due to these factors under dark conditions. The low level of residual oxygen may have resulted in partial nitrate production.

At 100 μmol photons m^{-2} s^{-1}, the ammonia removal and nitrate production in the hydrogel (Figure 3-2B′) and light-shielding hydrogel (Figure 3-2B″) was faster than that in the dispersion (Figure 3-2B). In the dispersion, the total nitrate production at the end of experiment was low. These results may be attributed to the photoinhibition of nitrifying bacteria and the resulting pH increase (> 11) in dispersion conditions. Notably, the previous result of the light intensity effect on nitrifying bacteria with the same sludge and experiment condition as in this section, the photoinhibition of nitrifying bacteria already started to confirm at 100 μmol photons m^{-2} s^{-1} under dispersion condition (Nishi et al., 2020). It was shown that photoinhibition of nitrifying activity leads to an increase in pH, negatively affecting both microalgae and bacteria in microalgae-nitrifying bacteria consortium.

In the dispersion, nitrate concentration at the experimental endpoint decreased with an increase in light intensity. It became almost negligible above 450 μmol photons m^{-2} s^{-1} (Figure 3-2C). The nitrate production for the hydrogel and light-shielding hydrogel at 100 μmol photons m^{-2} s^{-1} (Figure 3-2B′, B″) was found to be comparable after 24 h, and did not decrease essentially up to 450 μmol photons m^{-2} s^{-1} (Figure 3-2C′, C″). At 1600 μmol photons m^{-2} s^{-1}, nitrate concentration at the experimental endpoint for the hydrogel (Figure 3-2D′) and the light-shielding hydrogel (Figure 3-

2D″) was reduced to approximately half of that at 450 μmol photons m^{-2} s^{-1}. However, complete nitrification was observed for the light-shielding hydrogel, while the hydrogel showed NO_2^- accumulation. The higher light-tolerance ability of the light-shielding hydrogel was due to the light-shielding material, whereas that of the hydrogel may be due to the self-shielding effect of the concentrated bacteria. However, the self-shading ability of the hydrogel was not sufficient for light as strong as 1600 μmol photons m^{-2} s^{-1} because of the NO_2^- accumulation.

The effects of light intensity on ammonia removal performance were first detected at 100 μmol photons m^{-2} s^{-1} for the dispersion (Figure 3-2B) and at 450 μmol photons m^{-2} s^{-1} for the hydrogel and light-shielding hydrogel (Figure 3-2C′, C″). This effect increased at 1600 μmol photons m^{-2} s^{-1} for all sludge conditions (Figure 3-2D, D′, and D″). The ammonia uptake by microalgae, ΔN_{algae}, peaked after 3 h and then decreased in all sludge conditions under light irradiation. This phenomenon is assumed because the oxygen concentration, which was initially zero under all conditions, started to increase with the beginning of light irradiation through photosynthesis by the microalgae. Therefore, firstly, the nitrification by nitrifying bacteria was not occurred, and the decrease in ammonia by microalgal uptake. Moreover, the imbalance between photosynthesis and nitrification increased the pH at this moment. Thereafter, nitrification reactions started to occur as the oxygen concentration increased. It was the competition between microalgae and bacteria in ammonia consumption, coupled with an increase in pH, that led to a decrease in ammonia uptake. Finally, ammonia uptake by microalgae was negligible after 24 hours because the pH was high and much of the ammonia was consumed. As mentioned above, the immobilized bacteria in hydrogel could obtain tolerance to external stimuli such as pH and temperature (Zhang et al., 2018). Therefore, nitrifying bacteria in the hydrogel and light-shielding hydrogel were less affected by high pH values than microalgae owing to the pH tolerance of the bacteria immobilization in the hydrogel.

The nitrification rates calculated based on the results are shown in Figure 3-3. The highest nitrification rates were observed at 100 μmol photons m^{-2} s^{-1} for all sludge conditions. Above 100

μmol photons m^{-2} s^{-1}, although the nitrification rates for the dispersion and hydrogel conditions decreased with increasing light intensity, those for the light-shielding hydrogel remained stable up to 450 μmol photons m^{-2} s^{-1} and decreased at 1600 μmol photons m^{-2} s^{-1}. The nitrification rates at 1600 μmol photons m^{-2} s^{-1} for the light-shielding hydrogel (0.8 mg-N L^{-1} h^{-1}) were approximately nine and two times higher than those for the dispersion and hydrogel (0.09 and 0.4 mg-N L^{-1} h^{-1}), respectively ($p< 0.05$ for all comparisons). These results imply, again, that the proposed method is effective for mitigating the photoinhibition of nitrifiers. Therefore, it is expected that the use of light-shielding hydrogel even under intense light irradiation such as sunlight may allow the nitrifying bacteria to achieve higher nitrification performance without photoinhibition. This technique of "immobilization" and "light-shielding" for microbes has potential for application not only to nitrifying bacteria, but also to other microorganisms that are less light tolerant.

The nitrogen mass balance and NH_4^+-N removal efficiency at the experimental endpoint are shown in Figure 3-4. The proportion of nitrogen oxides (NO_2^--N and NO_3^--N) reached a peak at 100 or 450 μmol photons m^{-2} s^{-1} and decreased at higher light intensities. In the dispersion, NO_2^- accumulation was observed at ≥ 100 μmol photons m^{-2} s^{-1}. Hydrogel exhibited almost no NO_2^- accumulation at 100 and 450 μmol photons m^{-2} s^{-1}, whereas this accumulation of 8.8% was observed at 1600 μmol photons m^{-2} s^{-1}. The NO_2^- accumulation of intermediate compounds may derive from nitrifying bacteria photoinhibition, depending on bacterial species, light intensity, light wavelength, and exposure time (Guerrero and Jones, 1996). Because NOB is more sensitive to light than AOB, NO_2^- accumulated under intense light (Kang et al., 2018; Vergara et al., 2016). As mentioned in Chapter 2, Guerrero and Jone (1997) reported that the cytochrome-c related to the energy conversion pathway in AOB and NOB is damaged by high photon energy at 408 nm.

The NO_2^- accumulation also could prevent the growth of microalgae. According to González-Camejo et al. (2020), NO_2^- accumulation of 20 mg L^{-1} in microalgae–bacterial consortia reduce the biomass productivity of microalgae by 19% and nitrogen recovery efficiency by 80%. Therefore, it is necessary to avoid NO_2^- accumulation in the consortia to prevent process

destabilization and reduction in the nitrogen removal performance. Moreover, NO_2^- accumulation leads to the emission of N_2O, a greenhouse gas. According to Castro-Barros et al. (2016), a gradual NO_2^--N concentration increase up to 100 mg-N L^{-1} increases N_2O emission. In this experiment, there was no observation of NO_2^- accumulation sufficient to produce such N_2O emission under any light condition.

Ammonia removal efficiencies reached at 100 μmol photons m^{-2} s^{-1} for all conditions, that is, 94.1%, 100%, and 100% for the dispersion, hydrogel, and light-shielding hydrogel, respectively. The ammonia removal efficiency subsequently decreased with increasing light intensity for all conditions, resulting 74% at 1600 μmol photons m^{-2} s^{-1} even for the light-shielding hydrogel. The low ammonia removal efficiency under dark conditions was due to the lack of oxygen supply from microalgae, which prevented nitrification. It has been reported that the microalgae *Chlorella sorokiniana* exhibited a maximum specific growth rate at a light intensity of 7500 lx, and this decreased at 10000 lx (equivalent to approximately 170 μmol photons m^{-2} s^{-1}) (Asadi et al., 2019). Ammonia uptake by microalgae also decreased below 100 μmol photons m^{-2} s^{-1} for all conditions, suggesting that nitrifying bacteria and microalgae were negatively affected by higher light intensity. Notably, it is worth noting that AOB and NOB exhibit the potential to recover from light damage during the dark period (Yoshioka and Saijo, 1984). Thus, in contrast to the present results, it seems likely that higher ammonia removal efficiency could be achieved with intermittent light exposure (i.e. 12 h/12 h of light/dark cycle) using the light-shielding hydrogel rather than continuous light exposure to nitrifying bacteria.

The obtained results in this study are summarized in Table 3-2 and are compared with the previous studies conducted under the experimental conditions similar to this study. Most of the experiments in the previous study were conducted at relatively low light intensities, and their results exhibited high ammonia removal efficiencies of above 73.3%. Among the previous study, only Vergara et al. (2016) carried out ammonia removal experiment using the consortium under the strong light intensity of 1250 μmol photons m^{-2} s^{-1} and reported high removal efficiency of 75%. However,

it should be noted here that although they conducted the experiment under high light intensity condition, they did pH control and aeration to stabilize their process. Whilst no pH control and aeration are necessary in the proposed process in this study. However, ammonia removal rates still exhibits comparable value to those of the previous studies, confirming the effectiveness of the proposed light-tolerant microalgae-bacteria consortium using the light-shielding hydrogel in extreme environments such as high light intensity.

3.3.3 Light transmission within the light-shielding hydrogel

To understand the property of the high mitigation potential for the photoinhibition of nitrifying bacteria observed in the light-shielding hydrogel, it is necessary to determine the potential light intensity reduction inside the hydrogel beads. Prior to this, the potential light intensity reduction due to self-shading of dispersed microalgae and nitrifying bacteria was estimated using the already reported data. According to Eqn. (3-8), The minimum light exposure of nitrifying bacteria in serum bottles was calculated for the strongest light exposure of 1600 μmol photons m^{-2} s^{-1}. It was found that nitrifying bacteria and microalgae were exposed to 128 μmol photons m^{-2} s^{-1} of light intensity even at the bottom of the reactor (the expected lowest light intensity exposure) in the dispersion condition. It seems that this light intensity has been greatly reduced, however, the microorganisms move to various positions in the reactor by agitation in this experiment. Therefore, the average light intensity throughout the reactor was obtained by integrating the calculated light intensity, resulting in a light intensity of 591 μmol photons m^{-2} s^{-1}. This value was sufficient light intensity for photoinhibition of nitrifying bacteria to occur as it was higher than 500 μmol photons m^{-2} s^{-1}.

The attenuation of the exposed light intensity for the nitrifying bacteria inside the light-shielding hydrogel was examined. Since it was difficult to measure the actual attenuation in light intensity from the surface to the center of the hydrogel beads, the light transmission through a solution mimicking 1% alginate and 0.1% carbon black hydrogel composition was measured and estimated

the attenuation inside the hydrogel. When the incident light intensity was 1600 μmol photons m^{-2} s^{-1}, the transmitted light intensity through this mixture solutions with thicknesses of 1.0, 1.5, and 2.0 mm against the light path direction were 403, 208, and 124 μmol photons m^{-2} s^{-1}, respectively (Figure 3-5). This corresponds to the transmittance of 25.2%, 13.0%, and 7.8% of the applied 1600 μmol photons m^{-2} s^{-1}. Whereas, if 1% alginate solution without carbon black, more than 99% of the light is transmitted under the same condition. It is to be noted that the light intensity experienced by the nitrifying bacteria was significantly decreased because of the light absorption by the light-shielding materials present in the light-shielding hydrogel (carbon black). The obtained equation for the fitting curve from the experimental plots is as follows:

$$y = 1275.4\ e^{-1.176x}$$

where y is the light intensity (μmol photons m^{-2} s^{-1}), and x is the distance from the surface of the mixture solution (mm). Since this mixture solution has the same composition and concentration as the light-shielding hydrogel without the nitrifying bacteria, the distance x can be considered as the distance from the outer surface of the light-shielding hydrogel to a specific depth x inside the hydrogel in the radial direction. Hence, for example, if the incident light I_0 is 1600 μmol photons m^{-2} s^{-1}, the light intensity at a distance of 1.5 mm from the surface is attenuated to 200 μmol photons m^{-2} s^{-1} in the light-shielding hydrogel. This means that the light intensity inside the gel beads with a diameter of approximately 3.0 mm is much lower than the incident light intensity. These results indicate that the light-shielding hydrogel can effectively reduce the light intensity irradiation for nitrifying bacteria compared to the dispersion.

Notably, the light-shielding material and its concentration were not yet fixed and optimized in this study. More efficient light-shielding would be required for light-shielding hydrogel when the light intensity to be irradiated is quite intense. For example, in sunbelt region such as Mexico, the light intensity as high as 2500 μmol photons m^{-2} s^{-1} is reached (de-Bashan et al., 2008). Even under those high light irradiation conditions, sufficient light-shielding properties can be easily controlled by simply increasing the light-shielding material concentration. It is expected that the combination of

immobilization and light shielding of microorganisms can be applied to other light-sensitive microorganisms.

3.3.4 Effect of free ammonia

In addition to photoinhibition, free ammonia (FA) induced by pH increase likely constitutes another exogenous factor affecting process performance. According to Anthonisen et al. (1976), a reduction in the AOB and NOB activities was confirmed at FA concentrations of 8.23–123.53 mg L^{-1} and 1.0–10 mg L^{-1}, respectively. Qian et al. (2017) also reported that the activity of AOB and NOB is reduced by 15.9% and 29.2%, respectively, at an FA concentration of 16.8 mg L^{-1}. Both studies clearly show that NOB are more sensitive to FA than AOB, and microalgae are also reported to be affected by FA. FA concentrations below 15–20 mg L^{-1} did not significantly affect the specific growth rate of microalgae, those above 30–40 mg L^{-1} reduced the specific growth rates of *Chlorella pyrenoidosa* by approximately 50% (Tan et al., 2016). Moreover, the growth of *Chlorella pyrenoidosa* growth was completely inhibited when the FA concentrations reached maximum at 137.9–192.3 mg L^{-1} at high pH in the range of pH 9.1-9.6 (Tan et al., 2016). Thus, both nitrifying bacteria and microalgae could be affected by FA, with nitrifying bacteria being more sensitive to this effect. The estimated FA concentrations at the experimental endpoint were calculated using Eqn. (3-4) based on the obtained results in the present study was shown in Table 3-1. In the dispersion, FA concentrations at 450 and 1600 μmol photons m^{-2} s^{-1} reached above 20 mg L^{-1}, indicating that nitrifying bacteria were likely inhibited. Therefore, besides photoinhibition, such high FA concentrations would affect nitrification inhibition, resulting in NO_2^- accumulation at 450 and 1600 μmol photons m^{-2} s^{-1} in the dispersion. This result is consistent with previous study (Akizuki et al., 2019) in which both strong light intensity of at least 1573 μmol photons m^{-2} s^{-1} and resulting high FA concentrations of 3.3–59.4 mg L^{-1} induced nitrification inhibition.

Since the microorganism immobilized in hydrogel is more tolerant to external stimuli, it was suggested that the relatively higher nitrification efficiency for the proposed light-shielding hydrogel

compared to the dispersion may be due to its property. However, it has also been reported that immobilization has no clear effect on the tolerance of nitrifying bacteria against FA (Rongsayamanont et al., 2010). Therefore, to determine whether FA or photoinhibition plays a more important role in the reduction of nitrification performance under intense light irradiation, the ratio of NO_2^--N in the nitrogen mass balance was examined. At 1600 μmol photons m^{-2} s^{-1}, the FA concentrations for the hydrogel and the light-shielding hydrogel were 6.26 and 13.2 mg L^{-1}, respectively. As shown in Figure 3-4, the proportion of NO_2^--N for the hydrogel in the nitrogen mass balance was higher than that for the light-shielding hydrogel. Thus, despite higher FA concentrations, FA-sensitive NOB was more active in the light-shielding hydrogel. Assuming that the pH/FA tolerance of nitrifying bacteria by immobilization is equivalent for the hydrogel and light-shielding hydrogel, the effect of photoinhibition rather than that of FA was considered more significant in this study. Especially, since superior light-shielding can suppress the photoinhibition effect, the more dominant factor of nitrification activity inhibition, the NO_2^- accumulation was almost negligible in the light-shielding hydrogel despite higher FA concentrations than in the hydrogel.

3.3.5 Possible sequence of process breakdown under intense light irradiation

Finally, based on the obtained results of process breakdown in the dispersion condition in this study, it was proposed that a possible mechanism for the breakdown of microalgae-nitrifying bacteria consortia under intense light irradiation (Figure 3-6). If the appropriate light intensity is irradiated in dispersed microalgae-nitrifying bacteria consortia, the pH value can be maintained at a neutral. This is because the pH-decreasing effect by nitrification and the pH-increasing effect by microalgal photosynthesis can be balanced (Jia and Yuan, 2016). Increasing light intensity to levels that induce photoinhibition by nitrifying bacteria may increase pH. This imbalance between the two effects on pH leads to a breakdown of the process. This is explained by the following mechanism. First, intense light irradiation lead to photoinhibition and start stopping nitrification (Figure 3-6 I),

and pH-decreasing effect become weak. The pH-increasing effect by microalgal photosynthesis to become dominant (Figure 3-6 II), thereby causing a pH increase in the reactor. High pH readily result in increasing FA concentrations (Figure 3-6 III), which inhibits the activity of not only nitrifying bacteria but also microalgae (Figure 3-6 IV). In this mechanism, photoinhibition plays an essential role as a trigger, with subsequent FA inhibition becoming more severe. These integrated effects ultimately lead to process breakdown. The understanding of this mechanism of process breakdown in microalgae-nitrifying bacteria consortia can help to achieve stable ammonia removal in outdoor operation.

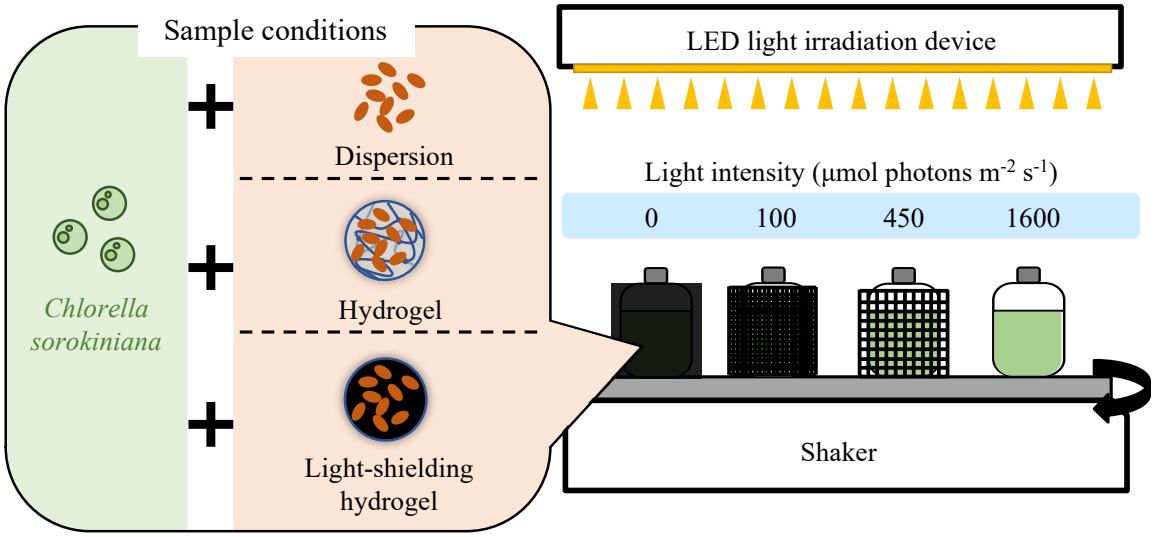

Figure 3-1 Concept of the light irradiation batch test (24 h).

Four light irradiation conditions were used: 0, 100, 450, and 1600 μmol photons m^{-2} s^{-1}.

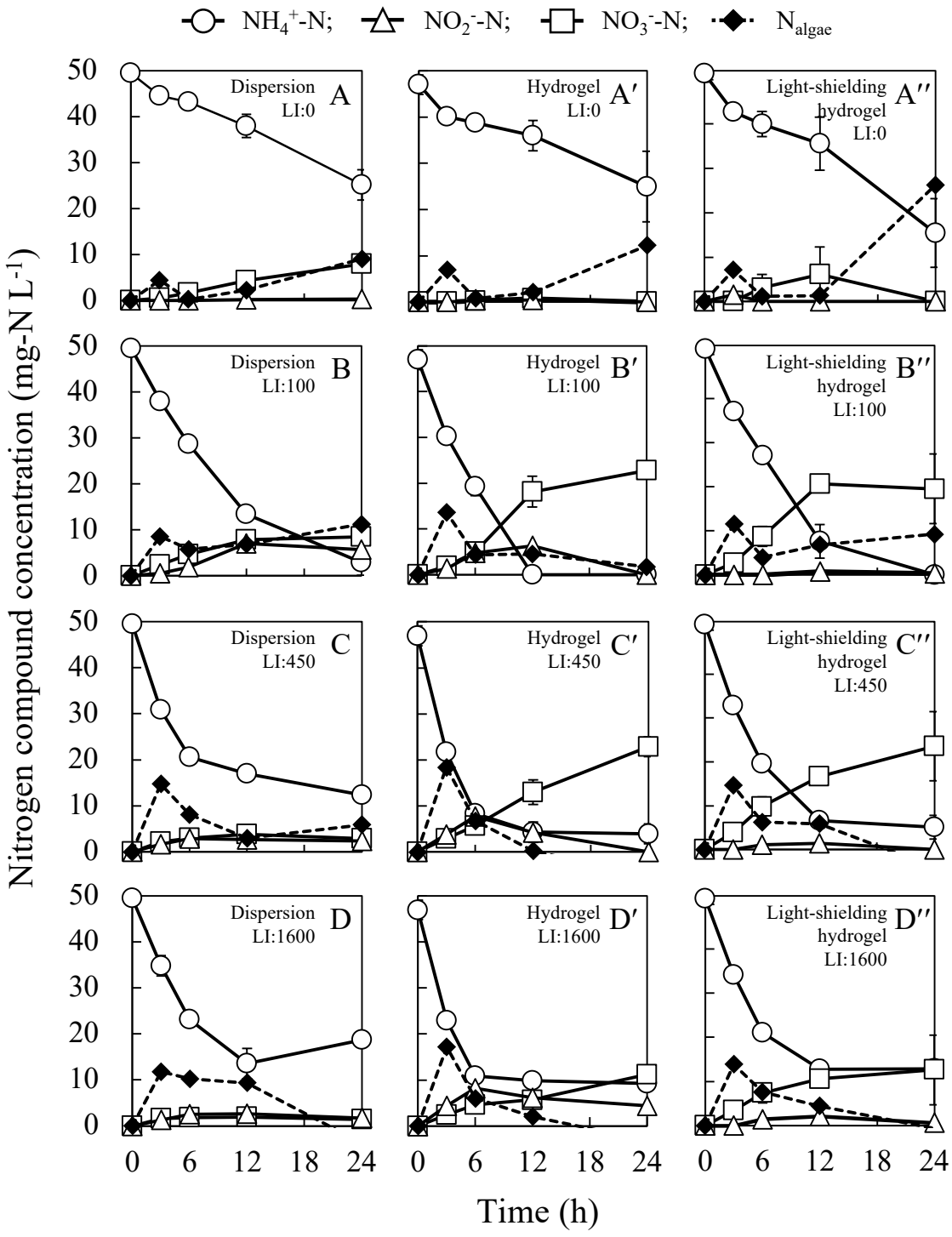

Figure 3-2 Time course of nitrogen compounds and nitrogen uptake by microalgae for dispersion, hydrogel, and light-shielding hydrogel conditions.

(left) Dispersion; (middle) Hydrogel; (right) Light-shielding hydrogel.

A: 0, B: 100, C:450, D: 1600 (μmol photons m^{-2} s^{-1}).

Error bars indicate the standard deviations of triplicate samples.

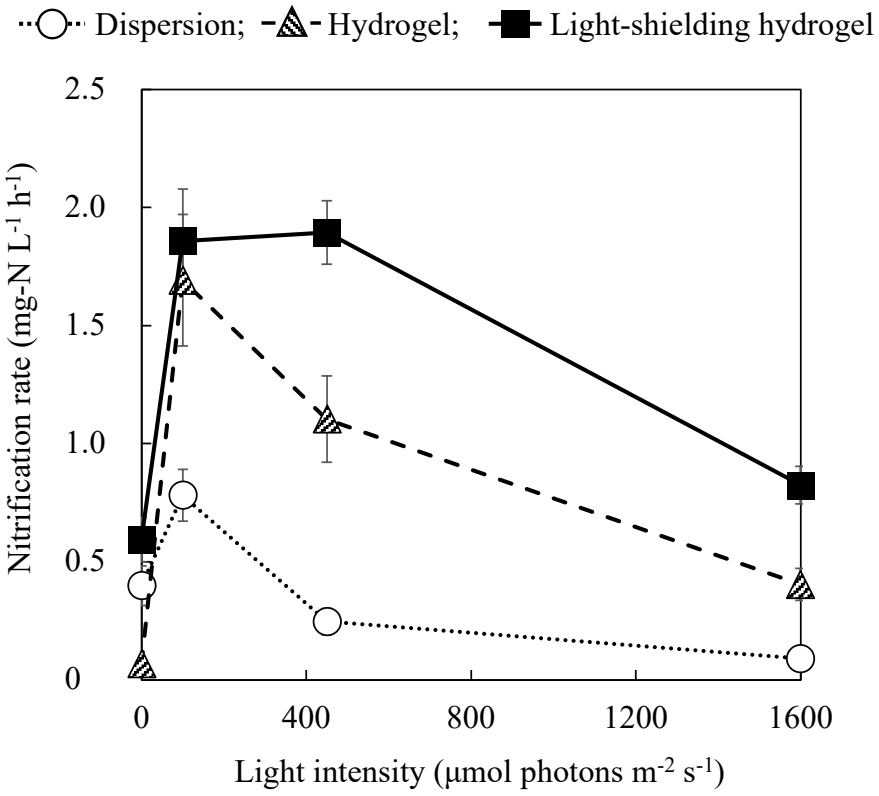

Figure 3-3 Effect of light intensity on the nitrification rate for dispersion, hydrogel, and light-shielding hydrogel sludge.

Error bars indicate the standard deviations of triplicate samples.

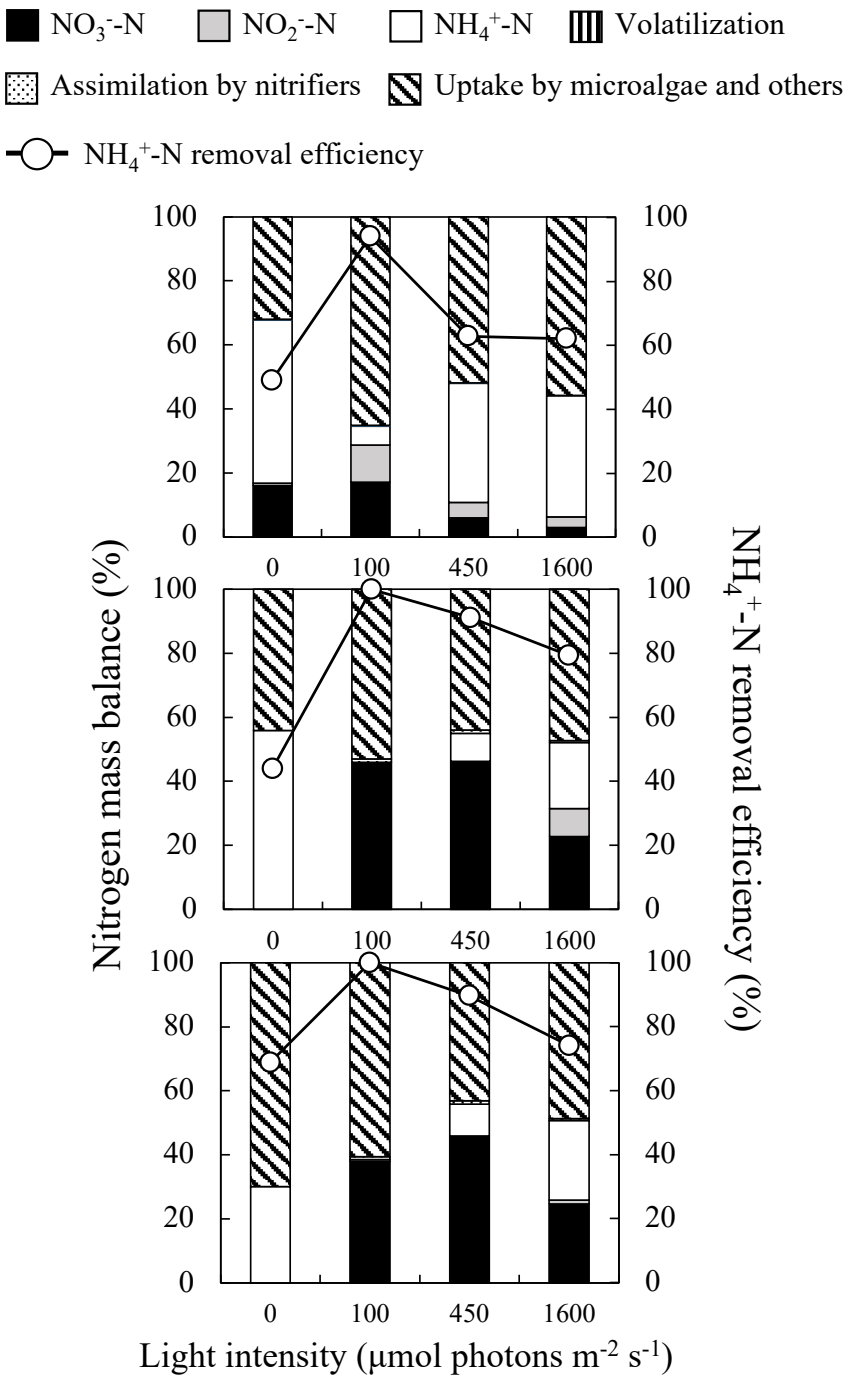

Figure 3-4 Nitrogen mass balance and ammonia removal efficiency.

(top) Dispersion; (middle) Hydrogel; (bottom) Light-shielding hydrogel.

Error bars indicate the standard deviations of triplicate samples.

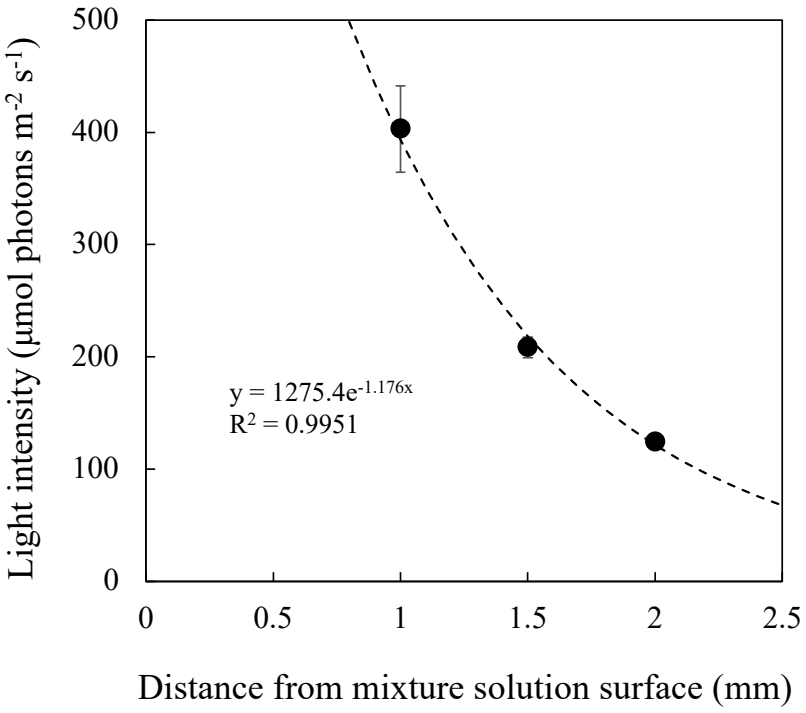

Figure 3-5 Light transmission through a solution mimicking 1% alginate and 0.1% carbon black hydrogel composition to estimate attenuation of the exposed light intensity inside light-shielding hydrogel.

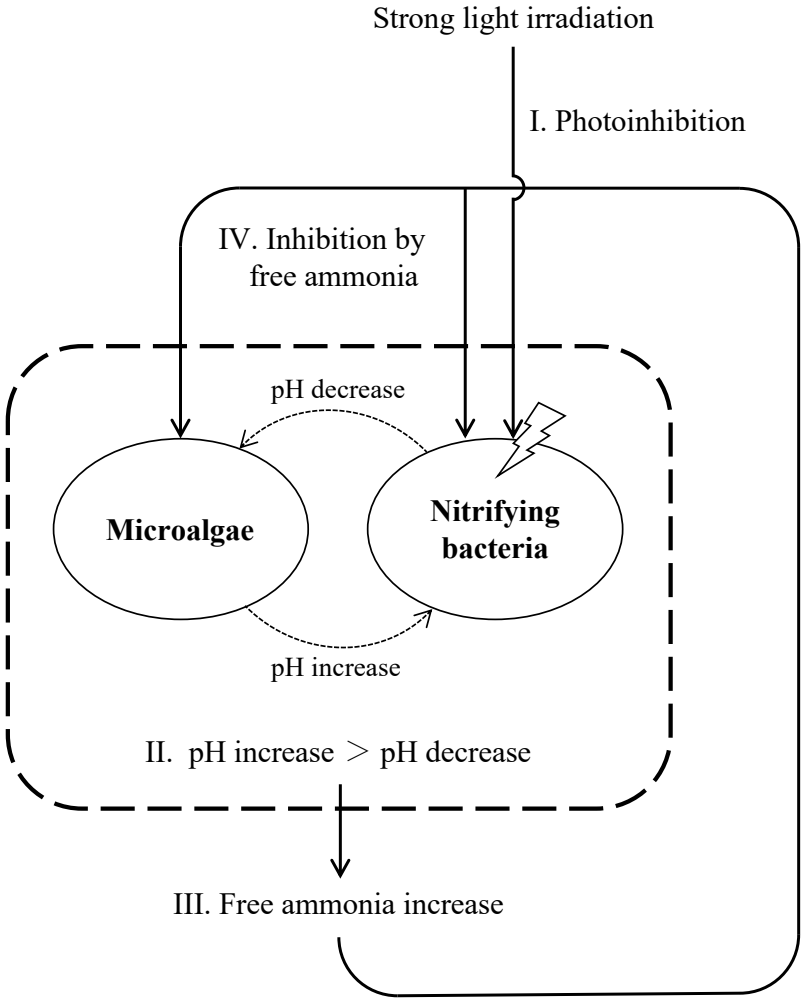

Figure 3-6 Breakdown of the consortia system triggered by strong light irradiation.

I–IV shows the time phase of the proposed process breakdown.

Table 3-1 pH value, DO concentration, and free ammonia concentration of each condition at the experimental endpoint (24 h).

		Light intensity (μmol photons m^{-2} s^{-1})			
		0	100	450	1600
pH	Dispersion	8.02±0.10	11.3±0.06	11.4±0.005	11.4±0.01
	Hydrogel	6.74±0.06	8.89±0.09	8.68±0.17	9.25±0.06
	Light-shielding hydrogel	6.56±0.003	8.09±0.40	9.58±0.19	9.99±0.02
Dissolved oxygen (mg L^{-1})	Dispersion	1.61±0.04	4.31±0.12	3.97±0.15	3.84±0.10
	Hydrogel	0.857±0.31	3.30±0.06	3.14±0.20	2.63±0.19
	Light-shielding hydrogel	0.707±0.09	2.18±0.38	2.99±0.28	2.86±0.17
Free ammonia (mg L^{-1})	Dispersion	1.72	3.49	22.1	22.6
	Hydrogel	0.11	0.0	1.15	6.26
	Light-shielding hydrogel	0.04	0.0	4.23	13.2

Table 3-2 The summarization of the microalgae-bacterial consortia studies.

References	Microalgae-bacterial consortia	Substrate	Reactor	Light intensity (μmol photons m^{-2} s^{-1})	Light/dark cycle (h/h)	Temp. (℃)	Aeration	pH control	Initial NH_4^+ (mg-N L^{-1})	Ammonia removal efficiency (%)
van der Steen et al. (2015)	Microalgae (*Scenedesmus quadricauda, Anabaena variabilis, Chlorella* sp., *Chlorococcus* sp., *Spirulina* sp.) - Nitrifying bacteria	Artificial WW	PSBR	66	24/0	23.1	+	−	66	84.8
Karya et al. (2013)	Microalgae (*Scenedesmus quadricauda*) - Nitrifying bacteria	Artificial WW	PSBR	60	24/0	30	−	+	26.7	100
Vergara et al. (2016)	Microalgae (*Chlorella sorokiniana*) - Nitrifying bacteria	Artificial WW	Batch	0	24/0	27	+	−	118	ca.100
				250	24/0	27	+	−	118	ca.100
				500	24/0	27	+	−	118	ca.100
				1250	24/0	27	+	−	118	ca.75
Akizuki et al. (2019)	Microalgae (*Chlorella* sp., *Scenedesmus* sp., Diatoms) - Nitrifying bacteria	ADE WW	Batch	140	24/0	25	−	−	102	73.3
Jia and Yuan (2018)	Algae (cyanobacteria and green algae) - Activated sludge	Artificial WW	PSBR	ca. 20 (1000 lx)	24/0	25	−	+	102	ca. 95
This study	Microalgae (*Chlorella sorokiniana*) - Nitrifying bacteria (Dispersion)	Artificial WW	Batch	0	24/0	25	−	−	49.5	49.1
				100	24/0	25	−	−	49.5	94.1
				450	24/0	25	−	−	49.5	75.1
				1600	24/0	25	−	−	49.5	62.2
	Microalgae (*Chlorella sorokiniana*) - Nitrifying bacteria (Hydrogel)	Artificial WW	Batch	0	24/0	25	−	−	46.9	46.9
				100	24/0	25	−	−	46.9	100
				450	24/0	25	−	−	46.9	91.6
				1600	24/0	25	−	−	46.9	80.4
	Microalgae (*Chlorella sorokiniana*) - Nitrifying bacteria (Light-shielding hydrogel)	Artificial WW	Batch	0	24/0	25	−	−	51.3	69.9
				100	24/0	25	−	−	51.3	100
				450	24/0	25	−	−	51.3	90.1
				1600	24/0	25	−	−	51.3	75

Artificial WW (Artificial wastewater); ADE WW (Anaerobic digestion effluent wastewater); PSBR (Photo-sequencing batch reactor)

Chapter 4

Evaluation of different inoculum biomass ratios on light-tolerant microalgae-nitrifying bacteria consortia

4.1 Introduction

Light-tolerant microalgae-nitrifying bacteria consortia in which only nitrifying bacteria are immobilized on light-shielding hydrogel was developed and performed high ammonia removal efficiency and nitrification performance even under strong light irradiation in Chapter 3 (Nishi et al., 2022). Furthermore, the advantage of this technology is not only to mitigate photoinhibition but also to realize the separation of microalgae and nitrifying bacteria by a simple method, which was difficult to do in the past. This is because the beads size of light-shielding hydrogel is normally around 2.0–4.0 mm and the cell size of microalgae is extremely smaller than that, thus easily separable using a sieve.

Besides, the effects of external factors such as C/N ratio (Zhu et al., 2019), inoculum biomass ratio (Fan et al., 2020), and aeration time (Zhang et al., 2020) have also been investigated to improve the treatment performance using microalgae-bacteria consortia. The inoculum ratio of microalgae and nitrifying bacteria biomass is one of the important factor for ammonia removal due to their different roles. Su et al. (2012) reported that the biomass ratio of 5:1 (algae: bacteria) among the different inoculum ratios had the highest TN removal efficiency of 91.0%, and the various biomass ratios affected the microbial community and contributed to TN removal performance. However, the effect of the biomass ratio on the “light-tolerant microalgae-nitrifying bacteria consortia” using light-shielding hydrogel has not yet been clarified. Evaluation of the effect of biomass on light-tolerant microalgae-nitrifying bacteria consortia is important to achieve higher ammonia removal performance in severe environments. Furthermore, no studies have yet investigated the effect of biomass ratio under strong light irradiation in conventional dispersed microalgae-nitrifying bacteria

coexistence consortia.

This section aims to examine the effect of biomass ratio on ammonia removal performance and microalgal growth in microalgae-nitrifying bacteria consortia using light-shielding hydrogel. The effect of biomass ratio on TN removal and ammonia removal performance was evaluated by multivariate analysis based on the obtained results and previous studies. This study provides prospects for availability of the proposed process and its improved removal performance under intense light environments.

4.2 Materials and methods

4.2.1 Microorganism and synthetic wastewater

Microalgae *Chlorella sorokiniana* (NIES-2173) was obtained from the National Institute for Environmental Studies (NIES), Tsukuba, Japan. Nitrifying sludge was collected by Yokohama Hokubu Sludge Treatment Center in Kanagawa, Japan.

The light-shielding hydrogel was encapsulated nitrifying sludge with alginate hydrogel adding light-shielding material following the procedure in Chapter 2. First, nitrifying sludge was concentrated by centrifugation at 3000 rpm for 10 mins. 1% sodium alginate was dissolved in concentrated nitrifying sludge. And 0.1% carbon black powder was also suspended in above mixture solution. The mixture solution dripped into 1% calcium chloride solution. The drops of mixture solution were formed beads approximately 3.0 mm immediately. After dripped, beads were reacted in calcium chloride solution in 3 h. These formed beads were named “light-shielding hydrogel”.

The synthetic wastewater was used in this experiment following composition; 2.4 g L^{-1} $(NH_4)_2SO_4$, 1.9 g L^{-1} NH_4Cl, 2.8 g L^{-1} KH_2PO_4, 2.0 g L^{-1} $MgSO_4$, 2.0 g L^{-1} NaCl, 17.5 g L^{-1} $NaHCO_3$, 1.28 g L^{-1} $CaCl_2 \cdot 2H_2O$, and trace metal solution (3 mL). The components of the trace metal solution were following composition; 1.0 g L^{-1} $Na_2EDTA \cdot 2H_2O$, 200 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 36 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 10.4 mg L^{-1} $ZnCl_2$, 4.0 mg L^{-1} $CoCl_2 \cdot 6H_2O$, and 2.5 mg L^{-1} $NaMoO_4 \cdot 2H_2O$.

4.2.2 Experimental setup

The experimental condition was shown in Figure 4-1. Nitrifying bacteria was prepared in two conditions, “light-shielding hydrogel” and “dispersion” as control. The serum bottles (100 mL of working volume) were used in this experiment. The inoculum biomass ratio of microalgae to nitrifiers was set six conditions: 10:0 (only microalgae), 9:1, 7:3, 5:5, 1:9 and 0:10 (only nitrifiers). The initial total biomass concentration was adjusted to 0.3 g-SS L^{-1} in each condition. The synthetic wastewater was diluted until the initial NH_4^+-N concentration was 50 mg-N L^{-1}. The light intensity of 500 μmol photons m^{-2} s^{-1} using white LED light irradiation device (Iida Lightning Co., Ltd., Japan) was irradiated in a 12:12h of light/dark cycle. All bottles were shaken at 180 rpm, and at 25 ± 2 °C for 72 h. Before the experiment, the pH value was adjusted to 8.0. DO concentration was not adjusted following the experimental conditions of Sephri et al. (2020). All experiments were performed in triplicate.

4.2.3 Analysis

All samples were filtered through a 0.45 μm filter (GC-50, Advantec, Taiwan) before analysis. The pH value was measured with a pH meter (9625-10D, Horiba, Japan). The dissolved oxygen (DO) concentrations were measured using a DO meter (9520-10D, Horiba, Japan). The SS concentration was measured after filtrated and dried at 105°C. Chlorophyll *a* concentration was measured with a Turner fluorometer (Model 10-AU, Turner Design, United States) after filtered samples was extracted with *N,N*-dimethylformamide (Suzuki and Ishimaru, 1990). The nitrogen compounds such as NH_4^+-N, NO_2^--N, and NO_3^--N were analysed using high-performance liquid chromatography (HPLC; Shimazu, Japan) with a cation column IC YS-50 (Shodex, Japan) and anion column IC NI-424 (Shodex, Japan).

4.2.4 Calculations

The specific growth rate (μ; d^{-1}) of microalgae was calculated from chlorophyll *a* concentration according to the following equation:

$$\mu = \frac{\ln (C_2 - C_1)}{(t_2 - t_1)} \quad (4\text{-}1)$$

Where C_1 is the concentration of chlorophyll *a* at time t_1 (mg L^{-1}), C_2 is the concentration of chlorophyll *a* at time t_2 (mg L^{-1}), and t_1 and t_2 are the experimental time 1 and 2 (day), respectively.

Free ammonia (FA) was calculated as follows (Anthonisen et al., 1976):

$$\text{FA (mg L}^{-1}) = \frac{17}{14} \times \frac{[\text{NH}_4^+\text{-N}] \times 10^{pH}}{\exp\left(\frac{6344}{273+T}\right) + 10^{pH}} \quad (4\text{-}2)$$

Where $[NH_4^+\text{-}N]$ is the NH_4^+-N concentration (mg-N L^{-1}), and *pH* and *T* are the pH value and temperature (°C), respectively.

The amount of volatilised ammonia was calculated using the following equation (modified Zimmo et al. (2004)):

$$\text{Amount of volatilised ammonia (g)} = \sum_{i=1}^{n} \{ SA \times (3.3 \times FA + 4.90) \} \quad (4\text{-}3)$$

Where *SA* is the surface area which is 2.12×10^{-3} m^2 per bottle. *FA* is the free ammonia concentration (mg L^{-1}), and *n* is the experimental time (day).

The amount of nitrogen assimilated into nitrifying bacteria ($N_{nitrifier}$) thoughout the experiment was calculated stoichiometrically accoding to this nitrification reaction (Mara, 2004):

$$\begin{aligned} &\text{NH}_4^+ + 1.32\ \text{O}_2 + 1.98\ \text{HCO}_3^- + 0.98\ \text{H}_2\text{O} \\ &\rightarrow 0.021\ \text{C}_5\text{H}_7\text{NO}_2 + 0.98\ \text{NO}_3^- + 2.02\ \text{H}_2\text{O} + 1.88\ \text{H}_2\text{CO}_3 \end{aligned} \quad (4\text{-}4)$$

The amount of ammonium uptake by microalgae and others was calculated as follows:

$$\begin{aligned} &\text{Amount of ammonia uptake by microalgae and others (mg L}^{-1}) = \\ &[NH_4^+\text{-}N]_{initial} - ([NH_4^+\text{-}N]_{end} + [NO_2^-\text{-}N]_{end} + [NO_3^-\text{-}N]_{end} + \text{Volatilized ammonia} + N_{nitrifiers}) \end{aligned} \quad (4\text{-}5)$$

Where $[NH_4^+\text{-}N]_{initial}$ is the initial NH_4^+-N concentration (mg-N L^{-1}). $[NO_2^-\text{-}N]_{end}$, $[NO_2^-\text{-}N]_{end}$, and $[NO_3^-\text{-}N]_{end}$ are the NH_4^+N, NO_2^--N, and NO_3^--N concentrations (mg-N L^{-1}) at the end of experiment, respectively. Volatilized ammonia is the amount of volatilized ammonia in bottles (g-N). $N_{nitrifiers}$ is the amount of nitrogen assimilated into nitrifying bacteria (mg L^{-1}).

The ammonia removal efficiency was calculated at the endpoint as follows:

$$\text{Ammonia removal efficiency (\%)} = \left(1 - \frac{[NH_4^+\text{-}N]_{end}}{[NH_4^+\text{-}N]_{initial}}\right) \times 100 \qquad (4\text{-}6)$$

Where $[NH_4^+\text{-}N]_{end}$ is the final NH_4^+-N concentration (mg-N L^{-1}) and $[NH_4^+\text{-}N]_{initial}$ is the initial NH_4^+-N concentration (mg-N L^{-1}).

To examine the relationships between different variables, multiple regression analysis was carried out using the data obtained from present study and previous studies. In detail, multiple regression analysis was conducted on the relationship between TN removal efficiency or ammonia removal efficiency as dependent variable and other factors as the independent variable. The obtained regression model's goodness was evaluated by the coefficient of determination (R^2) and adjusted R^2 and *p*-value.

4.3 Results and Discussion

4.3.1 pH and Dissolved oxygen

The time course of pH for each experimental condition was shown in Figure 4-2. The presence of light/dark periods during this experiment results in a pH-increase in light period and pH-decrease in dark period for both dispersion and light-shielding hydrogel conditions. Microalgae consumed autotrophically inorganic carbon through photosynthesis during the light period (Kumar et al., 2014), resulting in an increased pH in this experiment. Two factors cause the pH to decrease during the dark period after light exposure: (1) respiration of both microbial and (2) recovery of nitrifying bacteria activity, which leads to nitrification and a decrease in pH (Merbt et al., 2012). In

dispersion condition, the pH in only 0:10 was stable at pH 8.0, however, the pH in other biomass ratio conditions reached approximately 13.0 after 12 h and remained at a higher pH thereafter. This reason for stable high pH could be nitrifying bacteria was easily stopped or prevented from nitrification by light irradiation of 500 μmol photons m^{-2} s^{-1}. On the contrary, the pH in 0:10 and 1:9 was stable at 8.0 for light-shielding hydrogel condition. Since light-shielding hydrogel can mitigate photoinhibition of nitrifying bacteria, the pH was maintained close to neutral value by nitrification even in co-culture with microalgae. One of the reasons for the high pH in this experiment was that $NaHCO_3$ (> 0.8 g L^{-1}) was used as a carbon source, and the synthetic wastewater was prone to increasing pH. Since high pH makes it easier to produce free ammonia, there is a risk of further microbial effect not only by higher pH but also by free ammonia (mentioned section 4.3.3).

The DO concentration at the start of the experiment was approximately 4.0 mg L^{-1} for both dispersion and the light-shielding hydrogel. The DO concentrations at the end of the experiment were higher than 7.0 mg L^{-1} under both sludge conditions except 0:10. This increase in DO concentration was caused by oxygen supply by microalgae because of the closed reactor used. The DO concentration of 2.0–2.5 mg L^{-1} for complete nitrification are recommended in wastewater treatment plants (Yoo et al., 1999). Since the DO concentration was above 7.0 mg L^{-1} in the consortium reactors except for 0:10 (only nitrifying bacteria), it was concluded that the presence of microalgae was sufficient to supply the required oxygen for nitrification in this study. At a biomass ratio of 0:10, the lack of oxygen supply made DO concentration reduced to 2.3 mg L^{-1} and 0.64 mg L^{-1} in the dispersion and light-shielding hydrogel, respectively. Relatively high DO concentrations of 0:10 in dispersion might not be consumed by oxygen by photoinhibition. On the other hand, the oxygen in light-shielding hydrogel was used as nitrification without photoinhibition, resulting in lower DO concentrations at the end of the experiment.

4.3.2 Algae growth with different biomass ratio

The specific growth rate of microalgae was calculated from the chlorophyll *a* concentration and shown in Figure 4-3. Specific growth rate increased with decreasing microalgae proportion under both dispersion and light-shielding hydrogel conditions. These results are affected by the light transmission efficiency in the reactor. The extinction coefficients were 0.1045 $m^2 g^{-1}$ for microalgae and 0.003 $m^2 g^{-1}$ for nitrifying bacteria, respectively, and thus the extinction coefficient for microalgae is higher than that for nitrifying bacteria (Vergara et al., 2016). It means that the high microalgae proportion in the biomass interferes with the light transmission efficiency in the reactor, subsequently interferes with the growth of microalgae as a result. Whereas, even for the same microalgae proportion (biomass ratio), the specific growth rates for the light-shielding hydrogel conditions are higher in all the biomass ratios except for the 0:10. One most likely reason could be the effect of pH. As shown in Figure 4-2, in the dispersed case, pH went up to above 12 already after 12 h for the biomass ratios from 10:0 to 5:5, and for the 1:9 it reached to above 12 after 36 h. On the other hand, in the light-shielding hydrogel, pH went up to above 12 after 36 h for the biomass ratios from 10:0 to 5:5, and for the 1:9 it did not reach until the end. Since high pH and resulting free ammonia (FA) is known to inhibit microalgae growth, low growth rate for the dispersion is reasonable. Another possible reason is that the same amount of nitrifying bacteria is encapsulated in the gel bead with much concentrated form in the case of the light-shielding hydrogel, light transmission would be improved than that for the dispersed. This kind of light transmission improvement can be observed when coagulant and flocculant are added to dirty water and then the resulting aggregate formation create space for light to pass through. The better light transmission through the reactor would be another possible reason for the higher specific growth rates in the light-shielding hydrogel.

The maximum specific growth rates were 1.0 d^{-1} and 1.3 d^{-1} for dispersion and light-shielding hydrogel at 1:9, respectively. The maximum specific growth rate of microalgae in the microalgae-bacteria consortia was 0.62 d^{-1} in continuous experiments (van der Steen et al., 2015) and 0.14 d^{-1} at

a biomass ratio of 5:1 (algae: bacteria) in batch experiments (Amini et al., 2020). The maximum specific growth rates in both bacterial conditions in this experiment exceeded the rates in these previous studies by approximately 2.1 to 9.5 times. Amini et al. (2020) and Sepehri et al. (2020) reported that the specific growth rate increases with the microalgae ratio. However, the finding from this study are not consistent with these reports. The reason for these differences might be attributed to the low light intensity of 2000 lx in previous studies and the high light intensity of 500 μmol photons m^{-2} s^{-1} (approx. 34000 lx) in the present study.

4.3.3 Ammonia removal and nitrification with different biomass ratio

The nitrogen mass balance and ammonia removal efficiency at the end of the experiment for each biomass ratio were shown in Figure 4-4. The production of NO_2^- and/or NO_3^- became higher as the ratio of nitrifying bacteria increased in both the dispersion and light-shielding hydrogel. In the dispersion, although the production of nitrogen oxides (NO_2^- and NO_3^-) became higher as the ratio of nitrifying bacteria increased, high percentages of NO_2^- accumulation were observed. This could be due to the photoinhibition of nitrifying bacteria by strong light irradiation with the intensity of 500 μmol photons m^{-2} s^{-1}. It has been known that NOB is more sensitive to strong light irradiation than AOB (Merbt et al., 2012), which may have resulted in the incomplete nitrification for the dispersion condition. In the light-shielding hydrogel, detectable accumulation of NO_2^- was not observed up to the 1:9 condition, and higher NO_3^- proportions were observed as compared to the dispersion. It indicates that light-shielding hydrogel mitigates photoinhibition of nitrifying bacteria so that more light sensitive NOB are also protected from light and that contributes to achieve complete nitrification even under high light intensities in this study. However, NO_2^- accumulation was confirmed only at a biomass ratio of 0:10 in the light-shielding hydrogel. This result was not likely due to the photoinhibition, rather the lack of oxygen due to the DO concentration decreasing to 0.64 mg L^{-1} at the end of the experiment without the presence of microalgae.

The maximum ammonia removal efficiencies for both the dispersion and light-shielding hydrogel were 70.5% and 100% at 1:9, respectively. The use of light-shielding hydrogel had the highest ammonia removal efficiency because the pH in the reactor remained neutral throughout the experiment, allowing the microalgae to uptake ammonia without pH inhibition. As shown in Figure 4-4, for the light-shielding hydrogel condition at the 1:9, uptake by microalgae and others occupy higher proportion as compared to the same condition for the dispersion. With the use of other biomass ratios, almost 50–60% of ammonia removal efficiencies were achieved.

The low ammonia removal efficiency can be attributed to two factors. One of the factors is the lack of oxygen for complete nitrification due to the absence of microalgae for both sludge conditions at 0:10. Another factor could be high pH and resulting free ammonia (FA). The consortia were negatively affected by the FA produced during the experiment because of high concentration of ammonia and pH deterioration to alkaline pH due to CO_2 uptake by microalgae. Free ammonia concentrations were calculated using Eqn. (4-2), and the changes in FA concentrations with time during this experiment was shown in Figure 4-5. As a result, under the condition with the biomass ratio of 0:10 in the dispersion, and the 0:10 and 1:9 in the light-shielding hydrogel, the FA concentration were below 5.0 mg L^{-1} throughout the experiment. The FA concentration for the 1:9 under the dispersion is also relatively low of less than 20 mg L^{-1}. Meanwhile, FA concentrations in the other biomass ratios showed a sharp increase up to approximately 30 mg L^{-1} after 6 h of the experiment. Free ammonia can affect the growth of microalgae and nitrifying bacteria. The microalgae *Chlorella pyrenoidosa* was demonstrated the activity decreases in specific growth rate of more than 50% at FA concentrations above 30 mg L^{-1} (Tan et al., 2016). Nitrifying bacteria were also reported to have decreases in activity of 29.2% for NOB and 15.9% for AOB above 16.82 mg L^{-1} (Qian et al., 2017). Thus, the high FA concentrations in this study affected both microbes.

In chapter 3, it was suggested that high light intensity induces a high pH, which promotes the production of free ammonia, eventually leading to the breakdown of the consortia process (Nishi et al., 2022). Microalgae-nitrifying bacteria consortia under high light intensities may not perform

well with high microalgae proportion because of the increased pH. Thus, the optimum biomass ratio was decided to the 1:9 with the highest ammonia removal efficiency in this experiment. Even with the optimum biomass ratio, the dispersion condition showed lower ammonia removal efficiency of 70.5% and on the contrary, the light-shielding condition exhibited 100% of the ammonia removal. This also demonstrates the effectiveness of the light-shielding hydrogel.

4.3.4 Influence of process variables on performance and efficiency

Regression equations obtained by multiple regression analysis from the data obtained in this study and those in the previous studies are shown in Table 1. To obtain the removal efficiency of TN and ammonia, since the amounts of microalgae and bacteria, the initial NH_4 concentration, the experimental time, and the FA concentration were likely the most important independent variables, and their effects were analyzed. It should be noted that there were no statistically significant differences in the data used for the multiple regression analysis for the only algae (10:0) or only bacteria (0:10) conditions, and thus they were excluded from this calculation (data not shown). This means that the obtained regression equations are applicable only for microalgae-bacteria consortia. The relationship between the measured and predicted removal efficiencies using the obtained regression model is shown in Figure 4-6. The plots in Figure 4-6 show that the measured values for both TN and ammonia removal efficiency fit well with the theoretical line ($y = x$). This means that the obtained regression model is able to predict reasonably close value to the measured values. The regression model for TN removal efficiency shows a high R^2 value of 0.90 and a p-value of < 0.001 for all coefficients, indicating a good fit to the data. From the obtained coefficients of microalgae and bacteria, it was found that the amount of microalgae has a greater influence than that of bacteria for TN removal efficiency. This is reasonable because microalgae directly remove nitrogen such as ammonia and nitrate by assimilation, but in contrast nitrifying bacteria does not directly contribute to remove nitrogen since they merely transform ammonia to nitrate which is still another form of

nitrogen existing in the wastewater. Therefore, it is natural that the amounts of microalgae exhibited a potent influence on TN removal efficiency. However, it is clear from the coefficients that the presence of bacteria also contribute to increase the TN removal rate. This can be explained as follows; if only microalgae are used, the maintenance of appropriate pH range for the consortia becomes difficult and results in pH increase. Then, the increased pH could also arise the increase in of FA concentration, and eventually lead to a process failure. It means the presence of bacteria contributes to diminish the negative coefficient of FA (–0.94) on TN removal efficiency. The obtained relationship between the coefficients of biomass and that of FA is consistent with the study conducted by Akizuki et al. (2021), who reported that biomass amount and FA are negatively correlated.

On the contrary, the results for ammonia removal efficiency showed that the amounts of bacteria, rather than microalgae, affected the most. However, some of obtained coefficients were not significant ($p > 0.12$), although the R^2 value of the regression model was high. In summary, to obtain the high TN and ammonia removal efficiencies, it is confirmed that the amounts of microalgae and bacteria were more important than the other variables according to the multiple regression analysis. However, this analysis only tells the importance of the biomass amount and does not tell about the effect of biomass ratio of microalgae to bacteria.

To evaluate the effect of the ratio on the total biomass, the relationships between the proportion of bacteria in the microalgae-bacteria consortia and the TN and ammonia removal efficiencies are shown in Figure 4-7. The results show that lower bacteria proportion (higher microalgae proportion) tends to exhibit higher TN removal rates in the previous studies and also in the dispersion condition in this study (Figure 4-7 a). The regression model in Table 4-1 also show that the coefficient for microalgae is higher than for bacteria, and the higher microalgae ratio in the ratio improves the TN removal efficiency. This finding is consistent with Su et al. (2012), which argues that assimilation is the primary mechanism for TN removal in microalgae-bacteria consortia (Su et al., 2012). On the other hand, the light-shielding hydrogel condition in this study showed exceptionally higher TN removal efficiency at 90% of bacteria proportion (biomass ratio of

microalgae to bacteria of 1:9). As mentioned above, this indicates that the maintenance of appropriate pH under this condition allowed the microalgae to effectively assimilate ammonia.

Regarding the ammonia removal efficiency shown in Figure 4-7 b, for both the dispersion and light-shielding hydrogel in this study showed that the highest results were obtained at the highest bacteria proportions of 90% except for bacteria only condition. Sepehri et al. (2020), Su et al. (2012) and Nguyen et al. (2020) also reported extremely low ammonia removal efficiency in bacteria only, all of which were mainly attributed to lack of oxygen. The regression model in Table 4-1 also shows that the coefficient for bacteria was higher than that for microalgae which is opposite to the TN removal. This study and Sepehri et al. (2020) showed that nitrification becomes dominant as compared to microalgae assimilation in ammonia removal because of higher ammonia removal efficiencies at higher bacteria proportion as shonw in Fig. 4-4. However, other previous studies have reported no change in ammonia removal efficiency with different bacteria proportions that could be due to their low light irradiation as compared to the very high intensity light in this study. The effect of light intensity on the appropriate bacteria proportion (biomass ratio of microalgae to bacteria) needs to be studied in more detailed in the future.

In this chapter, appropriate biomass ratio in the light-tolerant microalgae-bacteria consortia under strong light irradiation for ammonia removal in a batch process was clarified as mentioned above. However, to apply the finding to a continuous process, maintenance of the biomass ratio by separating and recovering fast growing microalgae from the process is inevitable. This has been a big challenge for the conventional consortia process (Fallahi et al., 2021), because of almost close cell size between microalgae and bacteria, and also because of their well mixing status in dispersion. However, the overgrown microalgae in our proposed consortia can be easily separated using separators such as a mesh because of the significant size difference between the light-shielding hydrogel and microalgae and also because of essentially different existing location of bacteria and microalgae which are inside and outside of hydrogel. This unique feature is expected to contribute to realize stable maintenance of biomass ratio when the consortia is applied for ammonia removal in a

continuous process.

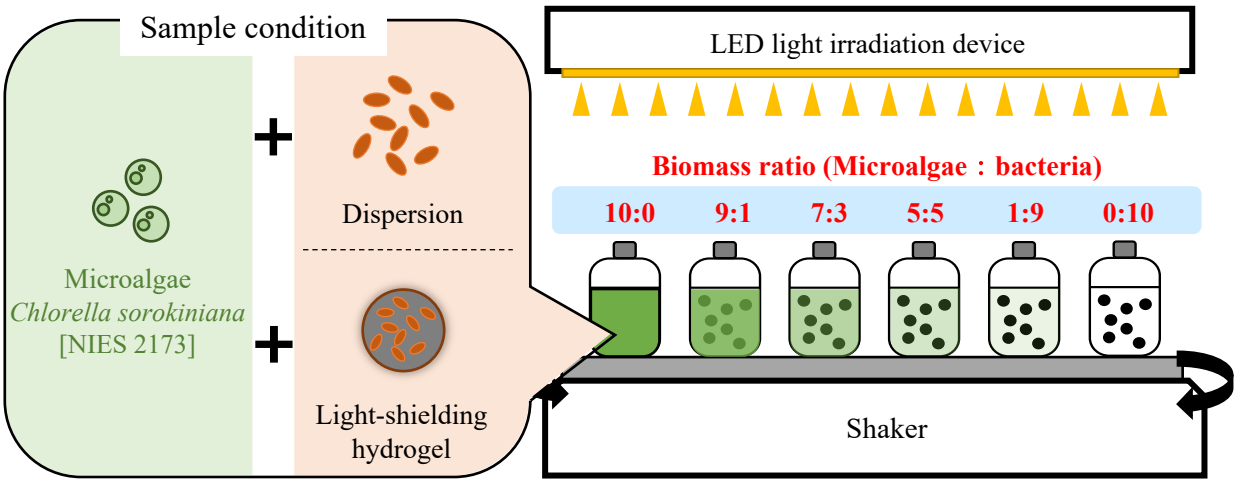

Figure 4-1 Experimental conditions of the microalgae-nitrifying bacteria consortia.

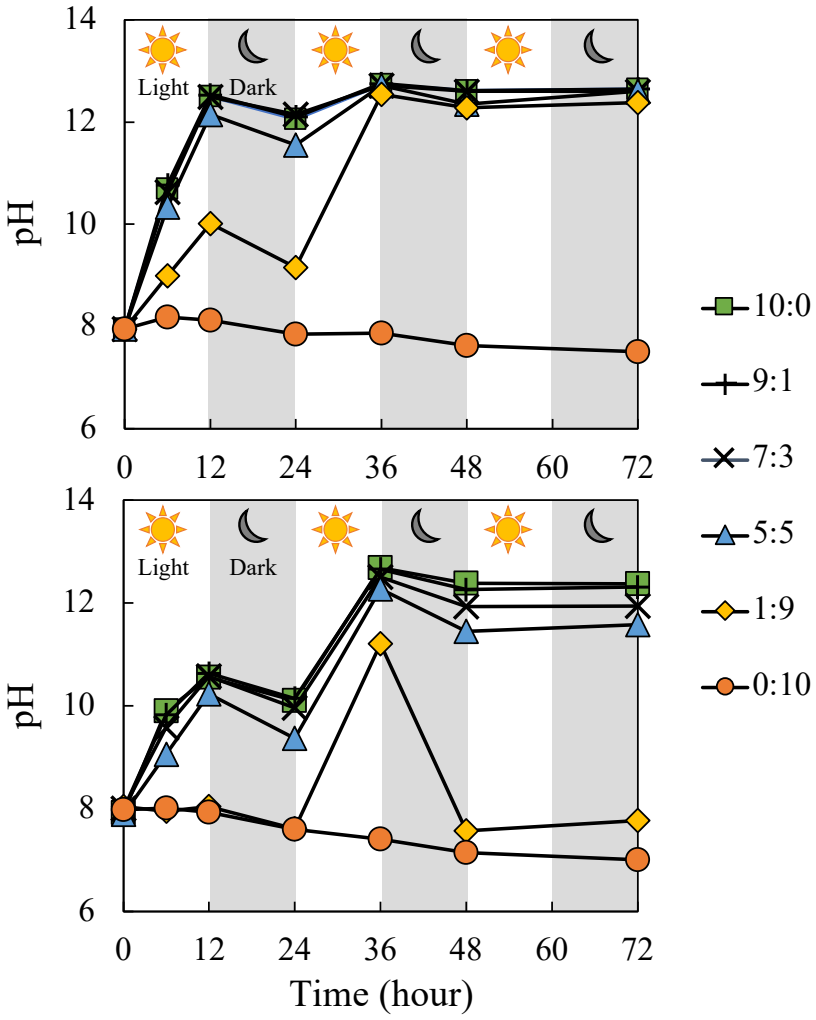

Figure 4-2 pH changes during experiment.

(above) Dispersion; (below) Light-shielding hydrogel.

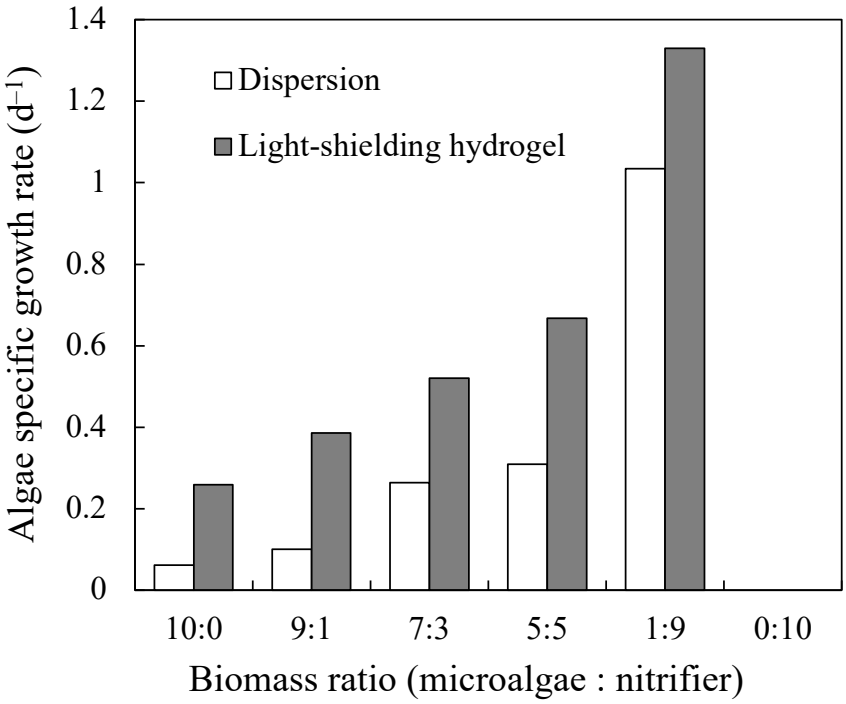

Figure 4-3 Algae specific growth rate at different biomass ratio.

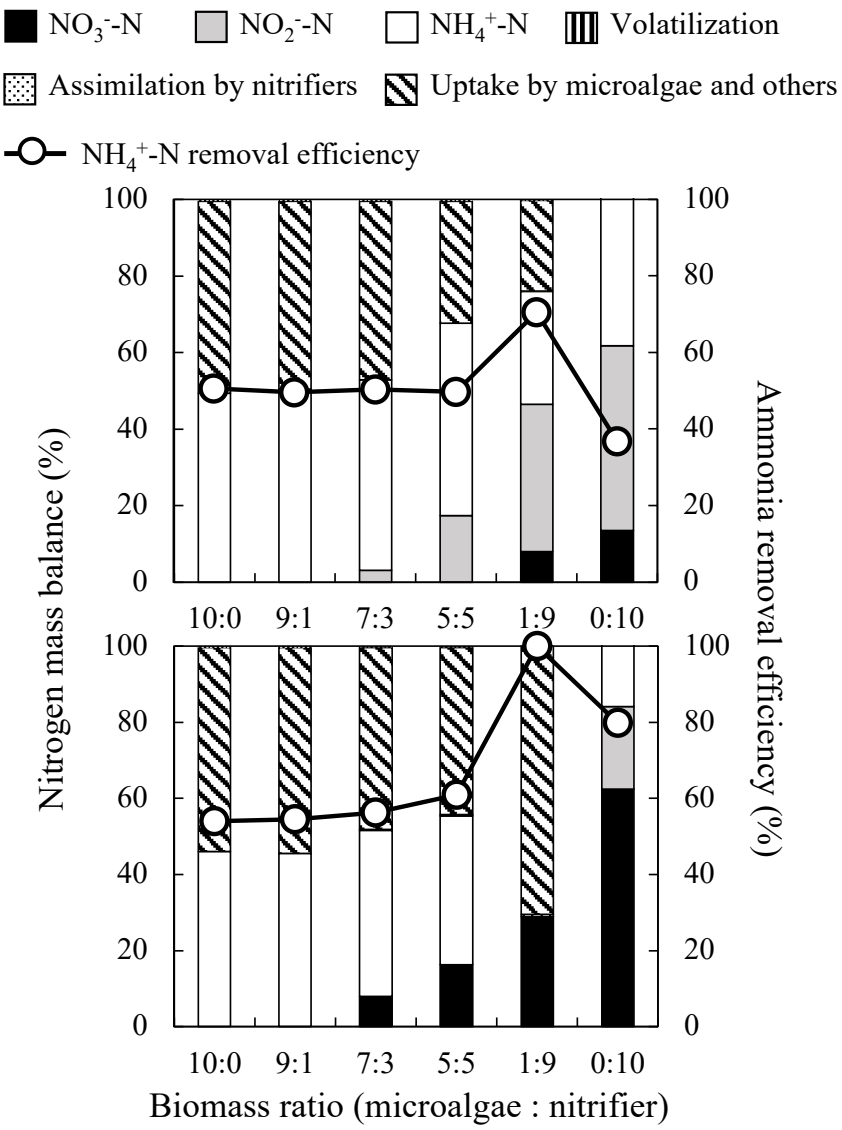

Figure 4-4 Nitrogen mass balance and ammonia removal efficiency at the end of experiment.

(above) Dispersion; (below) Light-shielding hydrogel.

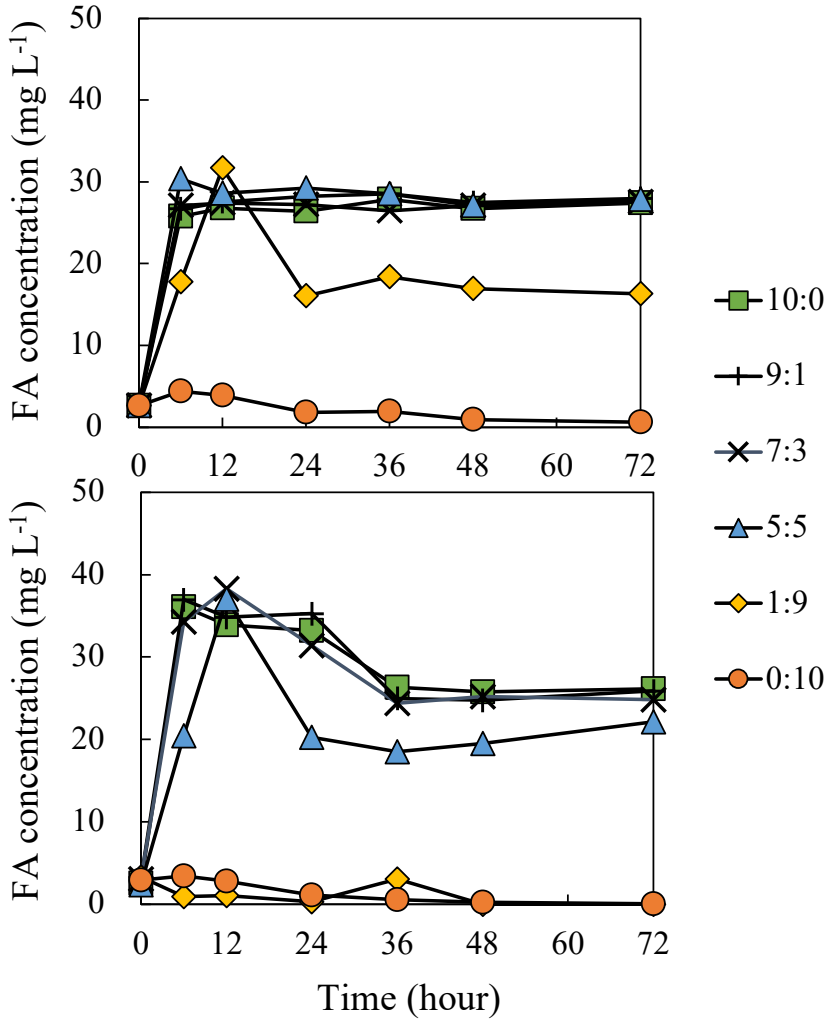

Figure 4-5 Free ammonia concentration during experiment.

(above) Dispersion; (below) Light-shielding hydrogel.

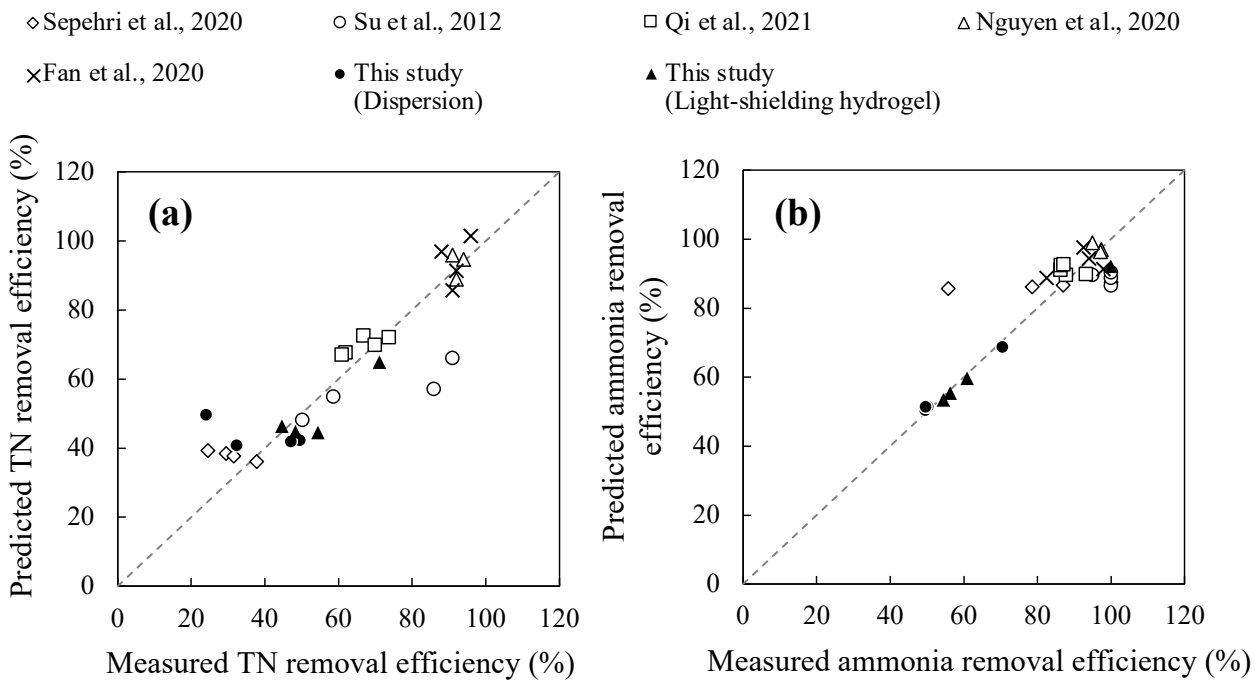

Figure 4-6 Relationship between measured data and predicted data on TN removal efficiency (a) and ammonia removal efficiency (b).

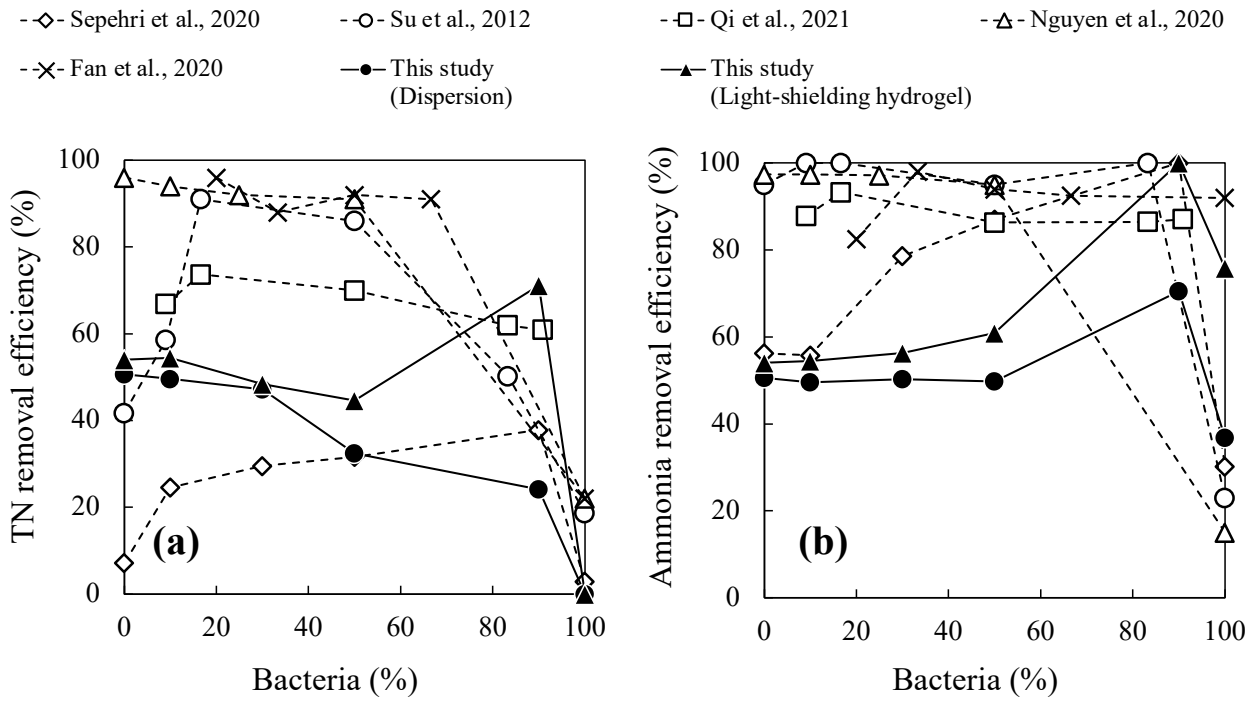

Figure 4-7 Influence of bacteria proportion in microalgae-bacteria consortia on TN removal efficiency (a) and ammonia removal efficiency (b).

Table 4-1 Results of the multiple regression analysis.

Dependent variable	Independent variable	Coefficient	Standard error	P	R^2	R^2 (adj)	F
TN removal efficiency	Constant	45.1	5.13	<0.001	0.80	0.76	18.0
	Algae	59.1	9.27	<0.001			
	Bacteria	45.3	9.97	<0.001			
	Initial NH4	0.33	0.05	<0.001			
	Time	-3.03	0.65	<0.001			
	FA	-0.94	0.24	<0.001			
Ammonia removal efficiency	Constant	87.9	3.95	<0.001	0.80	0.75	17.6
	Algae	5.5	7.14	0.45			
	Bacteria	12.6	7.68	0.12			
	Initial NH4	0.08	0.04	0.09			
	Time	-0.58	0.50	0.26			
	FA	-1.47	0.19	<0.001			

Chapter 5
General discussion

5.1 Achievements in chapters 2–4

To overcome the challenging issue of the photoinhibition of nitrifying bacteria in the microalgae-bacteria consortia for a treatment of ammonia containing wastewater under strong light irradiation, an advanced light-tolerant microalgae-nitrifying bacteria consortia was established by developing the new technology of light-shielding hydrogel through chapter 2-4. First, light-shielding hydrogel was prepared by combining immobilization and shading to protect the nitrifying bacteria from light. It was found that the nitrification performance of nitrifying bacteria immobilized in light-shielding hydrogel remains the same as in the dark condition, and is considerably higher than the dispersion even at quite high light intensities (1600 μmol photons m^{-2} s^{-1}). The results for the dispersed nitrifying bacteria proved that NOB was more sensitive to light than AOB, and photoinhibition induced NO_2^- accumulation. The use of light-shielding hydrogel prevented the NO_2^- accumulation and showed that it enabled to effectively mitigate photoinhibition of nitrifying bacteria (Chapter 2).

The prepared light-shielding hydrogel was then applied to the microalgae-bacteria consortia under different light intensities to evaluate its ammonia removal and nitrification performance. The microalgae *Chlorella sorokiniana* of 0.3 g-SS L^{-1} provided the dissolved oxygen required for complete nitrification even under weakest light irradiation of 100 μmol photons m^{-2} s^{-1}. The light-shielding hydrogel successfully contributed to maintain a stable pH balance in the consortia and resulted in the high ammonia removal efficiency of above 75% even under strong light irradiation of 1600 μmol photons m^{-2} s^{-1}. Furthermore, photoinhibition mechanism of nitrifying bacteria was found to be the destabilization of the pH balance in the reactor at the initial stage due to photoinhibition of nitrifying bacteria, leading to process breakdown caused by the subsequent accumulation of high

concentration of free ammonia (Chapter 3).

As a preliminary study to use one of the advantages of microalgae-nitrifying bacteria consortia with light-shielding hydrogel, i.e., facile separation of biomass, the effect of inoculum biomass ratio on the ammonia removal performance was evaluated. The 1:9 biomass ratio exhibited the highest ammonia removal efficiencies among the six different biomass ratios and the values were 70.5% and 100% for the dispersion and light-shielding hydrogel, respectively. The lower microalgae proportion increased the microalgal specific growth rate up to 1.3 d^{-1} due to the improved light transmission in the reactor. Multiple regression analysis using the results from the previous studies and this study indicates that the biomass amount of microalgae are more important than that of bacterial for TN removal efficiency (Chapter 4). In contrast, the biomass amount of nitrifying bacteria is more important for ammonia removal efficiency. Since the obtained findings in this study are the fundamental knowledge/information for future practical application, the scale-up of the process and the design of continuous outdoor operation were discussed in the following.

5.2 Examination of continuous experiment towards practical application

Some modifications/improvements are needed to use the light-tolerant microalgae-nitrifying bacteria consortia in continuous experiments as described below. i) Due to the lack of durability, alginate hydrogel, which is a natural and biodegradable polymer, is not suitable as immobilizing material to be used for long-term operation. Thus, it needs to be changed to other material such as a non-degradable synthetic polymer or to the combination of such synthetic polymer and natural polymer. ii) Since optimum biomass ratio of microalgae and nitrifying bacteria is confirmed in this study, new design of continuous experimental system which can control the ratio to maintain its optimum value is needed. However, no such continuous system has been designed so far because it has been almost impossible to separate microalgae and nitrifying bacteria in the conventional consortia process.

Therefore, the examination of new immobilizing materials with high durability and the design of a continuous experimental system which enable to control the biomass ratio by continuously separating microalgae and light-shielding hydrogel, and simultaneously recovering microalgae biomass are discussed in the following sections.

5.2.1 Improvement of durability for light-shielding hydrogel

Natural polymers such as alginate and κ-carrageenan are not suitable as immobilizing material to encapsulate nitrifying bacteria for long-term operation at least as only component because they are biocompatible but easily degraded by microorganisms. In the experiment in chapter 3, the elastic modulus of light-shielding hydrogel, indicating the mechanical strength of the hydrogel, decreased from 42.5 kPa to 35.1 kPa though the batch experiment in the microalgae-nitrifying bacteria consortia (data not shown). From these results, the weakness of alginate hydrogel was obvious. To improve the low durability, microbial immobilization using highly durable synthetic polymers such as polyacrylamide (PAM), polyethylene glycol (PEG), and polyvinyl alcohol (PVA) has been investigated in several studies (Bouabidi et al., 2019). Although these synthetic polymers are suitable for a long-term operation due to their persistence, there is a disadvantage that the cross-linking/polymerization reagents for such polymers can be toxic to the microorganisms (Takei et al., 2011). Recently, immobilization methods using PVA and sodium alginate (SA) composites are often applied for activated sludge, nitrifying bacteria, and ANAMMOX to reduce toxicity during immobilization. Furthermore, PVA is a relatively inexpensive material, making it easy to use for wastewater treatment. Therefore, PVA-SA is considered as an appropriate option to prepare light-shielding hydrogel suitable for a continuous operation in terms of durability and cost.

5.2.2 Proposal of new continuous and biomass ratio controllable system

The Anaerobic-anoxic-aerobic (A2O) process is one of the conventional nitrification-

denitrification methods as shown in Figure 5-1 (top). For example, in China, the A2O process has been implemented in 1167 locations of WWTPs, accounting for 26.31% of the total number (Liang et al., 2020). The A2O process performs nitrification and denitrification by cycling through Anaerobic, Anoxic, and Aerobic reactors. The energy requirements in the A2O process are mainly for agitation power in Anaerobic and Anoxic, aeration power in Aerobic, and power for sludge circulation and reactor mixing. The HRAP reactor used in the conventional microalgae-nitrifying bacteria consortia shown in Figure 5-1 (middle) is operated in a single reactor. The energy requirement in the process is for agitation power and pump power in algae recycling. However, wastewater treatment by HRAP requires a longer hydraulic retention time (HRT) of 3.5-5 days to achieve high removal rates as compared to the shorter HRT of 6-24 h in A2O process (Saúco et al.., 2021). Therefore, although conventional consortia using HRAP are cost- and energy-efficient, their relatively low nitrogen removal performance is a challenge. From the results of Chapter 4, it is clear that the nitrogen removal performance varies with biomass ratio, suggesting that an approach using some kind of operation to control biomass ratio is an effective way to improve nitrogen removal performance. Therefore, a new biomass ratio controllable reactor is proposed by modifying a conventional HRAP as shown in Figure 5-1 (bottom). To achieve separation and recovery of microalgae from light-shielding hydrogel, it is necessary to have the equipment for hydrogel separation, biomass recovery and liquid return. A simple separator such as a sieve or mesh to separate light-shielding hydrogel from microalgae can be installed at the outlet of the HRAP reactor. This is possible due to considerable size difference between microalgae (< 8μm) and light-shielding hydrogel (> 3 mm). Based on the monitored data of biomass ratio in the reactor, the required amount of supernatant from the sedimentation tank is circulated to the reaction tank after solid-liquid separation by filtration or centrifugal separation. This operation allows for the reduction of overgrown microalgae in the reactor. On the contrary, if there is a shortage of microalgae in reaction tank, microalgae can be added by returning the algal biomass directly from the sedimentation tank to reaction tank without removing microalgae. This operation will allow continuous biomass ratio control in the reactor and is expected to contribute to improving

nitrification performance as well as shortening its HRT. However, since this system involves additional costs compared to the conventional microalgae-nitrifying bacteria consortia, whether its economics is a more effective approach than the conventional A2O process will be discussed in the next section.

5.3 Energy estimation for full-scale experiment

As shown in Figure 5-1, the proposed new HRAP process needs additional facilities for microalgae recovery and filtrate return in addition to the conventional HRAP reactor. Table 5-1 shows the advantages and disadvantages of different microalgae harvesting methods such as coagulation/flocculation, filtration and centrifugation. The proposed HRAP requires a low-energy water-recycling facility for microalgae harvesting. From the Table, it appears that algae harvesting by filtration is the most appropriate for this proposed HRAP process because of the availability of water recycle and its advantages. There are two types of membrane-based filtration: tangential flow filtration (TFF) and dead-end filtration (DEF) (Singh and Patidar, 2018). The TFF, well known as cross flow filtration, has high removal efficiencies of 70-89%. Meanwhile, DFE is not an economical method because it easily causes membrane fouling. Therefore, a new modified HRAP reactor combining TFF as a filtration was proposed.

Predicted energy consumption and energy reduction based on the previous studies are shown in Table 5-2. Energy consumption in wastewater treatment plants such as the Anaerobic-aerobic (AO) process and the A2O process is 0.39-0.67 kWh m^{-3} in all regions (Hernández-Sancho et al., 2011). Another study reported that the energy consumption for the A2O process was 0.48 kWh m^{-3} (Li et al., 2021). On the other hand, the energy consumption for wastewater treatment with HRAPs by microalgae-bacteria consortia was 0.25 kWh m^{-3} (Kohlheb et al., 2020). The energy consumption for TFF to harvest microalgae was reported to be 0.2 kWh m^{-3} (Soomro et al., 2016). However, in this case, all the wastewater was filtrated to harvest whole microalgae in the effluent from the reactor. To

estimate energy consumption for the modified HRAP with microalgae returning based on the reported values, three different scenarios were considered: HRAP with no TFF (0%), HRAP with TFF treating half volume of effluent (50%), and HRAP with TFF treating full volume of effluent (100%).

Assuming daily treatment amount of wastewater is at 1000 m^3 day^{-1}, estimated energy consumptions are 480 and 250 kWh day^{-1} for the A2O and HRAP process, respectively. The energy consumption of the proposed HRAP was 250 kWh day^{-1} with 0% TFF operation, 350 kWh day^{-1} with 50% TFF operation, and 450 kWh day^{-1} with 100% TFF operation. The results show that if no biomass control is needed and no TFF operation is required, expected energy reduction of the proposed process as compared to the conventional A2O process could be approximately 50%. However, in the extreme case of full operation of TFF, energy reduction could be only 6%. Since TFF needs to be operated only when the biomass control is required, energy consumption could be within the range of these values. The analysis suggests that if the proposed advanced light-tolerant microalgae-nitrifying bacteria consortia was applied for ammonia containing wastewater treatment under strong light irradiation by using the modified HRAP process, it can be operated more energy-efficiently than the conventional A2O process with mechanical aeration. Additionally, it is expected to provide higher removal performance of ammonia than the conventional HRAP process due to the maintenance of optimum biomass ratio.

5.4 Future study

This study focused on the development of a light-tolerant microalgae-nitrifying bacteria consortia under intense light irradiation. Although the batch experiments were conducted as a fundamental investigation of the process in this study, the photoinhibition mitigation effect of nitrifying bacteria and the nitrogen removal performance by microalgae-nitrifying bacteria consortia in continuous experiments have not been clarified. Furthermore, the effect of fluctuation in various environmental parameters such as temperature, solar radiation time, light intensity, and other factors

in outdoor condition is not examined yet. To evaluate the effectiveness of the proposed process for practical application, further continuous outdoor experiments should be conducted and appropriate operational conditions to achieve stable treatment performance needs to be clarified.

The effect of the ratio of microalgae to bacteria on nitrogen removal performance was investigated in chapter 4. However, it has been reported that nitrogen removal performance is affected not only by biomass ratio, but also by effluent C/N ratio, bacteria strains, and HRT (Fallahi et al., 2021). Since results from only batch experiments are insufficient to establish an overall predictive model, detailed information from continuous experiments under various conditions is required for an accurate multivariate analysis.

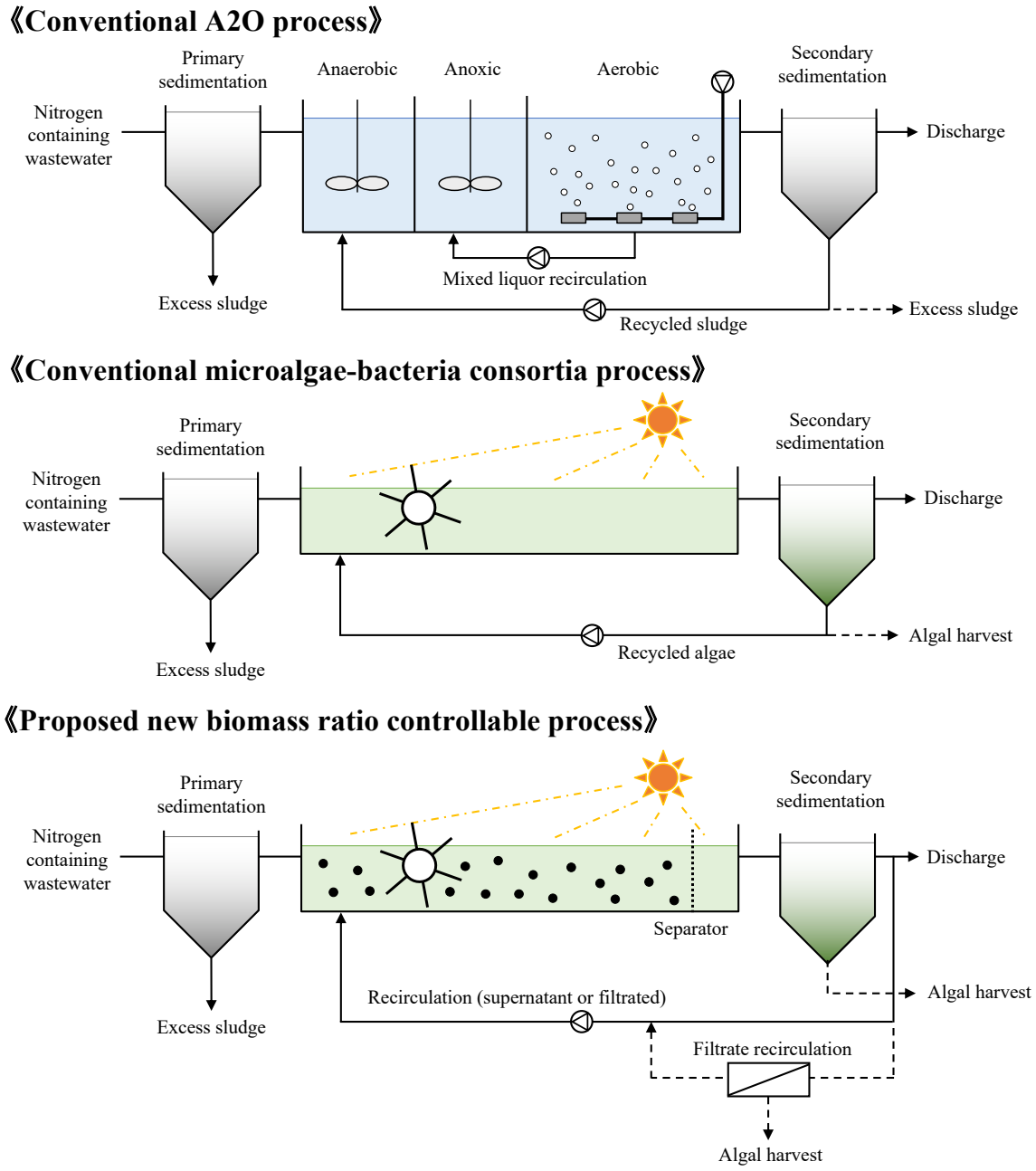

Figure 5-1 Conceptual diagram of conventional process and proposed process

(top) conventional A2O process;

(middle) conventional microalgae-bacteria consortia process;

(bottom) proposed new biomass ratio controllable process.

Table 5-1 Advantages and disadvantages of different microalgae harvesting methods (Singh and Patidar, 2018).

Harvesting method	Advantages	Disadvantages
Coagulation/flocculation	• Fast and easy process	• Chemicals may be expensive
	• Less cell damage	• Difficult to separate the coagulant from harvested biomass
	• Used for large scale	• Culture medium recycling is limited
	• Less energy requirements	• Highly pH dependent
	• Auto and bioflocculation may be inexpensive method	
Filtration	• Cost effective	• Slow, requires pressure or vacuum
	• Low energy consumption (natural and pressure filter)	• Membrane fouling/clogging and replacement increases operational and maintenance costs
	• Water recycles	
Centrifugation	• No chemical required	• High energy consumption (vacuum filter)
	• Fast and effective technique	• Expensive technique with high energy requirement
	• High recovery efficiency (>90)	• High operation and maintenance costs
	• Applicable to all microalgae	• Time consuming and too expensive for large scale

Table 5-2 Predicted energy consumption and energy reduction based on previous study.

Reference	Process	Energy consumption (kWh m^{-3})	Energy consumption (kWh d^{-1})	Energy reduction (%)
Li et al. (2021)	A2O process	0.48	480	100
Kohlheb et al. (2020)	HRAP	0.25	250	47.9
Soomro et al. (2016)	TFF for algae harvest	0.2	200	58.3
This study	Modified HRAP (0% filtration)	0.25	250	47.9
	Modified HRAP (50% filtration)	0.35	350	27.1
	Modified HRAP (100% filtration)	0.45	450	6.25

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