Contribution of Microalgae to Granulation of Algal-Bacterial Sludge and Nutrients Removal

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Abstract

With the global population growth, annual production of wastewater is increasing; eco-friendly technologies are encouraged in the practice of wastewater treatment in order to alleviate or mitigate its environmental pollution. One of such technologies is algal-bacterial aerobic granular sludge (AGS), in which algal and microbial cells can aggregate into a dense and compact structure, yielding high biomass content with excellent settling ability and nutrients uptake, and outstanding capability with high energy saving potential to treat various wastewaters. Microalgae in algal-bacterial AGS are regarded to be highly capable of N and P removal from wastewater, which convert these nutrients into biomass that can be utilized as resources for renewable energy and fuels, thus further reducing greenhouse gases emission. Previous studies mainly focused on the formation of algal-bacterial AGS by inoculating microalgae with or without the bacterial granules as the seed sludge under different operation conditions. Up to now, however, the mechanisms involved in the formation of algal-bacterial AGS remain unclear, and the contribution of biological factors to the granulation of algal-bacterial symbiosis system hasn't been addressed yet.

This dissertation attempted to figure out the contribution of microalgae to the granulation of algal-bacterial symbiosis system and nutrients removal. In this study, microbial community of the algal-bacterial AGS was firstly investigated during the operation of two identical sequencing batch reactors (SBRs) seeded with two types of algal-bacterial AGS in order to understand the dynamic changes of dominant bacteria or microalgae during long-term operation. Then two microalgal strains were isolated respectively from the granules of the two SBRs on day 35 (under stable operation). After their nutrients removal and auto-aggregation capabilities being confirmed, the two isolated microalgae species were inoculated into another two SBRs with activated sludge to examine their contribution to the granulation process.

The main results from this study can be summarized as follows.

(1) During the operation of two types of mature algal-bacterial AGS for 90 days, the dominant classes of bacteria were found to be *Alpha-, Beta-, Gamma-,* and *Delta-proteobacteria* and *Flavobacteria*, which remarkably decreased during the later stage of operation of both SBRs. In terms of algal community, *Cyanobacteria* and *Unclassified* groups dominates the reactors. Besides, some significant changes in both communities were discerned, which might contribute to the instability of algal-bacterial AGS and its breakage during the long-term operation.

(2) On day 35, two microalgal strains (A1 and A2) were isolated from the algal-bacterial AGS in the above two SBRs. Both isolated microalgae belonged to the genus *Chlorella*, not the expected class (*Cyanobacteria*). With a faster biomass growth and higher auto-aggregation index (82.4% on day 5), strain A1 exhibited excellent nutrients removal, achieving 93-96% of N and P removal when treating wastewater containing 50 mgNH_4-N/L and 5 mgPO_4-P/L, which were always 9-14% higher than strain A2. The total extracellular polymeric substances (EPS) content of the isolated strains had a strongly positive correlation (r^2=0.95 for A1 and r^2=0.92 for A2) with auto-aggregation capability under the test light/dark (12 h/12 h) cycle condition, while a weak correlation (r^2=0.42 for A1 and r^2=0.43 for A2) was obtained under only dark cultivation (0 h/24 h).

(3) When the two isolated microalgal strains (*Chlorella* sp.) were respectively inoculated into two SBRs with seed activated sludge, the inoculated microalgae were observed to help the fluffy activated sludge to transform into a little bit tight structure, initiating the formation of algal-bacterial granules. Both reactors exhibited similar efficiency in total nitrogen (TN) removal of ~ 59%. However, the total phosphorus (TP) removal was detected to decrease from 78% to 56%, and then improved up to ~82% during stages I and II, respectively. The class of *Anaerolineae* dominated the reactors when seeded only with activated sludge, which noticeably decreased after inoculation of the microalgal strains. In terms of algal community profile, the dominant genus was *Chlorella*, and the genus of *Leptolyngbya* was observed in R2 at the end of the operation. The dynamic changes of the bacterial and microalgal profiles shed light on the mechanisms regarding the formation and stable operation of algal-bacterial AGS.

Results from this study imply that inoculation of some proper microalgae may accelerate the establishment of algal-bacterial AGS system, providing a feasible approach for quick conversion of conventional activated sludge system to algal-bacterial AGS system. The contributions of other microalgal species and biological factors to algal-bacterial AGS formation and nutrients removal are still pending.

Keywords: Algal-bacterial aerobic granular sludge; Microalgae; Auto-aggregation; Nutrients removal; Granule formation

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Abbreviations and acronyms

AGS	Aerobic granular sludge
AOB	Ammonia oxidizing bacteria
AS	Activated sludge
Chl-a	Chlorophyll a
COD	Chemical oxygen demand
EPS	Extracellular polymeric substances
GAOs	Glycogen accumulating organisms
HRT	Hydraulic retention time
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
N	Nitrogen
NOB	Nitrite oxidizing bacteria
P	Phosphorus
PAOs	Phosphorus accumulating organism
PN	Proteins
PS	Polysaccharides
SBR	Sequencing batch reactor
SRT	Solids retention time
SVI	Sludge volume index
TP	Total phosphorus

Chapter 1 Introduction

1.1 Wastewater issue

Nowadays, the world has been tackled with drinking water issues, including water shortage and efficient solutions, thus it is required to be aware of how the countries are concerned and deal with various water issues and compare their policies and regulations on water. With respect to the developing countries, the circumstance of obsolete wastewater treatment technology heavily affects the resource of drinking water which is contaminated frequently with toxic chemicals and various bacteria.

According to the latest report by the United Nations World Water Development Report (2017), about 70% of the municipal and industrial wastewaters are treated in high-income countries, 38% in upper middle-income countries and 28% in lower middle-income countries. Worst of all, this ratio drops to only 8% in the low-income countries, in which 80% of wastewater globally is disposed to environment without treatment. This untreated wastewater not only contaminates the surface water bodies, but also causes serious soil contaminations that can lead to further underground water contaminations. In other words, there are more threats to the water circulation system in the environment. Consequently, water and soil contaminations exert significant impact on the surrounding environment and trigger the outrage of infectious disease as well.

1.2 An overview on aerobic granulation technology

1.2.1 Aerobic granular sludge (AGS)

Aerobic granular sludge (AGS) has been developed during the application of activated sludge (AS) system in wastewater treatment and the earliest research work were published in 1990s (Mishima and Nakamura, 1991; Morgenroth *et al*., 1997). Microbial communities in AS exist as flocs whereas these mixed microbial communities in AGS turn into aggregates and layered form, when operating sequencing batch reactors (SBRs) with bubbling aeration system and short settling time (Nancharaiah *et al.,* 2018). The advantages of AGS systems include 23% less electricity requirement compared to AS, less involvement of chemicals for nutrients removal, better settling properties and so on (Bengtsson *et al.,* 2019)

In the AGS systems, microbial cells can be aggregated into dense and compact structure, yielding high biomass content with excellent settling ability, and outstanding capability to treat extremely toxic organic and inorganic pollutants (Adav *et al.,* 2008). The compact structure of AGS allows to survive a diverse number of facultative and aerobic microbes including

ammonia-oxidizing, denitrifying and phosphate accumulating bacterial species, achieving efficient removals of nitrogen and phosphate (Wang *et al.,* 2019). According to these authors' report, the appropriated C/N ratio is important to obtain the compact structure of AGS with high nutrients removal efficiency. For instance, the highest removal efficiencies were 96% and 88% for chemical oxygen demand (COD) and ammonium nitrogen (NH_4 - N), respectively while a COD of 3,300 mg/L and ammonium of 165 mg/L (C/N = 8) could promote excretion of higher amount of protein in extracellular polymeric substances (EPS) resulting in better AGS formation.

The better sludge settling properties can be one of the cost-efficient solution for the wastewater treatment plants, and AGS always has very good settleability compared AS. In the AGS system, when the sludge volume index (SVI) was evaluated by the difference between 30 and 10 minutes of settling (SVI_{30}/SVI_{10}), the ratio of about 0.8 indicates formation of granules. Generally, when SVI_{30} in the AGS system with total suspended solids ranged between 30 mL/g to 60 mL/g, they increased up to 100 mL/g in the AS system (Bengstone *et al.,* 2018).

1.2.2 Algal-bacterial AGS

(1) State-of - the -art

Up to the present, many kinds of bacterial AGS have been developed, and most research works are centered on the development of efficient bacterial AGS systems with respect to rapid granulation, granular stability, pollutants or nutrients removal efficiencies, and mechanisms involved (Bengstone *et al.*, 2018; Nancharaiah and Reddy, 2018; Zhang *et al.*, 2019). Due to the merits of co-existence of algae and bacteria, recently the newly developed algal-bacterial AGS system has attracted many researchers' attention. In the algal-bacterial symbiosis system, aerobic bacteria have potential to oxidize and decompose organic matters in the wastewater into nutritional forms which can be then assimilated by microalgae; meanwhile microalgae can release oxygen to be utilized by bacteria to decompose organics (Ji *et al.*, 2018). Thus, compared to the general bacterial AGS, algal-bacterial AGS system is more appealing.

Huang *et al.* (2015) found that algal-bacterial granules could be formulated after 19 days' operation of sequencing batch reactor (SBR) under natural sunlight illumination. After that, most current research works focused on the rapid formation of algal-bacterial AGS (He *et al.*, 2018), granular stability and performance under various operation conditions like different light illumination intensities and salinity levels on algal-bacterial AGS formation and lipid accumulation (Meng *et al.*, 2019a, 2019b), performance of continuous-flow reactors (Ahmad *et al.*, 2017, 2019), use of algal-bacterial AGS to treat low carbon wastewater (from COD/N

of 8 to 1)(Zhao *et al.*, 2018), and stability under shaking with no air bubbling (Zhao *et al.*, 2019). However, the mechanisms of granulation in the algal-bacterial symbiosis system and its contributors are not well addressed.

(2) Contribution of algae

Algae are regarded to be highly capable to convert N and P from wastewater into biomass that can be utilized as resources for renewable energy and fuels, thus reducing total greenhouse gases emission (Beuckels *et al.*, 2015). Arcila and Buitrón (2017) studied the influence of solar irradiance on the formation of microalgae-bacteria aggregates for the use in high rate algal pond to treat municipal wastewater. Moreover, microalgae possess potentials for removing heavy metals and other micropollutants, very harmful to the healthy environment, which is considered as environmental and economic benefits of microalgae (Malik, 2002; Wu *et al.,* 2014; Kube *et al.,* 2018). One previous study on microalgal research reported that *Chlorella sorokiniana* GXNN 01 immobilized in calcium alginate had higher removal efficiency of N and P from synthetic wastewater under autotrophic, heterotrophic, mixotrophic and micro-aerobic conditions than free-living cells (Liu *et al.,* 2012).

Liang *et al.* (2013) reported that the algae-bacteria symbiosis system of *Chlorella vulgaris* and *Bacillus lincheniformis* exhibited higher NH_4-N (78%) and TP (92%) removal efficiency than the single bacteria or algae system. However, some monoculture of microalgal species has capability as their symbiosis system for treating similar N and P removal various wastewaters (Liu and Vyverman, 2015). They noted that the efficiency of pollutant removal and aggregate formation is strongly associated with the irradiance level, and a lower irradiance level mildly promoted microalgae-bacteria granule formation, mainly attributable the increased excretion of bound EPS. Cai *et al.* (2019) successfully obtained algae granules after 60 days' operation of SBR with a mixture of naturally grown microalgae under the designed selective pressure and hydrodynamic shear force. These researchers claimed that the formation of algae granules can ease the separation of algae biomass from wastewater and significantly improve the nutrients removal efficiency.

(3) Problems statement

Globally, various innovative technologies have been designed for wastewater treatment. However, it is a question of addressing every single impediment for further sophisticated development. Even though AGS biotechnology is apparently a part of this milestone, the conceptualization of AGS has lasted for three decades. Still, it is not clear whether bacteria,

microalgae, fungi and or protozoa drive mainly the granulation process. Challenging the uncertainty, it is required to identify whether these biological factors enhance the granulation process. What is more, microalgae harvest technology for the wastewater treatment system is considered as costly and requires huge investment efforts. In response to this urgency, it is meaningful to identify whether bacteria or microalgae play an important role in the granulation process.

1.3. Research objective and thesis structure

Nowadays, algal-bacterial AGS system for treating wastewater has raised attention. However, the biological interactions and the contribution of each individual biological organism to the formation of algal-bacterial granules remain unclear. Restated, very little information is available on the contribution of microalgae and bacteria to the formation of algal-bacterial AGS and the enhanced nutrients removal performance. Thus, it's necessary to illustrate the biological factors that effect on the granulation of algal-bacterial symbiosis system.

The main objectives of this research are: 1) to analyze the differences of biological structures in algal-bacterial AGS; and 2) to isolate microalgal strains with high nutrients removal efficiency and auto-aggregation ability. By operating SBRs, this research also aimed to find out whether microalgae is the contributor to the granulation process of the algal-bacterial symbiosis system.

The structure of the thesis and the relationship among the chapters are shown in Fig. 1-1.

(1) Chapter 1 Introduction

The current issue of the wastewater was addressed in addition to the summary of the achievements of the related state-of-the-art technologies such as AGS and algal-bacterial AGS system for wastewater treatment. The objectives of this study and the thesis structure were arrived at in this chapter.

(2) Chapter 2 Dynamics of microbial and algal community analysis in algal-bacterial AGS

The biological community characteristics were analyzed to define the dominant group(s) of bacteria and microalgae in algal-bacterial AGS. Then the discussion was carried out on whether the dominant group had correlation with the granulation of algal-bacterial AGS during the operation of SBRs.

(3) Chapter 3 Isolation of the microalgal strains from algal-bacterial AGS and examination on their performance

The isolation of microalgal strains from mature algal-bacterial AGS from R1 and R2 was performed. Their contents of extracellular polymeric substances (EPS) and nutrients removal

together with auto-aggregation capability were monitored in this chapter. The biomass growth, N and P removal, and auto-aggregation capability of the two isolated strains were compared and discussed.

(4) Chapter 4 Examination on the contribution of the isolated strains to the granulation process

Morphological and biological community changes of the activated sludge and the mixture of activated sludge with the isolated microalgal strains were explored during the re-cultivation of algal-bacterial AGS in the SBRs. In addition, the reactor performances such as nutrients removal and biomass growth in the two SBRs were evaluated.

(5) Chapter 5 Conclusion and future research perspectives

The results of this research were summarized with the future research being prospected.

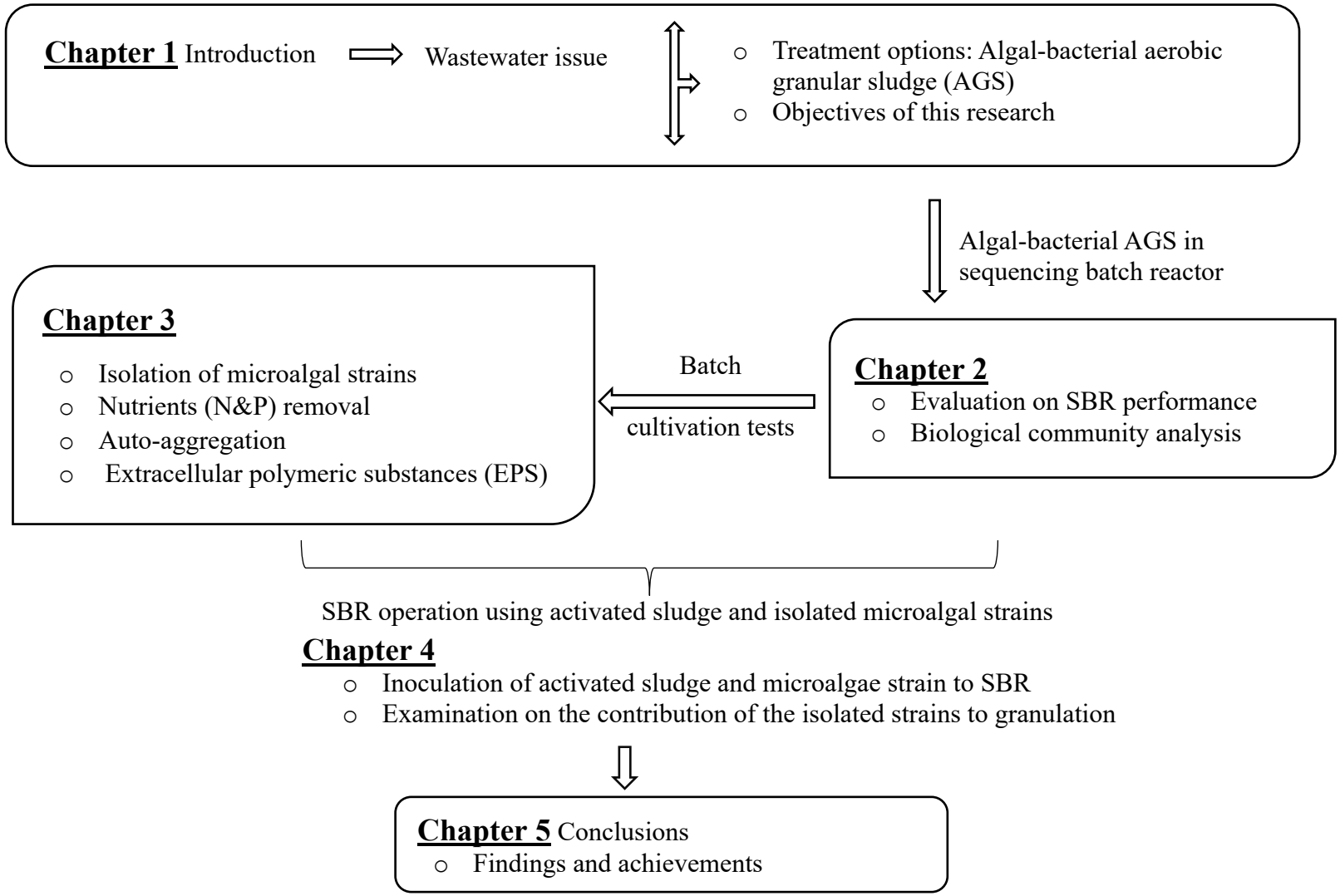

Fig. 1-1 The framework of this thesis

Chapter 2 Dynamics of bacterial and algal communities in algal-bacterial AGS

2.1 Background

Algal-bacterial consortium or symbiosis system is one of the innovative technologies for wastewater treatment, since each individual organism has its unique metabolites and functions to enhance efficiency of the treatment process. Exploration on microbial community in algal-bacterial AGS is important for a better understating of the granulation process and mechanisms involved. One previous study reported that the percentage of family members of N and P removal bacteria such as *Nitrospiraceae* and *Nitrosomonadaceae* were found to be higher in the AGS reactor in comparison to the reactor with symbiotic growth of bacteria and algae (Huang *et al.,* 2015). While during the formation of algal-bacterial granular consortia with bacterial aerobic granules under the summer natural sunlight exposure, He *et al.* (2018) observed few changes in the microbial community and the predominant algae were shifted from *Diatomea* to green algae *Chlorophyceae*. Most recently, Wang *et al*. (2019) noticed some significant difference in the bacterial structure, when operating AGS at different carbon to nitrogen (C/N) ratios. For instance, some microbes were increased or decreased, and the predominant microbes changed at various levels of taxonomy: at phylum level the *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Verrucomicrobia* were predominant; meanwhile, at family level *Rhodocyclaceae*, *Verrucomicrobiaceae*, *Comamonadaceae*, and *Flavobacteriaceae* became predominant.

The main purpose of this Chapter was to understand the dynamic changes of the biological community structure through the analysis of the most dominant bacteria or microalgae. It is expected to find out whether the dominant group is correlated with the granulation of algal-bacterial AGS during the operation of SBRs.

2.2 Materials and methods

2.2.1 Operation conditions for the SBR reactors

Two identical sequencing batch reactors (SBRs, R1 and R2) made of acrylic transparent plastic, each with a working volume of 0.5 L, were used in this study. R1 was seeded with the frozen algal-bacterial AGS which was originally collected from a lab-scale continuous-flow reactor (Ahmad *et al.,* 2017) and preserved at -20°C for 3 months. R2 was inoculated with the mature algal-bacterial AGS from a lab-scale SBR (Zhao *et al.,* 2018). Both mother algal-

bacterial AGS reactors (one in continuous-flow and another one in batch mode) have been operated for treating synthetic wastewater for 10 months before the granules being sampled. R1 and R2 were operated for 90 days at room temperature (25±2°C) with no light control (light illumination intensity was about 165 – 201 μmol/m^2·s when all room lights were on).

In this study, one operation cycle was 6 h, including 9 min influent filling, 120 min non-aeration, 214 min aeration, 3 min settling and 14 min effluent withdrawal. Both SBRs were operated at a hydraulic retention time (HRT) of 12 h with no control of sludge retention time (SRT). Synthetic wastewater was fed to the two SBRs, which was composed of 300 mg chemical oxygen demand (COD, NaAc)/L, 5 mg PO_4-P/L (KH_2PO_4), 50 mg NH_4-N/L (NH_4Cl), 10 mg Ca^{2+}/L ($CaCl_2$), 5 mg Mg^{2+}/L ($MgSO_4 \cdot 7H_2O$), 5 mg $Fe^{2+/}$L ($FeSO_4 \cdot 7H_2O$), and 1 ml/L of trace elements solution. The trace elements solution was prepared according to Huang *et al.* (2015).

2.2.2 Evaluation on SBR performance

Effluent samples were collected once every 5 days at the end of the aeration cycle and filtered through 0.22 μm membrane for the determinations of ammonia nitrogen (NH_4-N), nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N), phosphorus (PO_4-P) and dissolved organic carbon (DOC). Samples for the determinations of mixed liquor (volatile) suspended solids (ML(V)SS) and sludge volume index (SVI) were collected once every twenty days. All the measurements were conducted according to the Standard Methods (APHA, 2012).

2.2.3 Microbial community analysis

The granules for biological community analysis were sampled from the two SBRs (R1 and R2) on days 5, 35 and 85, respectively, thus totally six samples were collected from the two reactors and used for this analysis. Total DNA was extracted from each granule sample using the PowerMax ®Soil Kit (QIAGEN GmbH, Germany) following the manufacturer's protocol. The extracted DNA was kept at -20°C until further process. Genomic DNA was detected by 1% agarose gel electrophoresis. The specific primers set with barcode 338F 5′-ACTCCTACGGGAGGCAGCA-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′ for bacteria, and P23SrV-1F 5′- GGACAGAAAGACCCTATGAA-3′ and P23SrV-1R 5′-TCAGCCTGTTATCCCTAGAG-3′ for algae were used for amplification of the hypervariable region of 16S rRNA gene and 18S rRNA gene, respectively. PCR was performed in ABI GeneAmp® 9700 (Applied Biosystems, USA) under the following conditions: 95°C for 3 min,

followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 45 s and a final extension step at 72°C for 10 min. After purification using the QIAquick PCR Purification Kit (Qiagen, Germany) and quantification using QuantiFluor™-ST (Promega, USA), the PCR products of all samples were taken for high-throughput sequencing on an Illumina platform (Illumina PE250, USA). MOTHUR (version: 1.31.2) was used for analyzing the biodiversity of bacteria and eukaryote in the granules. The Illumina MiSeq was used to analyze the biological community characteristics.

2.3 Results and discussion

2.3.1 Performance of the two SBRs

At the beginning of the operation of R1, the MLSS, SVI_5 and mean granule diameter were 3.50 g/L, 36.98 mL/g-MLSS and 0.61 mm, respectively; while in R2, they were 3.29 g/L, 30.40 mL/g-MLSS and 1.75 mm, respectively (Table 2-1).

The granular size gradually increased up to 2.1 mm (in R1) and 8.0 mm (in R2) on day 70, and after that a decreasing tendency of granular size was noticed in both reactors (Fig 2-1). Biomass concentration seemed to be relatively stable under the designed operation conditions, as MLSS was determined as 3.54 g/L and 4.03 g/L in R1 and R2 on day 85, respectively. However, the sludge settleability became worse to some extent, with their SVI_5 values varied between 65 -100 mL/g-MLSS in R1 and 88-128 mL/g-MLSS in R2, respectively. The increase in SVI_5 may also indicate the beginning of breakage of algal-bacterial AGS.

During the 90 days' operation of the two SBRs, NH_4-N, PO_4-P, and DOC removal efficiencies were 95 - 99 %, 63 - 76 %, and 82 - 87%, respectively.

2.3.2 Biological community diversity

During the whole operation, totally six samples were collected from the two SBRs, which were sampled on days 5 (R1-5, R2-5), 35 (R1-35, R2-35), and 85 (R1-85, R2-85), respectively.

After quality control, 419,399 sequence could be converted into 786 operational taxonomic units (OTUs), and 487,527 sequence into 345 OTUs bacterial and algal profiles, respectively for the samples collected on day 5 and 35. Moreover, 226,622 sequence could be converted into 360 OTUs and 185,902 sequence into 145 OTUs bacterial and algal profiles, respectively for the samples collected on day 85 (when the breakage of the algal-bacterial AGS was observed). In order to obtain identification information corresponding to each OUT, the RDP classifier was used (Wang *et al.,* 2007). The OTUs were identified with 97% identity and were assigned to 8 classes, 10 orders, 13 families and 22 genera in the bacterial profile. In addition,

algal profile was assigned to 2 classes and 5 genera. Taxa with relative abundance < 1% and unclassified groups were named as 'others'.

The five indexes are summarized in Table 2-2 to evaluate the differences of alpha-diversity in the bacterial and algal communities. The coverage value is to evaluate the presenters of the sampled gene sequences, and high coverage estimators (≥ 99%) are the evidence of the well-generated gene sequences. Besides, abundance-based coverage estimation (ACE) and Chao values are for community richness, and the Shannon and Simpson indexes can be used to represent their diversity (Lucas *et al.,* 2017; Kim *et al.,* 2017).

Clearly seen from Table 2-2, the bacterial profiles have the highest community richness and diversity compared to the algal profiles. And all the samples have the high coverage value of 0.99.

The relative abundances of algal-bacterial AGS samples are shown in Figs. 2-2a and 2-2b, and Figs.2-3a and 2-3b at the class and genus levels of bacterial and algal profiles, respectively. As shown in Fig. 2-2a, obvious differences in microbial community were observed in the granules from the two SBRs along with the operation. According to the relative abundance, *Alpha-, Beta-, Gamma-,* and *Delta-proteobacteria* occupied 27%, 20%, 22%, and 7% (with a subtotal of 76%) of the total OTUs in the granules from R1, which were 23%, 10%, 40%, and 8% (with a subtotal of 81%) in R2 on day 5, respectively. These bacteria are the major classes in gram-negative bacteria with the capability of growing at very low nutrients level and high abundance in diverse environments. Therefore, microbes in these classes can be found to be dominant, constant or alterable in activated and granular sludge (Zhang *et al.,* 2012; He *et al.,* 2016). On day 35, except some slight increase in *Delta- proteobacteria*, *Alpha-, Beta-*, and *Gamma-proteobacteria* were found to decrease to some extent. This observation agrees with the finding by *Wang et al.* (2019) who claimed that during the granulation process, the classes within the phylum *Proteobacteria* decreased from 60.3% (day 1) to 31.5% (day 60) when the influent C/N ratio was increased to 8 (with COD of 3,000 mg/L and NH_4-N of 165 mg/L). However, Zhang *et al.* (2018) reported that *Alpha-proteobacteria* increased from 7.5% to 29.5% (non-phototrophic) and 24.2% (phototrophic) in algal-bacterial AGS, which together with other classes like *Beta-* and *Gamma-proteobacteria* dominated the reactor. In this study, the other classes were found to be *Flavobacteria* (11% in R1 and 14% in R2), and *Sphingobacteria* (4% in R1 and 2% in R2) on day 5. As shown in Fig. 2-2a, an obvious increase in relative abundance was observed on *Flavobacteria* (21% in R1 and 20% in R2) on day 35, during which *Sphingobacteria* were noticed to slightly increase to 5% in R1 and 4% in R2, respectively. According to Wang *et al.* (2019), microbes in these classes mainly responsible

for secretion of EPS are relatively stable in various environments, which is meaningful for the stability of granules. The increase of these classes in this study signals the improved stability of the algal-bacterial AGS, which may also contribute to the excellent performance of the 2 reactors during this period to some extent.

However, on day 85 it was found that the microbial population decreased substantially, and the largest decrease was noticed in the classes of *Alpha-, Delta-proteobacteria*, and *Flavobacteria* (to 3%, 1%, and 0%) in R1. While in R2 almost no *Flavobacteria and Delta-proteobacteria* with only 2% of *Alpha-proteobacteria* were detected. On the other hand, in this study, microbes belonging to class of *Anaerolinea* showed an increasing tendency amounting to 8% and 24% of total OTUs at class level, which were not detectable in the granules from R1 and R2 on days 5 and 35, respectively. The representatives of the *Anaerolineae* class are obligate anaerobes which can be found in different types of natural and artificial environments and are also the major microbial population for anaerobic digestion, but their role in the environment is ambiguous due to their unculturable nature or scarcity of isolates (Xia *et al.,* 2016). As reported, these filamentous bacteria are usually existing in WWTPs at low concentrations, and the increase of these bacteria may lead to high risk of sludge bulking (Xu *et al.,* 2018). Therefore, in this work the remarkable increase of the microbial population of class *Anaeroline* might be the major reason for the breakage of algal-bacterial AGS.

In terms of algal community in the bacterial profile, the dominant class was *Cyanobacteria*, and its percentage increased from initial 4% to 32% in R1, and from initial 0% to 16% in R2 on day 85 (Fig. 2-3a). This remarkable increase of *Cyanobacteria* to some extent is in agreement with the observation by He *et al.* (2018) who found that *Cyanobacteria* increased from 1.4% to 7.5% during the granulation of algal-bacterial symbiosis system. While *Flavobacteria* may play a major role in the lysis of *Cyanobacteria* (Zhang *et al.,* 2018), thus the disappearance of *Flavobacteria* might be the reason for the increase of *Cyanobacteria* in this study.

Fig. 2-2b shows the microbial communities of bacterial profile at genus level. The genera of *Delftia, Pseudomonas, Stenotophomonas and Cloacibacterium* were only observed in R1, while *Thauera* and *Flavobacterium* were only in R2. As demonstrated, the granules from R1 and R2 shared 2 genera, *Pseudoxanthomonas* (12% in R1 and 19% in R2), and *Brevidomonas* (16% in R1 and 18% in R2) on day 5. Their dominance percentage was found to slightly decrease on day 35, about 1.0-2.0% in both reactors. However, except the genus *Zoogloea,* all the above genera disappeared and instead*, Nitrosomonas, Nitrospira, Candidatus Nitrogtoga, Azoarcus,* and *Candidatus Competibacter* appeared on day 85.

The most detected genera in this work are those frequently reported in AGS bioreactors treating different types of wastewater in previous studies. For instance, the genus *Flavobacterium* is able to produce cyclic-diguanylate and then induce gene expression which is responsible for EPS production (Wan *et al.,* 2015). This genus has been also found in the wastewater treatment plant where an enhanced biological phosphorus removal process was applied (Ryu *et al.,* 2007). The genera of *Thauera,* and *Azoarcus* are denitrifies which are often detected in the sludge from industrial WWTPs like coking wastewater treatment in steel industry (Ma *et al.,* 2015). In addition, the genera of *Candidates Competibacter* glycogen accumulating organisms (GAOs) were found to increase in this work (7% in R1 and 12% in R2). GAOs may compete with polyphosphate accumulating organisms (PAOs) for organic substrate, although no relationship has been found between GAOs presence and poor P removal during a three years' survey on 28 Danish full-scale WWTPs (Mielczarek *et al.,* 2013).

As for the classes detected in the algal profile (Fig. 2-3a), *Cyanobacteria* was slightly increased from 32% to 49 % in R1 during the whole operation: However, in R2 it was about 44%, 81% and 32% on day 5, 35 and 85 respectively. Meanwhile, the unclassified group showed a similar trend in R1, but, in R2 it is decreased from 16% to 6% on days 5 and 35, respectively, then increased up to 61%. This observation is possibly due to their different operation mode (continuous-flow or batch), because the two seeded algal-bacterial granules were obtained under almost similar laboratory conditions (Ahmad et al., 2017; Zhao et al., 2018). The genus of *Leptolyngbya* and *Merismopedia* were the dominant ones in both reactors (Fig. 2-3b). The genus of *Merismopedia* in the two reactors, however, was found to drop to 0.0% on day 85 even though it was noticed to increase from 13% to 26% in R1, and from 24% to 48% in R2 when the operation lasted from day 5 to day 35, respectively. During the operation the genus of *Lyptolyngbya* was observed to increase from 18% (day 5) to 44% (day 85) in R1, and from 20% (day 5) to 30% (day 85) in R2, respectively. These changes may be associated with the stable reactor performance (on day 35), and at the same time the percentage of relative abundance was increased. In contrast, when the breakage of algal-bacterial AGS (on day 85) started these microalgae disappeared, indicating that the microalgae belonging to this genus are playing important role in the granulation process. Moreover, the genus *Prosthecobacter* was also detected to decrease from 7% (day 5) to 2% (day 85) in R1, and from 3% (day 5) to 2% (day 85) in R2, respectively. Another 2 genera of algae, *Jaaginema* and *Opitutus* were only observed in R1. The former increased from 1% (day 5) and 5% (day 85), while the latter (about 2%) was only detected on day 85.

As reported by Tsolcha *et al.* (2018), the consortium *Leptolyngbya* based mixotrophic culture possessed capability to remove organic and inorganic compounds from various agro-industrial wastes and wastewaters. In addition, the results from Baresova *et al.* (2017) indicated that the cellular organic matter from *Merismopedia tenussima* may function as cationic polymer flocculant which could be applied for algae harvesting for biofuel production. Based on above results, the two dominant algal strains were isolated, and in the following experiments special attention was paid to their nutrients removal and auto-aggregation capability to shed light on their contribution to the performance of algal-bacterial granules.

2.4 Summary

In summary, during the 90 days' operation of the two SBRs, significant changes in dominant population of bacteria in the algal-bacterial AGS were observed at class, family and genus levels. During the operation of the two reactors seeded with frozen granules and mature granules, respectively, the bacterial profiles have the highest community richness and diversity compared to the algal profiles. Moreover, the dominant class of algal community in the bacterial profile was *Cyanobacteria*, which remarkably increased during the later stage of operation of both reactors. As granule size became bigger, the number of existing filamentous microbes such as class of *Anaerolinea* tended to be higher. The relative abundances percentage of this class was higher in R2 (24%), with bigger sized granules (8 mm) in comparison to R1(8%) with small sized granules (2.1 mm) at the end of the operation. In addition, the disappearance of genus *Merismopedia* and of class *Flavobacteria,* and the appearance of class *Anaerolinea* might contribute to the instability of algal-bacterial AGS and its breakage during the long-term operation.

More in-depth research works are still necessary to address the contribution of the biological factors (bacteria and algae) to the stable operation of algal-bacterial symbiosis system.

Table 2-1 Changes in of the ML(V)SS and SVI_5 during 90 days' operation

Operation	**MLSS (g/L)**		**MLVSS (g/L)**		**SVI_5 (mL/g)**	
days	R1	R2	R1	R2	R1	R2
0	3.51	3.29	3.06	2.94	36.98	30.40
10	4.85	3.93	4.12	3.20	41.19	48.28
20	3.09	3.24	2.30	2.35	51.78	52.39
40	5.17	.6.56	4.44	5.28	75.36	88.41
60	5.39	5.41	4.75	4.67	64.94	127.54
70	3.5	3.16	2.59	2.18	74.29	101.27
80	3.54	4.03	3.28	3.59	84.75	104.22
90	4.40	4.01	3.57	3.17	100.00	106.43

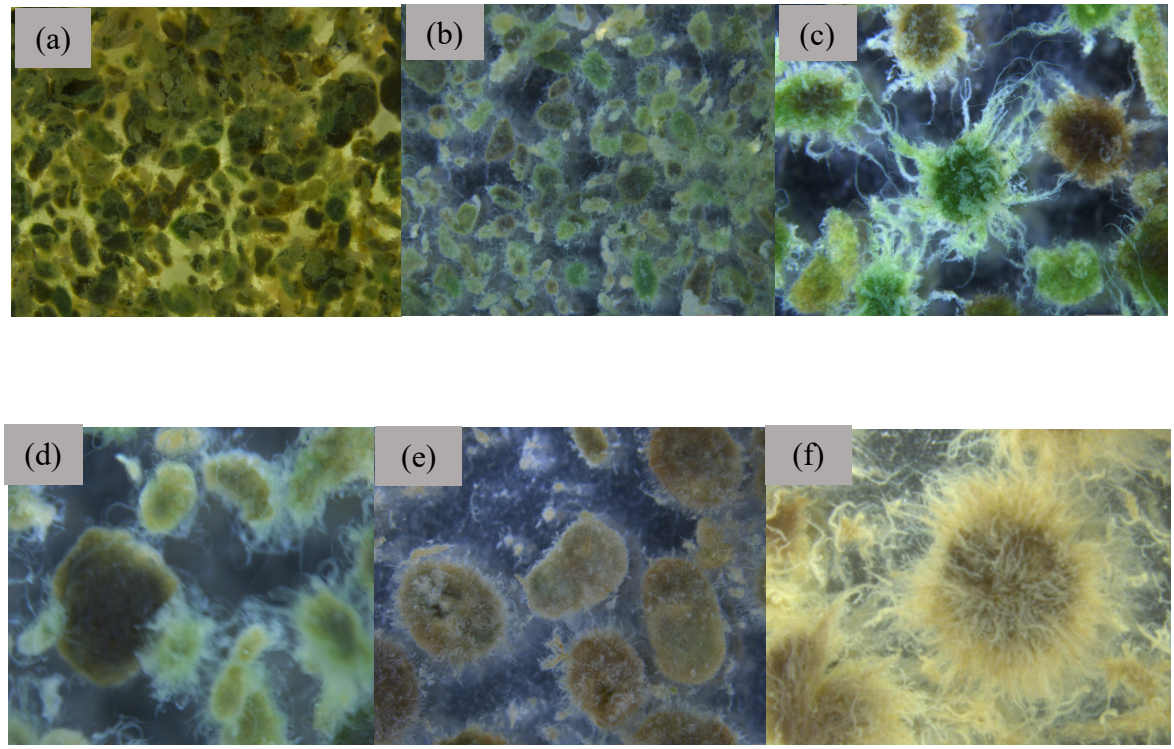

Fig. 2-1 Changes in morphology of the algal-bacterial AGS during the SBR operation: Ø= 0.6–1.2 mm (a), 1.3-1.7 mm (b), 1.8-2.1mm (c) in R1, and Ø=1-3mm (d), 4-6mm (e), 7-9 mm (f) in R2

Table 2-2 Changes in bacterial and algal diversities in algal-bacterial AGS during 90 days' operation of the two SBRs.

Sample ID	Shannon [a]		Simpson [b]		ACE [c]		Chao [c]		Coverage [d]	
	Bacteria	Algae	Bacteria	Algae	Bacteria	Algae	Bacteria	Algae	Bacteria	Algae
R1-5	3.46	2.42	0.068	0.138	336	120	337	123	0.99	0.99
R1-35	3.41	2.23	0.069	0.169	347	137	341	137	0.99	0.99
R1-85	3.70	1.97	0.067	0.278	339	120	340	119	0.99	0.99
R2-5	3.14	2.34	0.082	0.150	331	117	306	117	0.99	0.99
R2-35	3.18	1.64	0.079	0.311	296	139	305	145	0.99	0.99
R2-85	3.77	2.40	0.058	0.168	336	124	343	126	0.99	0.99

Note: R-5, -35, and -85 denote that the granule sampling was conducted on day 5, 35 and 85, respectively.

[a] Community diversity: A larger number denotes more diversity.

[b] Community diversity: A larger number denotes less diversity.

[c] Community richness: A larger number denotes greater richness.

[d] Sampling depth.

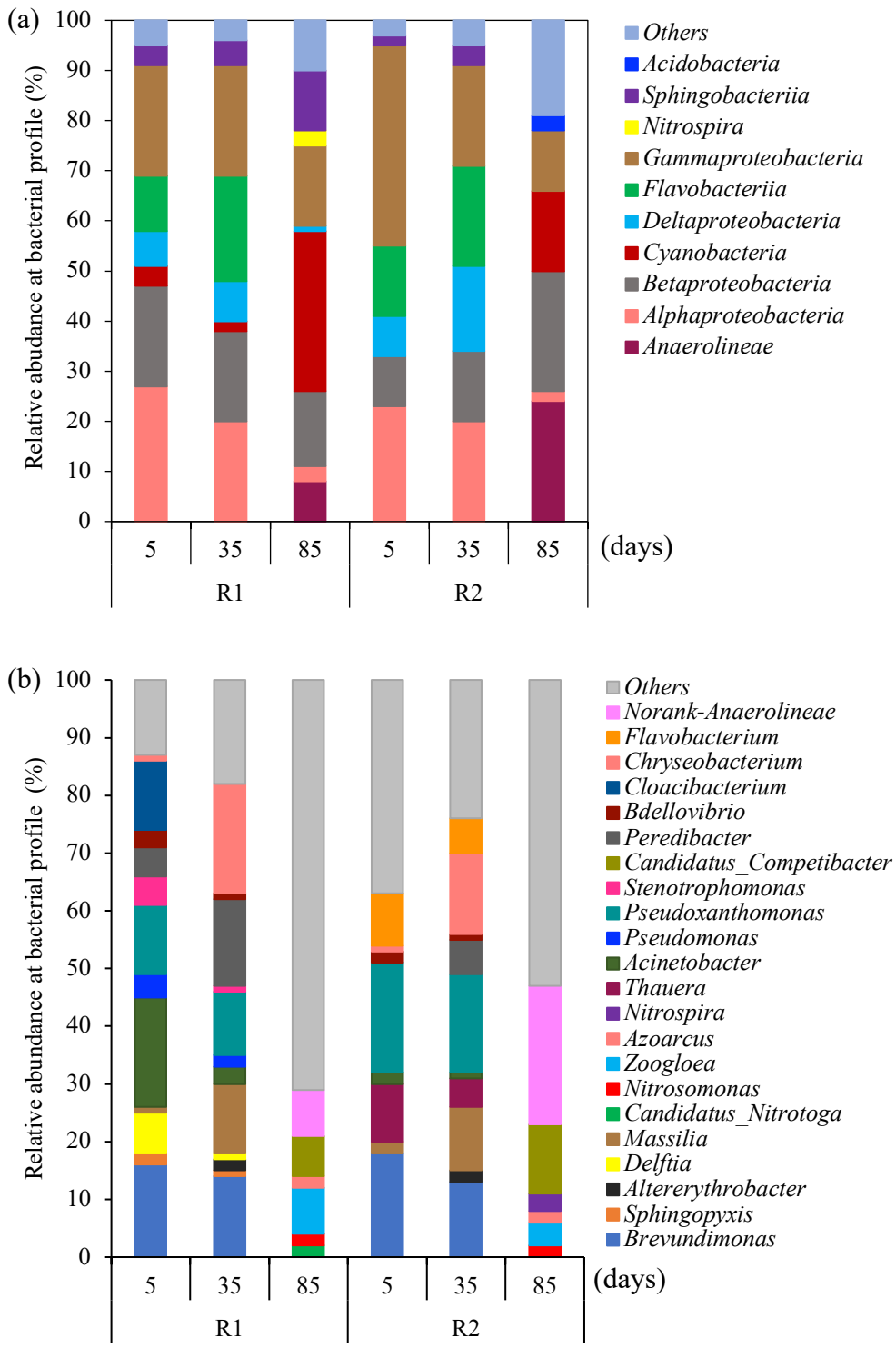

Fig. 2-2 Changes in biological community of algal-bacterial AGS on days 5, 35, and 85: Bacterial community profile at class level (a), and genus level (b).

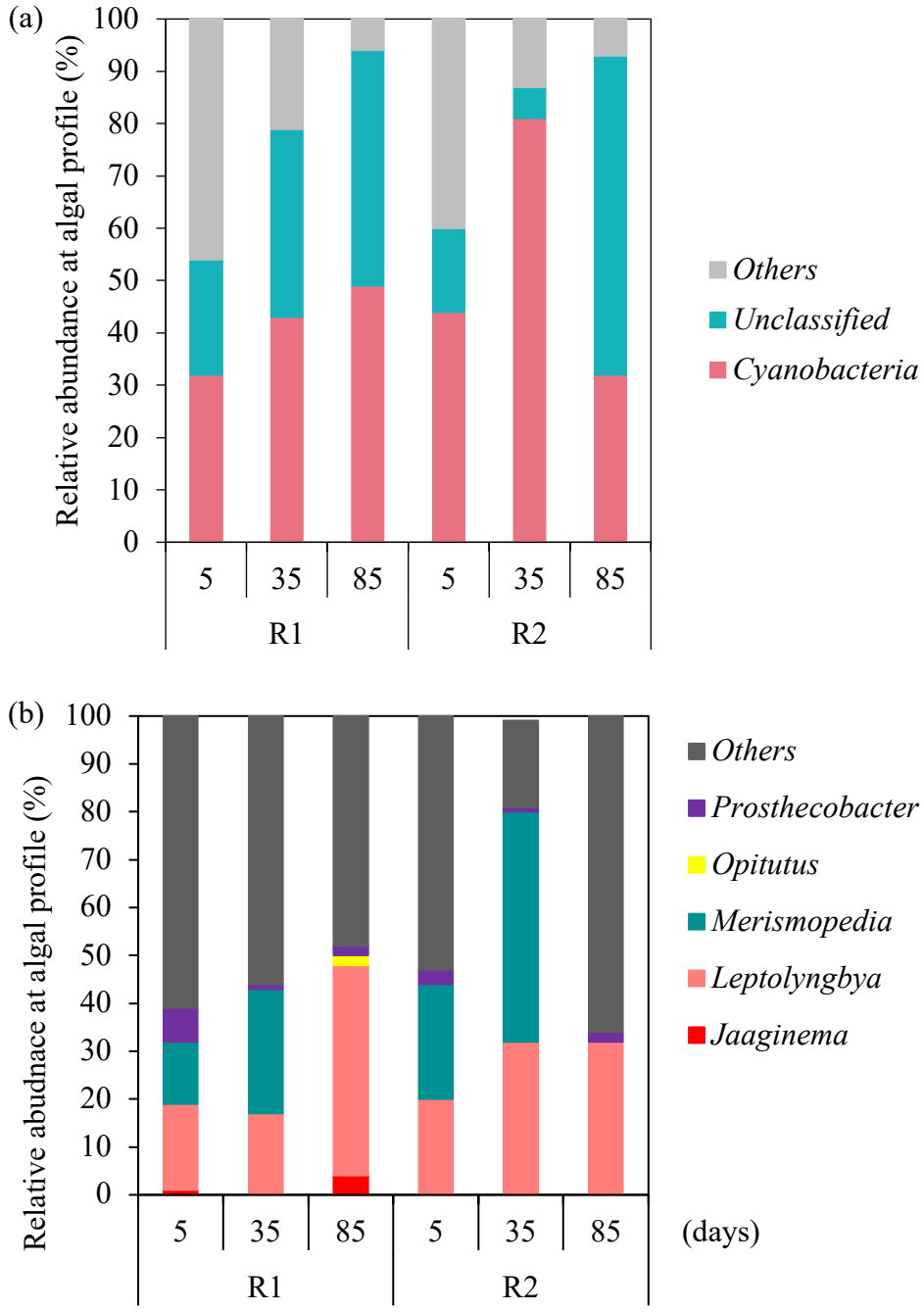

Fig. 2-3 Changes in biological community of algal-bacterial AGS on days 5, 35, and 85: Algal community profile at class level (a), and genus level (b).

Chapter 3 Isolation of the microalgal strains from algal-bacterial AGS and examination on their performance

3.1 Background

Eutrophication usually occurs in water bodies due to the improper treatment and disposal of various wastewaters containing high levels of organics, nitrogen and phosphorus. Algae can be employed to treat such pollutions, because of its high capability to convert N and P from wastewater into biomass that can be utilized as resources for renewable energy and fuels, thus reducing total greenhouse gases emission (Beuckels *et al.*, 2015). Previous studies have reported that different types of wastewater can be used as medium for cultivation of algae with promising results being received. Murwanashyaka *et al.* (2017) investigated nutrients removal efficiency of *Chlorella sorokiniana* with seven different concentrations of N and P when cultivating under heterotrophic conditions. The primary concentration for N and P was 15.45 mg-N/L and 3.35 mg-P/L, furthermore each nutrient concentration was doubled, and final concentration was 988.77 mg-N/L and 214.40 mg-P/L. Their results revealed that the removal efficiency was related with the initial concentration of nutrients, and the highest removal efficiency (99%) was detected at initial concentrations of 123.6 mg-N/L and 26.8 mg-P/L. *Galdieria sulphuraria* is unicellular red algae which have ability to remove organic carbon and nutrients from primary-settled urban wastewater. This strain was tested by cultivating in a 700 L photobioreactor (PBR) which was operated under batch operation by feeding primary-settled wastewater. The removal efficiencies ranged between 46–72%, 63–89%, and 71–95% for organic carbon, ammonia nitrogen, and phosphate, respectively (Henkanatte-Gedera *et al.,* 2017). In the real wastewater treatment systems, even though microalgae can exist in co-cultures, some specific species may have superior capacity for pollutants removal from wastewater (Kube *et al.,* 2018). Thus, it is important to investigate such specific and capable microalgal strains from algal-bacterial AGS. According to the biological community structures defined in Chapter 1, this chapter aims to isolate the most dominant microalgal strains with high nutrient removal efficiency and auto-aggregation ability, which might be the major contributors to the granulation process of algal-bacterial AGS system.

3.2 Materials and methods

3.2.1 Isolation and identification of microalgal strains

The performance of the laboratory SBR became relatively stable since day 30 and onwards. On day 35, mainly two microalgal strains were isolated using enrichment culture method. BG

11 medium was used to isolate the microalgal strains, and the medium contained (in g/L) $NaNO_3$ (1.5), $K_2HPO_4 \cdot 3H_2O$ (0.04), $MgSO_4 \cdot 7H_2O$ (0.075), $CaCl_2 \cdot 2H_2O$ (0.036), citric acid (0.006), ferric ammonium citrate (0.006), Na_2-EDTA-Mg (0.001), Na_2CO_3 (0.002), and 1 ml/L trace metal mix A5+Co. The trace elements solution contained (in g/L) H_3BO_3 (2.86), $MnCl_2 \cdot 4H_2O$ (1.81), $ZnSO_4 \cdot 7H_2O$ (0.22), $Na_2MoO_4 \cdot 2H_2O$ (0.039), $CuSO_4 \cdot 5H_2O$ (0.079), and Co $(NO_3)_2 \cdot 6H_2O$ (0.049) (Rippka *et al.*, 1981). The medium was autoclaved at 121°C for 20 min prior to use.

Five ml of algal-bacterial AGS was collected at the end of the aeration period and completely crushed with a glass rod. Then 1 mL of crushed sample was inoculated into a 250 ml Erlenmeyer flask containing 100 mL BG11 and cultivated on a shaker (EYELA multi shaker MMS 210) at 130 rpm and room temperature (25±2°C). After 24 h cultivation, the enriched sample was diluted in sterile 0.9% NaCl solution up to 10^3, 10^4 and 10^5 times, and 0.1 mL aliquot was spread on Petri dishes with BG11 and then cultivated at (28 ± 2)°C until green colonies being observed. Two LED lights (NLM 10SG-DC/AC, Nikki Trading Corp., Japan) were installed inside the incubator with a light intensity of 149 -154 μmol/m^2·s measured by a pocket digital lux meter (ANA-F11, Tokyo Photo- electric Co., Ltd., Japan).

Single green colonies on the plate were taken and put into screw-capped tubes preloaded with BG11 medium and incubated for 24 h under the same light condition. Again, serial dilution was conducted, and 0.1 mL of the aliquot was spread on petri dishes with BG11 medium. This procedure was repeated three times to purify the strain.

After purification, molecular identification was conducted, and the total cellular DNA was extracted from the microalgal strains using the NucleoSpin® Microbial DNA following the manufacturer's protocol. The 23S rRNA partial gene sequence comparison was carried out by BLAST homology search system on the DDBJ website (http://www.ddbj.nig.ac.jp).

3.2.2 Cultivation of the isolated microalgal strain

After being isolated from the algal-bacterial granules sampled from the laboratory SBR, the microalgal strains were cultivated by using the same synthetic wastewater fed to the laboratory SBR. The synthetic wastewater was composed of 300 mg chemical oxygen demand (COD)/L (NaAc), 5 mg PO_4-P/L (KH_2PO_4), 50 mg NH_4-N/l (NH_4Cl), 10 mg Ca^{2+}/L ($CaCl_2$), 5 mg Mg^{2+}/L ($MgSO_4 \cdot 7H_2O$), 5 mg Fe^{2+}/L ($FeSO_4 \cdot 7H_2O$), and 1 ml/L of trace element solution. The trace elements solution was prepared according to Huang *et al.* (2015).

Firstly, the algal strains were pre-cultured for 3 days in the synthetic wastewater on a shaker (EYELA multi shaker MMS 210) at 130 rpm and a light/dark cycle of 12 h/12 h according to the photoperiod condition proposed by Lee *et al.* (2015). Secondly, 10 mL of algal biomass was inoculated into a 250 mL Erlenmeyer flask containing 100 mL of the synthetic wastewater, resulting in an initial algal biomass concentration of 10% of 100 mL medium. The flasks were placed in an incubator and shaken at 130 rpm and temperature of 28±2 °C under illumination at a light/dark cycle of 12 h/12 h. In parallel, the control flasks were placed on a shaker (EYELA multi shaker MMS 210) at 130 rpm and room temperature (25±2 °C), and the shaker was covered with a black plastic bag to avoid light exposure and ensure cultivation in darkness. All of the above inoculation and cultivation were performed in triplicate.

3.2.3 Examination on nutrients removal and auto-aggregation capability

Every day all 100 mL culture samples from the two parallel flasks were used for evaluation of dry biomass, chlorophyll a (Chl.a), auto-aggregation test, extracellular polymeric substances (EPS), and nutrients consumption.

As for the examination on nutrients removal, a volume of 50 mL algae suspension was filtered through 0.22 μm filter and the filtrate were used for analysis of PO_4-P and NH_4-N according to the Standard Methods (APHA, 2012).

Dry weight in addition to Chl.a concentration and optical density (data not shown) were used to represent algae growth. Algal dry weight was determined according to the method described elsewhere (Moheimani *et al.*, 2013). Briefly, 10 mL of algal suspension was sampled from the flask and filtered through the pre-weighted Advantec GF/F filter. The filter was then dried in a drying chamber (Convection oven MOV-112F, SANYO) at 105°C for 24 h. Finally, the dried filter was re-weighted using an electronic balance (HR-100AZ, Japan).

Algal specific growth rate was calculated according to Eq. (3-1) (Liu and Vyverman, 2015).

$$\text{Specific growth rate } (\mu, d^{-1}) = \ln (DW_{t2}/DW_{t1})/(t_2-t_1) \quad (3\text{-}1)$$

where DW is the dry mass of sampled algae (mg/L), t_1 and t_2 are the two sequential sampling time, and t_2-t_1 is the sampling interval (d).

Nutrient (N or P) removal rate was computed according to Eq. (3-2) (Aslan and Kapdan, 2006).

$$R= (P_0-P_t)/(t_t-t_0) \quad (3\text{-}2)$$

where R (mg/L-d) represents the nutrient removal rate, S_0 (mg/L) is the initial (t_0) nutrient (N or P) concentration, S_t (mg/L) is the N or P concentration at the time t_t, and t_t (day) is the time that N or P concentration reaches equilibrium (no significant change occurs).

In this study, the specific nutrient removal rate (Rs, mg/(mg-(Chl-a)•d)) was calculated by dividing the N or P removal rate by the initial Chl-a concentration (at the beginning of the experiment); namely, $Rs=R/(Chl\text{-}a)_{t0}$. The initial concentration of Chl-a ($(Chl\text{-}a)_{t0}$) was 1.093 mg/L in this work.

Algal cells were harvested daily and the aggregation capability was measured using the method developed by Eboigbodin and Biggs (2008) with modifications. The pellets were washed 3 times with 0.9% NaCl solution and re-suspended in this solution. Then a 3 mL aliquot of suspended cells was carefully transferred to a cuvette and the optical density of the suspension was measured at 600 nm. The auto-aggregation index was calculated according to Eq. (3) by comparing the initial OD (OD_0) and the OD value after 5 h (OD_{5h}).

Auto-aggregation index (%) = $100\times(OD_0-OD_{5h})/OD_0$ (3-3)

3.2.4 EPS extraction and quantification

For the extraction of EPS, algal cells were harvested by centrifugation at 5,000 rpm for 15 min (Eboigbodin and Biggs, 2008). The supernatant was filtered through 0.22 μm membrane filter, and the obtained filtrate was used to determine the quantity of loosely bound EPS (LB-EPS). Then the cell pellets were used to extract tightly bound EPS (TB-EPS), which was carried out according to the method described by Adav and Lee (2008) with modifications. The cell pellets were re-suspended with 0.9% (w/v) NaCl to the original volume, then heated to 80°C and kept at this temperature for 30 min. The extracted solution was centrifuged at 10,000×g and 4°C for 20 min, then the supernatant was filtrated through 0. 22 μm membrane and the collected filtrate was used for analysis of the TB-EPS fraction. The proteins (PN) and polysaccharides (PS) contents were determined using phenol-sulfuric acid method (Dubois *et al.*, 1956) and Lowry-Folin method (Lowry *et al.*, 1951) with glucose and bovine serum albumin (BSA) as the standards, respectively.

3.3 Results and discussion

3.3.1 Algal biomass growth

On day 35 of the laboratory SBR operation, microalgal strains were isolated from the algal-bacterial AGS in R1 and R2 and the microalgal strains were designated as A-1 and A2, then they growth were evaluated in the synthetic wastewater under different lighting conditions. Upon the confirmation of biological community structures of algal-bacterial AGS, the isolation of genera of *Merismopedia* was priority due to its dominant genera. The assumption is in its important genera for the granulation process due to the disappearance of this genera when algal-bacterial AGS was started to breakage. From the result, however, the isolated microalgal strains belonged to the genus *Chlorella*, and its closest strain is *Chlorella* sp. NJD-3 which has been reported and isolated from a domestic wastewater treatment plant (Xiamen, China) with 100% gene sequences similarity (Shen *et al.*, 2017).

Dry biomass was determined to quantify the growth of microalgae. As shown in Fig.3-1, almost no lag phase was observed for the growth of the isolated strains under the test dark condition, which directly shifted to the exponential phase on day 0–2, yet with almost no obvious stationary phase being observed as the biomass concentration started to decrease after 3-day dark cultivation. Conversely, under the test light/dark (12 h/12 h) cycle condition, the strain A1 dry biomass weight was increased from the initial 42 mg/L to 314 mg/L, and the stationary phase remained till the end of the experiment (day 5). As for strain A2, during the light/dark (12 h/12 h) cultivation the biomass concentration increased from the initial 56 mg/L to 346 mg/L on day 4, then some decrease was observed on day 5, to 308 mg/L.

The maximum specific growth rate (μ_{max}, d^{-1}) was calculated from the changes of their dry weight during the cultivation (Table 3-1). Strain A1 had a higher maximum specific growth rate in the exponential phase, averagely 0.841 d^{-1} and 0.682 d^{-1} under the light/dark cycle of 12 h/12 h and 0 h/24 h, respectively, in comparison to strain A2 (0.573 d^{-1} and 0.444 d^{-1}, respectively). When compared to a previous work by Li *et al.* (2011), the final biomass concentrations (1.179 g/L and 1.06 g/L) of *Chlorella* sp. were higher than the results from this study. However, they obtained correspondingly lower specific growth rates (0.479 d^{-1} and 0.677 d^{-1}), most likely due to the different wastewater composition and culture conditions being applied. Most of microalgal growth is autotrophic, however, heterotrophic and mixotrophic growth conditions are also possible for the genera of *Chlorella* with different growth rates, depending on individual characteristics of the strain and source of wastewater. For example, the growth rates of *Chlorella vulgaris* (Tam and Wong, 1996) and *Chlorella zofingiensis* (Liu *et al.*, 2011) were 0.219 d^{-1} and 0.235 d^{-1}, respectively under autotrophic condition. In addition, the growth rates were also reported to range 0.479–0.948 d^{-1} and 0.3 d^{-1} under heterotrophic

and mixotrophic conditions, respectively, for the genera of *Chlorella* (Li *et al.*, 2011; Liu *et al.*, 2011; Wang *et al.*, 2010; Wang *et al.*, 2012).

From the growth pattern of the dry algal biomass, the strain *Chlorella* sp. A-1 had a relatively rapid growth under the light condition. While under the dark condition for 3 days, the growth rate was declined for both strains. The dark condition limited energy source, although the existing organic carbon source was enough to enhance the synthesis of cells, thus the growth of algal strain was inhibited to some extent. Probably mixotrophic condition is more suitable for the growth of the strains *Chlorella* sp. A1 and A2.

3.3.2 Auto-aggregation performance and its relationship with EPS content

The auto-aggregation properties of bacterial strains are known to play an important role during the granulation of AGS. The involved mechanisms somehow still remain unclear. Generally, the phenomenon of bacterial auto-aggregation might be the protection reaction from the stressful environment (Trunk *et al.*, 2018).

Fig. 3-2 demonstrates the changes in auto-aggregation capability and EPS content of the strains A1and A2 during 5-day cultivation. Strain A1 showed much higher auto-aggregation ability than A2 under the test light/dark cycles (12 h/12 h and 0 h/24 h), an obvious increase trend was noted along with the cultivation under the light/dark cycle of 12 h/12 h, gradually from average 58.6% (day 1) to 82.4% (day 5). This increasing trend agrees with that of *Enterobacter* sp. strain FL reported by Wang *et al.* (2018) who found that the auto-aggregation index was gradually increased from 14.3 to 54.3% when the bacterial cells were growing. In contrast, strain A2 exhibited a much lower auto-aggregation index, which also increased along with the cultivation, from 29.5% (day 1) to its highest (50.0%) on day 5 under a light dark cycle of 12 h/12 h. Under the dark condition (light/dark=0 h/24 h), the auto-aggregation remained relatively stable, about 50.3–53.5% for strain A1, and a slight increase from 26.0% (day 1) to 31.7% (day 3) was noted in strain A2, while it quickly reduced to 11.5% on day 5. In addition, under the dark condition (light/dark=0 h/24 h), the content of total EPS was 1.5 times lower than that under light/dark condition (Fig.3-3). The total EPS content of both algal strains was found to have a strongly positive correlation (R^2=0.95 for A1 and R^2=0.92 for A2) with their auto-aggregation capability under the test light/dark (12 h/12 h) condition, while a weak correlation (R^2=0.42 for A1 and R^2=0.43 for A2) was obtained under dark cultivation (0 h/24 h).

As Figs. 3-4a-b reveal, the both strains showed a higher content of loosely bound EPS (LB-EPS) than the tightly bound EPS (TB-EPS) under the light/dark (12 h/12 h) condition. More

specifically, the correlation (r^2) of loosely bound protein (LB-PN) or polysaccharide (LB-PS) with the strains biomass were 0.61 or 0.81 (for A1) and 0.55 or 0.87 (for A2), while that (r^2) of tightly bound protein (TB-PN) or polysaccharide (TB-PS) with the strains biomass were 0.90 or 0.97 (for A1) and 0.83 or 0.91 (for A2), respectively. This observation suggests that TB-EPS, especially TB-PS contributes to the aggregation capability. Wang *et al.* (2014) found that the content of biomass associated protein (similar to TB-PN in this study) was higher than LB-PN when evaluating the growth of the microalgal strains of *Chlorella* sp. and *Micractinium* sp. in different wastewaters. Moreover, in this previous study, although the reported initial content of PS in EPS of the *Chlorella* sp. was relatively higher compared to the isolated strain in this work, a similar increasing trend from day 0 to day 15 was observed in PS content along with the cultivation as in this current work (from day 0 to day 5). The different contents of EPS might be resulted from the different wastewaters and different EPS extraction methods applied in these two studies. In addition, the different algal strains might also contribute to the above difference to some extent. Besides, in comparison with the light/dark (12 h/12 h) condition, the contents of LB- EPS and TB-EPS under dark (0 h/24 h) condition were lower (Figs. 3-4a -b). It is known that EPS are metabolic products protecting the cells from the harsh environment, which can be utilized as carbon and energy sources (Xiao and Zheng, 2016). Seen from Chapter. 3.1.1. mixotrophic condition may be more suitable for the growth of the both strains than autotrophic growth. Possibly due to the much less accumulation of EPS under the dark condition (Fig. 3-3), less energy source was available for the strain's growth, thus the growth of the strain A-1 and A2 ceased after day 3 (Fig. 3-1).

As pointed out by Bahat-Samet *et al.* (2004), arabinose in the exopolysaccharide of *Azospirillum brasilense* plays a key role in cell aggregation. In this study, it has been observed that the total EPS content is positively correlated with the self-aggregation property and biomass growth of the isolated microalgal strain under the suitable conditions, and TB-EPS (or TB-PS) might be one crucial factor for the aggregation of the isolated microalgae and formation of algal-bacterial AGS. Still, which component of PS or PN plays the key role in the aggregation of the isolated microalgae remains unclear. The detailed composition of the microalgal EPS together with the mechanisms involved in the algal aggregation is still under investigation.

3.3.3 Nutrients removal efficiency

As for the nutrients removal, the sampling was daily performed to measure the concentrations of NH_4-N and PO_4-P in the culture medium during the cultivation, which were

initially 50 mg-N/L and 5 mg-P/L, respectively (Figs. 3-5 and 3-6). The strain A-1 achieved the highest P removal efficiency (96.2%) leaving only 0.18 mg-P/L in the final effluent after day 5 under the light/dark cycle of 12 h/12 h, and its average specific removal rate (Rs_P) was calculated as 0.87 mg-P/(mg-(Chl-a)·d), while under the same cultivation condition A2 removed 87.2% of P and average specific removal rate (Rs-p) was 0.79 mg-P/(mg-(Chl-a)·d).

In contrast, the P removal efficiency sharply decreased to 52.3% (for A1) and 34.74 % (for A2) when the microalgal strains were cultivated under dark condition (0h/24h, Fig. 3-4a). Under dark condition the final P concentration and Rs_P were 2.26 mg-P/l and 0.49 mg-P/(mg-(Chl-a)· d), respectively for strain A1. And for strain A2 the final concentration of P was 3.10 mg-P/l and Rs_P was 0,33 mg-P/(mg-(Chl-a)·d). Compared with the results from the microalgal strain of *Chlorella vulgarius* (Aslan and Kapdan, 2006) and *Chlorella* sp. (Wang *et al.*, 2014) which showed Rs_P at 0.15, 0.2, and 0.25 mg-P/(mg-(Chl-a)·d) when the initial concentration of P was 3.5, 4.0 and 7.7 mg-P/l, respectively, the isolated algal strain in this study is much more efficient as its highest Rs_P from the synthetic wastewater (5 mg PO_4-P/L) can be realized within 5 days. As for N removal, similarly, A1 exhibited about 9-14% higher removal efficiency than A2, achieving 92.6% (12 h/12 h) and 49.3% (0 h/24 h) in comparison to 83.6% (12 h/12 h) and 35.7% (0 h/24 h) by A2 after 5-day cultivation. (Fig. 3-6). The final N concentration was 3.18 and 7.65 mg-N/L at the end of the experiment, yielding an average specific N removal rate (Rs_N) of 8.47 and 8.18mg-N/(mg-(Chl-a)·d) under the light/dark (12 h/12 h) condition for strain A1 and A2 respectively. Meanwhile, in this study about half Rs_N 4.51 mg-N/(mg-(Chl-a)·d and 3.26 mg-N/(mg-(Chl-a)·d were obtained with a final N concentration of 25.3 mg-N/L and 32.16 mg-N/L under the dark cultivation condition for strains A1 and A2 respectively. As Wang *et al.* (2014) noticed, a higher initial N concentration can lead to a higher Rs_N, and even the same species of algae may have different nutrient removal rates at different initial N concentrations. These authors observed that the specific N removal rates for the strain *Chlorella* sp. were 3.8 and 1.8 mg-N/(mg-(Chl-a)·d) when grown for 14 days in diluted sludge centrate (160 mg-N/L) and primary effluent (28 mg-N/L), respectively. The results from this study are similar to the findings from previous studies, and the high nutrients removal efficiency may be associated with algal species, cultivation condition, media composition, initial nutrient concentration and light/dark cycle (Liu and Vyverman, 2015; Aslan and Kapdan, 2006; Lee *et al.*, 2015). Figs. 3-5 and 3-6 also show that light condition is crucial for the microalgal strain, and most of the nutrients (N and P) were removed during the first 4 days under the light/dark cycle of 12 h/12 h. Under dark cultivation

(0 h/24 h), nutrients (N and P) removal mainly occurred on day 1, likely attributable to the limited growth of microalgae. As shown in Fig. 3-1, compared to the light/dark (12 h/12 h) condition, the microalgal biomass did not increase much under dark cultivation till the end of the experiment, resulting in substantially lower N and P removals. This observation agrees with Kube *et al.* (2018) that N and P are essential elements for many functional components of algae, and suitable N and P concentrations (or N/P ratio) may favor more assimilation or uptake of these elements by algae, resulting in the increase of microalgae biomass.

3.4 Summary

The two isolated algal strains, A1 and A2 from R1 and R2, respectively, exhibited high efficiencies of N (84-93%) and P (87-96%) removal under the test light/dark (12 h/12 h) condition during 5 days' treatment of wastewater containing 50 mg-N/L and 5 mg-P/L. In addition, the two strains were identified as the genus *Chlorella* which can serve as a potential source of food and energy. In this study, the two microalgal strains, especially A1, possess excellent auto-aggregation ability which is strongly and positively correlated with the EPS content. This finding suggests that inoculation of some proper algal species into the bacterial granulation system may accelerate the formation and stable operation of algal-bacterial AGS, which was further investigated in Chapter 4.

Table 3-1 Parameters estimation on algal growth kinetics, maximum specific growth rate (μ_{max}) and nutrient removal rate (Rs) for isolated microalgal strains A1 and A2.

Light/dark cycle	μ_{max} (d^{-1})	Rs_{-N} (mg-N/(mg-(Chl.a)·d)	Rs_{-P} (mg-P/(mg-(Chl.a)·d)
A1 (12 h/12 h)	0.84	8.47	0.87
A1 (0 h/24 h)	0.68	4.51	0.49
A2 (12 h/12 h)	0.57	8.18	0.79
A2 (0h/24h)	0.44	3.26	0.33

Note: 12 h/12 h or 0 h/24 h denotes the light/dark cycle condition.

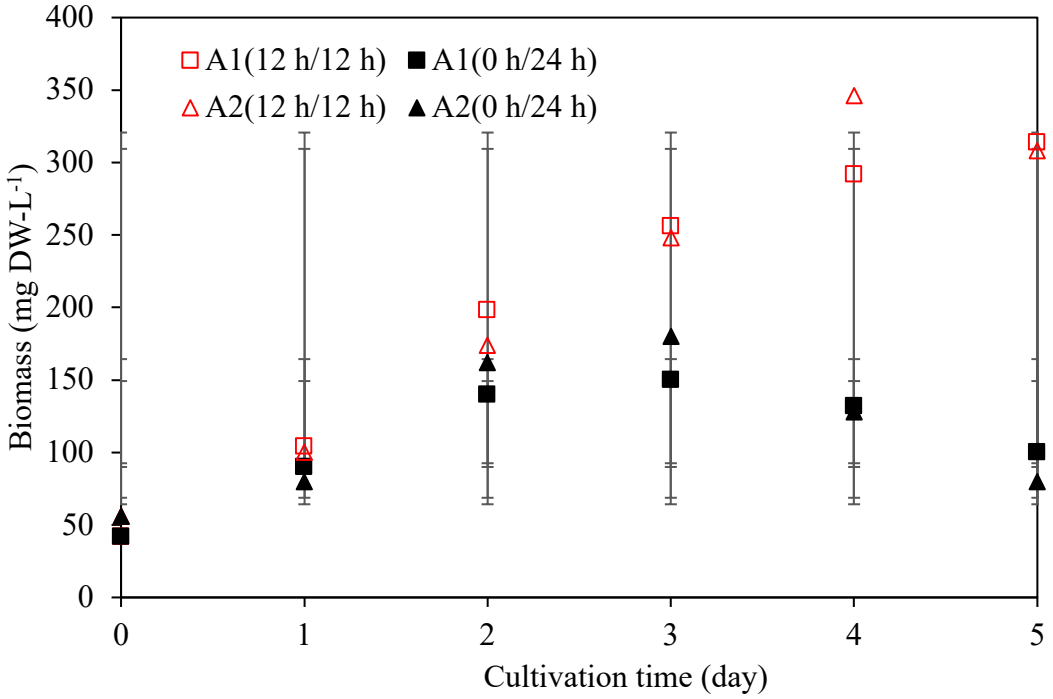

Fig. 3-1 Growth curves of microalgal strains A1 and A2 cultivated in synthetic wastewater for 5 days under different light conditions.

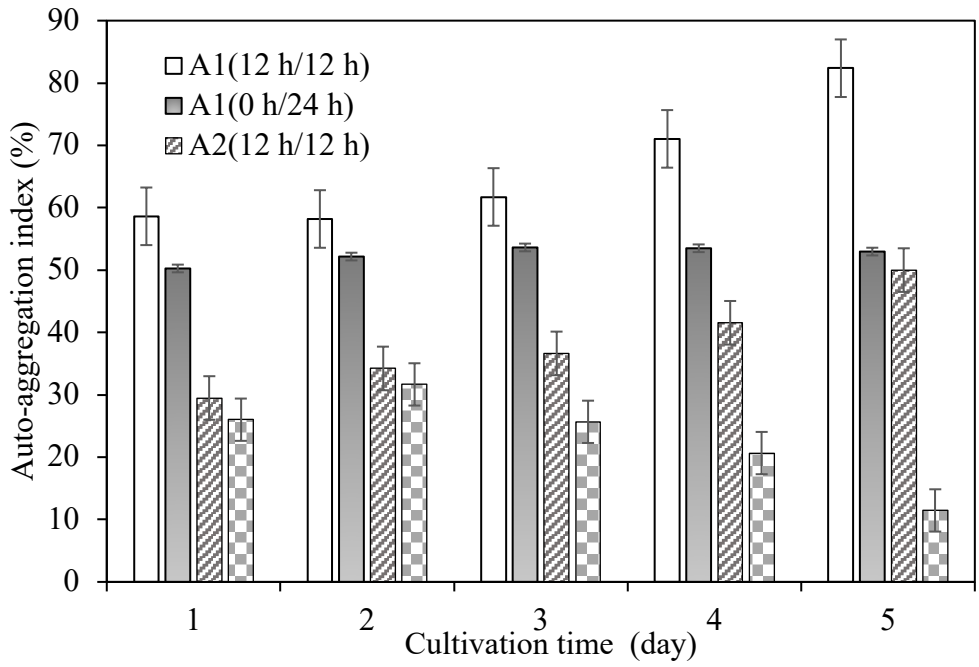

Fig. 3-2 Changes in auto-aggregation index of the isolated microalgal strains A1 and A2 under different light conditions.

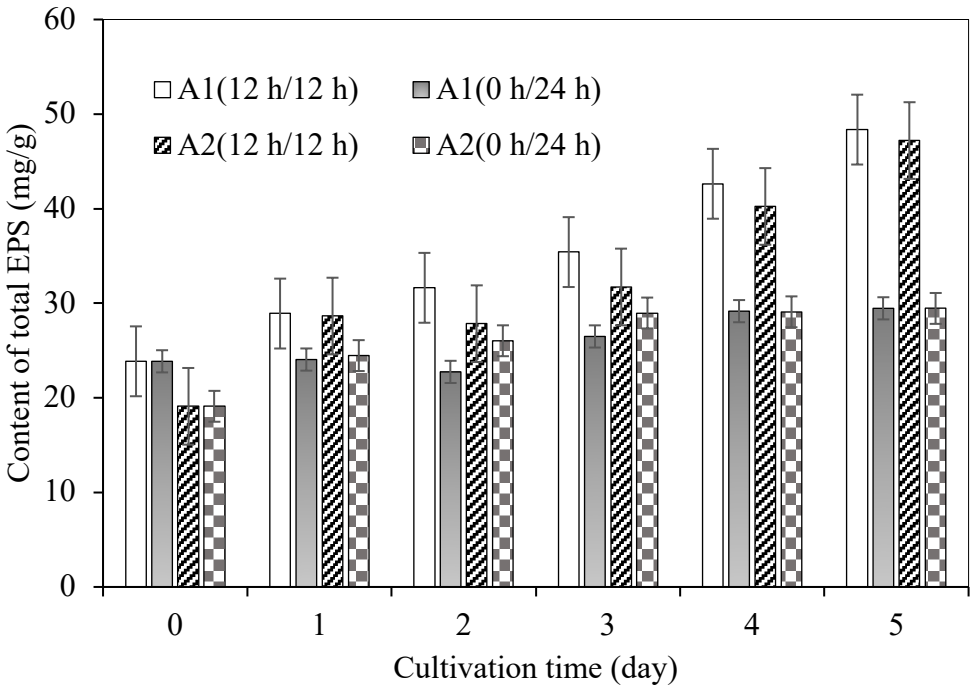

Fig. 3-3 Changes in EPS content of the isolated microalgal strains A1 and A2 under different light conditions.

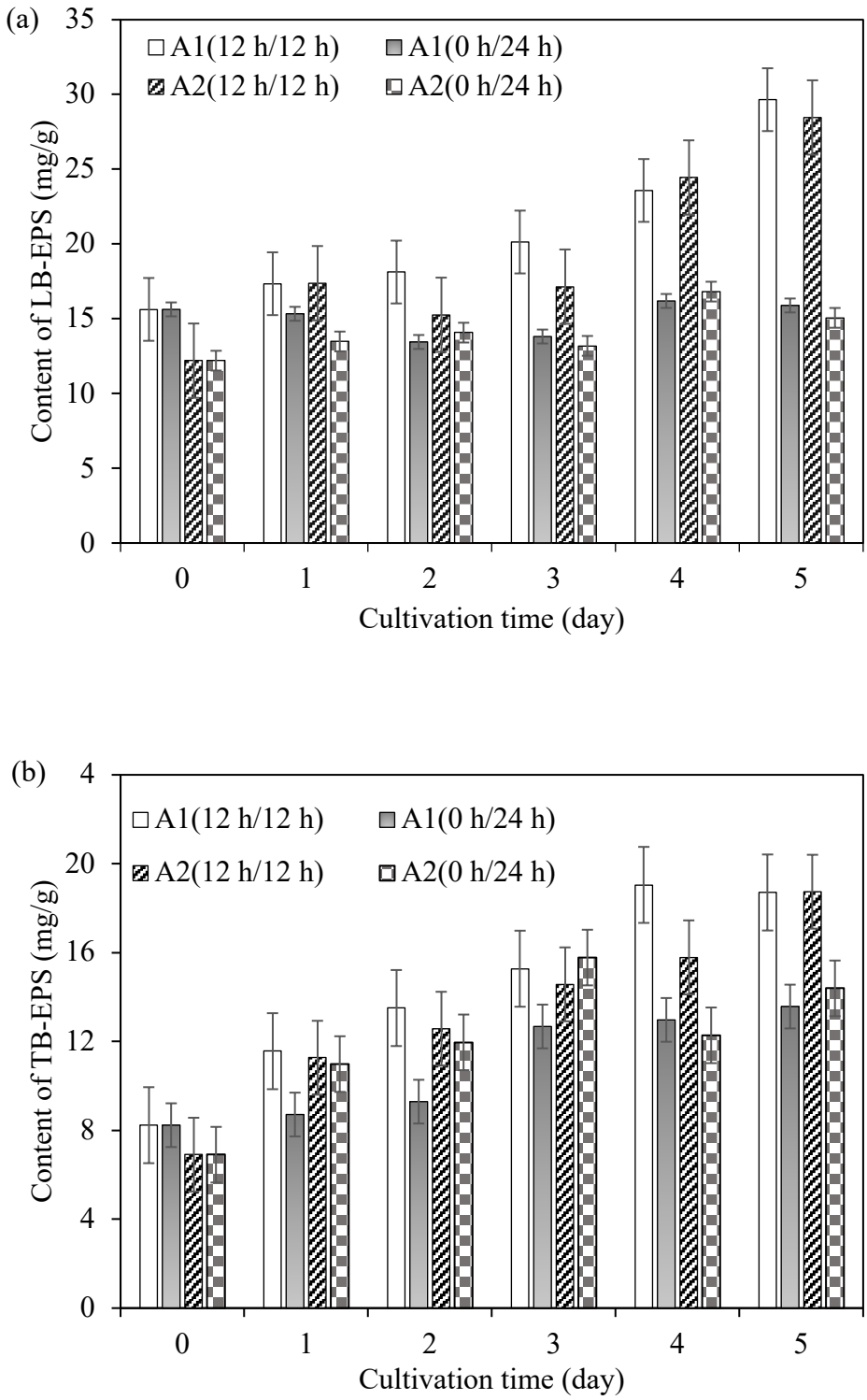

Figure 3-4 Changes in EPS content of the isolated microalgal strains A1 and A2 under different light conditions: LB-EPS (a), TB-EPS (b).

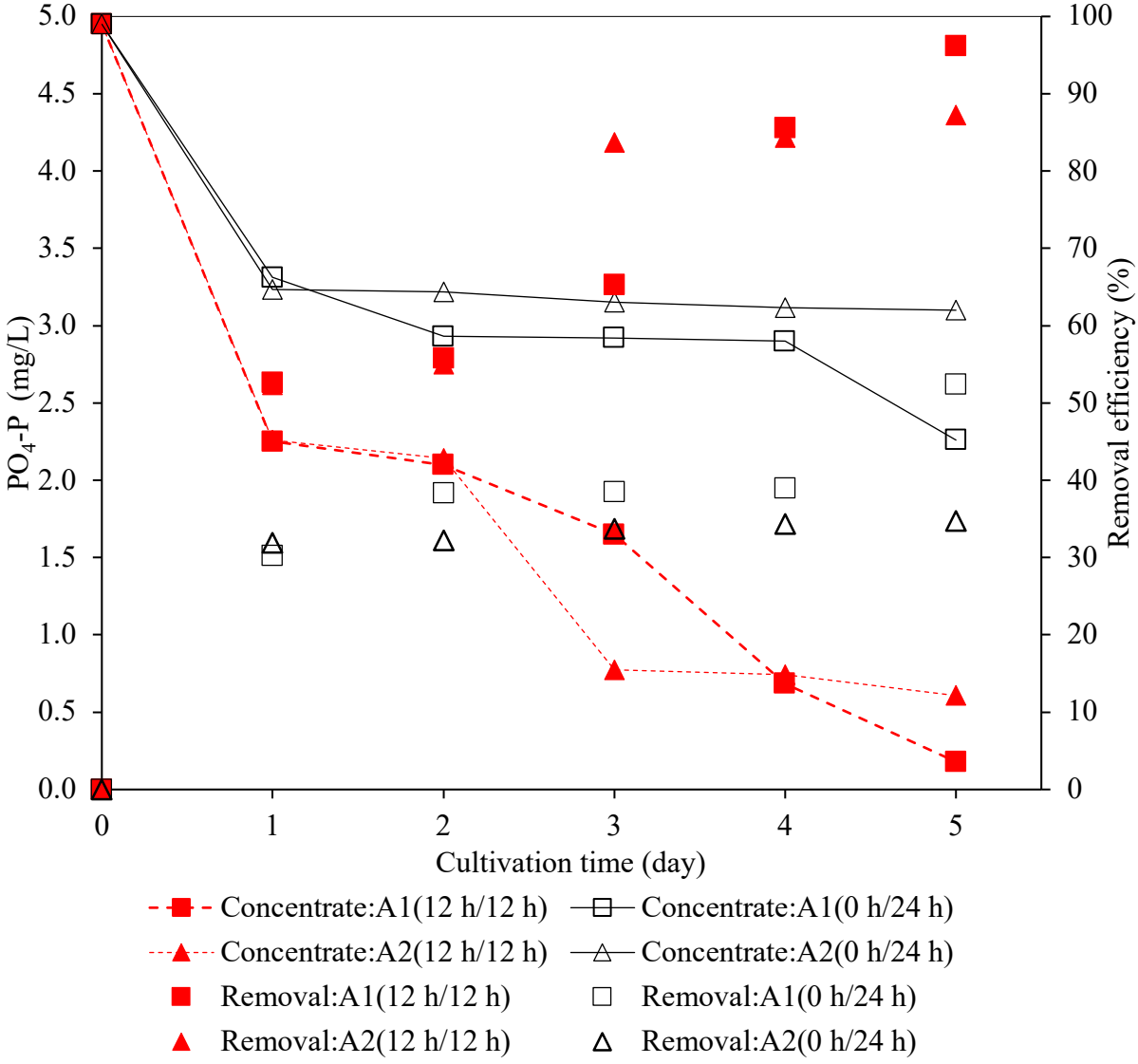

Fig. 3-5 Changes in PO_4-P concentration in the synthetic wastewater.

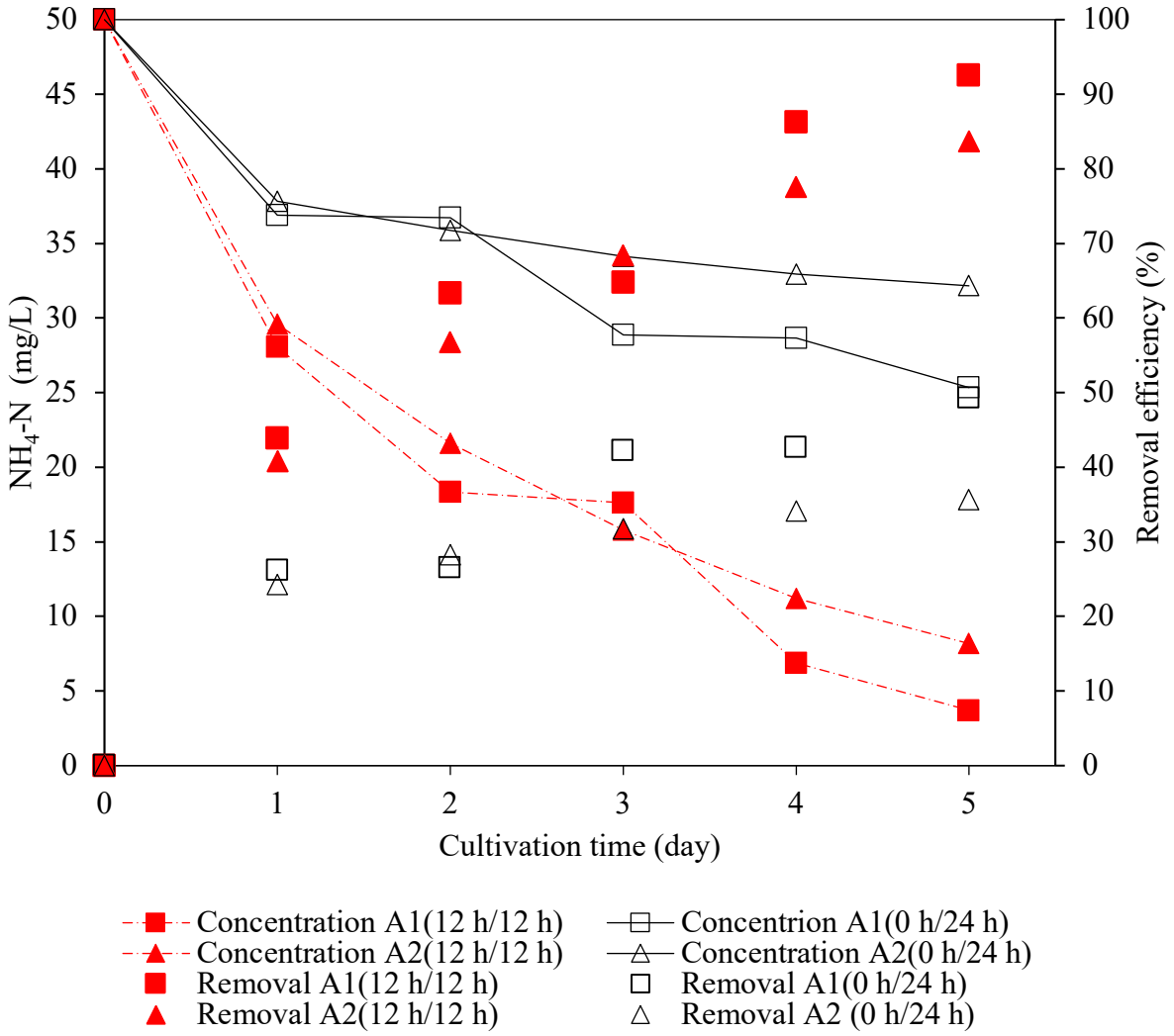

Fig. 3-6 Changes in NH_4-N concentration in the synthetic wastewater.

Chapter 4 Examination on the contribution of isolated strains to the granulation process

4.1 Background

There are numerous bacteria in the surrounding environment, and some of them have symbiotic relationship with algae, especially in aquatic systems in a natural way. In the algal-bacterial symbiosis system, aerobic bacteria have potential to oxidize and decompose organic matters in the wastewater into nutritional forms which can be then assimilated by microalgae; meanwhile microalgae can release oxygen that can be utilized by bacteria to decompose organics (Ji *et al.*, 2018). Liu *et al.* (2015) noted that the efficiencies of pollutant removal and aggregate formation was strongly associated with the irradiance level and lower irradiance level mildly promoted microalgae-bacteria granule. This phenomenon is proposed to be related with the increased excretion of bound extracellular polymeric substances (EPS). Moreover, Liu *et al.* (2017) demonstrated that aerobic granules can serve as an immobilized material of microalgae (*Chlorella* and *Scenedesmus*), and the total N and P removals were enhanced in the reactor with high concentration of algal biomass. Besides, He *et al.* (2018) claimed that the natural sunlight can induce the growth of water born algae in the reactor of AGS, and algal-bacterial consortia can be developed within 7 days. Most of the previous works focused on the formation of algal-bacterial AGS with or without inoculation of microalgae and the aerobic bacterial granules as the seeding sludge under different operation conditions. Up to now, however, the mechanisms involved in the formation of algal-bacterial AGS remain unclear, and the contribution of biological factors to the granulation of algal-bacterial symbiosis system hasn't been addressed yet. Thus, in this Chapter, the experiments were conducted to examine whether the two isolated microalgal strains contributed to the granulation process.

4.2 Materials and methods

4.2.1 Operation conditions for SBRs

Two identical SBRs made of acrylic transparent plastic, with a working volume of 0.5 L each, were operated in parallel with their average results being reported. The activated sludge was sampled from a sedimentation tank of the Shimodate Sewage Treatment Plant, Ibaraki Prefecture, Japan. The initial mixed liquor suspended solid (MLSS) concentration was 8.34 g/L with a sludge volume index (SVI_5) of 116.4 mL/g. The same synthetic wastewater as in Chapter 2.2.1 was fed to the reactors during the operation. In this study, one operation cycle

was 6 h, including 6 min influent filling, 120 min non-aeration, 222 min aeration, 5 min settling and 7 min effluent withdrawal, equivalent to a hydraulic retention time (HRT) of 12 h. During the operation, the settling period time was decreased from initially 30 min to 5 min since day 4, and correspondingly the aeration time was increased while shortening the settling time.

4.2.2 Inoculation of activated sludge and microalgae strains to form algal-bacterial AGS

The SBRs were operated for 2 stages.

(1) During stage I (bacterial AGS cultivation), each SBR was inoculated with 2.00 g-MLSS/L of activated sludge, and the operation was continued for 4 weeks at room temperature (25±2°C) with no control of the room lights (the illumination intensity was about 165–201 μmol/m^2·s when all room lights were on), and the sludge retention time (SRT) was controlled at about 30 days.

(2) Stage II or algal-bacterial AGS cultivation: On day 28, 0.52 g/L-MLSS of the microalgal strain A1 and A2 was added into R1 and R2, respectively and the MLSS of bacterial AGS of each SBR were about 1.95 g-MLSS/L. The reactors were operated under the light illumination at a light/dark cycle of 12 h/12 h, which were covered with a black plastic bag to avoid light exposure during the dark cycle. Four LED lights (NLM 10SG-DC/AC, Nikki Trading Corp., Japan) were installed and the light intensity was measured by a pocket digital lux meter (ANA-F11, Tokyo Photo- electric Co., Ltd., Japan). Two lights were illuminated from the side walls of the reactors and another two lights from the top of the reactors, creating light intensity of 149–154 μmol/m^2·s (about 5 cm from the SBR side walls) and 253–259 μmol/m^2·s (at the top of SBRs), respectively. During this stage, the mixed liquor was not discharged daily for SRT control; instead, once a week 80 mL of mixed liquor was manually removed during the aeration period in order to retain the microalgal biomass in the SBRs.

4.2.3 Evaluation on SBR performance and calculation

Effluent samples were collected once every 3 days at the end of the aeration cycle and filtered through 0.22 μm membrane with the determinations of ammonia nitrogen (NH_4-N), nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N), and phosphorus (PO_4-P). Samples for the determination of mixed liquor (volatile) suspended solids (ML(V)SS), sludge volume index (SVI), content of chlorophyll a were collected once every 7 days. All the measurements were conducted according to the Standard Methods (APHA, 2012).

The morphology of the activated sludge, algal strain, or granule was observed using Leica M205 C Microscope (Leica Microsystems, Switzerland).

Chl-a concentration was calculated according to Eq. (4-1) at four wavelengths: 750 nm, 664 nm, 647 mn, and 630 nm (APHA, 2012), and each absorbance was subtracted by A_{750}.

$C_a = 11.85(A_{664}-A_{750}) - 1.54(A_{647}-A_{750}) + 0.08(A_{630}-A_{750})$

Chlorophyll a (mg/g-MLSS) = C_a × Extract volume (L) / volume of sample (L) (4-1)

The overall removal efficiency and capacity were calculated by using Eqs. (4-2) and (4-3) (Cai *et al.,* 2016; Ahmed *et al.,* 2017), respectively.

$$\text{Removal (\%)} = 100 \times (1 - \rho_{eff} / \rho_{inf}) \quad (4\text{-}2)$$

in which ρ_{inf} (mg/L) and ρ_{eff} (mg/L) are influent TN or TP concentration and effluent TN or TP concentration, respectively.

$$\text{Removal capacity } (X, \text{ mg/g-MLVSS/d}) = 24 \times (\rho_{inf} - \rho_{eff})/(\text{MLVSS} \times \text{HRT}) \quad (4\text{-}3)$$

where X (mg/g-VSS/d) is the removal capacity of TN or TP, and ρ_{inf} (mg/L) and ρ_{eff} (mg/L) are the concentrations of TN or TP in the influent and effluent, respectively. MLVSS (g/L) is the MLVSS concentration in the reactor, HRT (h) is the hydraulic retention time of wastewater in the reactor, and 24 is the conversion unit from day to hour.

Nitritation and nitratation efficiency were calculated according to Lackner and Smets (2012) and Lei *et al.* (2013) by using Eqs. (4-4) and (4-5), respectively.

$$\text{Nitritation efficiency (\%)} = 100 \times \{[TAN]_0 - [TAN]_t\}/[TAN]_0 \quad (4\text{-}4)$$

$$\text{Nitratation efficiency (\%)} = 100 \times \{1 - [NO_2 - N]_t / \{[TAN]_0 - [TAN]_t\}\} \quad (4\text{-}5)$$

in which $[TAN]_0$ and $[TAN]_t$ are the total ammonia nitrogen concentrations (mg/L) at initial and time t, and $[NO_2 - N]_t$ is the nitrite nitrogen concentration (mg/L) at time t, respectively.

4.3 Results and discussion

4.3.1 Biomass growth of bacterial AGS or algal-bacterial AGS

The values of the MLSS and SVI_5 represent biomass growth and granular settleability as shown in Fig 4-1. The initial MLSS of both SBRs was about 2.0 g/L, which was increased to 2.35 g/L and 2.71 g/L in R1 and R2, respectively on day 7. Then the biomass concentration slightly decreased to 1.94, 1.6, and 1.89 g/L (in R1) and 2.10, 2.08, and 2.3 g/L in (R2) on days 14, 21 and 28, respectively. This decreasing tendency is probably caused by certain amount of biomass being washed out, when shortening the settling time in both SBRs. Meanwhile, the SVI_5 values were averagely 118.95 mL/g-MLSS and 108.74 mL/g-MLSS on day 14 in R1 and R2, respectively. After that it dramatically decreased to 46.58, and 39.37 m/g-MLSS on day 21

and 28, respectively in R1. As for R2, the SVI_5 value was higher in comparison to R1. However, some decreasing tendency to 60.09 mL/g-MLSS and 54.32 mL/g-MLSS was noticed on days 21 and 28, respectively. The smaller value of SVI indicates better settleability of the sludge in both reactors.

On day 28 about 0.52 g/L-MLSS of the microalgal strain A1 and A2 was cultivated into R1 and R2, respectively, and the MLSS concentrations in both reactors were about 1.95 g/L. Both reactors were operated for 4 days, no withdrawal of effluent and ingredients was added manually on daily basis. During these days, the MLSS was increased to 4.52 g/L and 4.71 g/L in R1 and R2, respectively, due to the mixed liquor was not discharged daily for SRT control. After 4 days, the normal operation was continued as Stage II, and during the first week the settling time was adjusted to 30 minutes, to avoid washing out the cells of microalgal strains. Then it was shortened to 5 minutes. In the first week of Stage II, the SVI_5 values gradually increased to 77.88 and 85.54 mL/g-MLSS, then decreased to 59.39 and 65.39 mL/g-MLSS in R1 and R2, respectively (Fig. 4-1). Besides, during the whole operation MLSS concentrations were averagely 2.5–2.9 g/L in both reactors.

The Chl-a content was used to indicate the growth of microalgae in the reactors. As illustrated in Fig. 4-2, the initial Chl-a was determined as 0.40 mg/g-MLSS (on day 28), which increased to 1.82 mg/g-MLSS (in R1) and 0.85 mg/g-MLSS after inoculation for 4 days. Then, the Chl-a showed continuously decreasing tendency to 0.85 (in R1) and 0.59 mg/g-MLSS (in R2) on day 45, and 0.25 (in R1) and 0.11 (in R2) on day 52. This is possibly caused by the daily discharge for SRT control and then, once a week 80 ml of mixed liquor was manually removed. After that, Chl-a gradually increased up to 0.75 and 0.73 mg/g-MLSS in R1 and R2, respectively on day 60 (the end of the operation). The SBRs operation was extended to another 1 week, to check whether the daily discharge of mixed liquor affected growth of microalgal strains: during this period the concentration of Chl-a was increased up to 2.50 (in R1) and 1.92 (in R2). The maximum specific growth rates (μ_{max}, d^{-1}) for microalgal strains in R1 and R2 were calculated from the changes of their Chl-a content during the operation. The strain A1, which was inoculated into R1 had a higher maximum specific growth rate (0.219 d^{-1}) in comparison to strain A2 (0.111 d^{-1}) which was inoculated into R2. This result agrees with Chapter 3, in which the strain A1 had a relatively rapid growth.

4.3.2 Performance on nutrients removal

Figs. 4-3, 4-4 and 4-5 demonstrate the nutrients profiles in the effluents from the two reactors during the 60 days' operation. From the N profile (Fig. 4-3a), it is seen that, both

reactors can achieve excellent NH_4-N removal excellent (> 99%) at the very beginning of the experiment. Nitritation and nitratation efficiencies were excellent, about 98-100% (Fig. 4-3b), reflecting the co-existence of ammonia oxidizing bacteria (AOB), and nitrite oxidizing bacteria (NOB) in both reactors.

Total nitrogen (TN) concentration was calculated as the sum of NH_4-N, NO_2-N and NO_3-N concentrations in the reactors in this study. TN removal efficiency showed gradually increasing tendency from 37.65 and 39.11% to 56.61 and 58.74% in R1 and R2, respectively (Fig. 4-5a) along with the operation. However, almost no TN removal efficiency was detected in R1 and R2 at the beginning of Stage II.

For the evaluation of P removal, the synthetic wastewater was prepared with KH_2PO_4. Thus, TP removal can be expressed by PO_4-P and its influent concentration was 5 mg-P/L. As for P removal efficiency, it gradually decreased from 75.2 and 78.08% to 56.04 and 59.04% in R1 and R2, respectively at the end of Stage I. Similarly, almost no removal efficiency was detected during the operation with no withdrawal of the mixed liquor. After that, in the first week of Stage II, P removal efficiency was 41.36% and 52.64% in R1 and R2, respectively, and the highest P removal efficiency was obtained in R1 (82.82%) with its effluent concentration of 0.85 mg-P/L, while 79.28% of P was removed by R2 (with an effluent P of 1.03 mg-P/L) at the end of Stage II (Fig. 4-4).

The changes in TP and TN removal capacities in both reactors are shown in Fig. 4-5b. The TN removal capacity of R1 was higher in comparison to R2. However, the capacity was observed to increase along with the operation in both reactors. For instance, TN removal capacity was increased from 26.15 to 52.94 mg TN/g-MLVSS/d in R1, and from 22.22 to 44.48 mg TN/g-MLVSS/d in R2 during Stage I. Then, microalgal strains were inoculated into the two reactors and operated only with aeration and non-aeration cycles for 4 days (chemicals added daily): during this period no TN removal was detected in R1 and R2, respectively. After these 4 days, a normal operation was continued as Stage II. In the first week, TN removal capacity quickly recovered up to 26.54 and 31.18 mg TN/g-MLVSS/d and then slightly increased to 35.39 and 32.28 mg TN/g-MLVSS/d in R1 and R2, respectively. However, this recovery was not completely when compared to Stage I, even though the removal capacity remained higher in R1 than in R2. As for TP removal capacity, it ranged between 5.23 – 7.29 mg TP/g-MLVSS/d in R1, while 3.84 – 5.26 mg TP/g-MLVSS/d in R2 during Stage I. As noticed, no TP removal capacity was intermittently detected when the operation was changed from Stage I to Stage II in R1 and R2. However, in both reactors this removal capacity was

soon recovered and increased to 1.93 and 2.91 mg TP/g-MLVSS/d and then further increased to 5.18 and 4.36 mg TP/g-MLVSS/d during Stage II.

From the results of nutrients removal, it is seen that TN was simultaneously removed in both reactors with no significant difference. However, the decreasing tendency was observed in TP removal during the operation of activated sludge, but when the operation continued, the removal efficiency or capacity of the mixture of activated sludge with microalgal strain *Chlorella* sp. A1 or A2 always tended to increase. This observation might be associated with the assimilation or uptake of P by microalgae. This might also be the reason why microalgae enhanced removal efficiency more than bacteria. In addition, the TP removal efficiency in R1 was higher than R2, which is in consistence with the results from Chapter 3, meaning that strain A1 has higher P removal efficiency (96.2%). Additionally, there is a discrepancy between strains A1 and A2 in terms of their biomass growth and nutrients removal efficiency, which have been evident during the reactor operation.

4.3.3 Contribution of the isolated A1 and A2 strains to the granulation process

Figs. 4-6 and 4-7 demonstrate the morphological changes of the activated sludge and the mixture of activated sludge with the strain *Chlorella* sp. A1and A2 during the re-cultivation of algal-bacterial AGS in the SBRs. As mentioned in Chapter 4.2.2, during stage I the activated sludge was cultivated in the SBRs for 4 weeks. The morphology of the initial seed activated sludge was observed to be with flat surface in a little yellowish color (Fig. 4-6a). After 2 weeks the flat surface became fluffy and branched from each other (Fig. 4-6b), and during the 4th week the sludge appearance turned to be fluffier with irregular structure and connected to each other by small filamentous, from where protozoa started to show up. After 4 weeks' operation, no apparent granules were observed in the SBRs. Then on day 28 the isolated strains *Chlorella* sp. A1 and A2 were inoculated into the operated SBRs (Stage II). Fig. 4-7a demonstrates the morphology of the mixture of the activated sludge and *Chlorella* sp. A1 and A2. As seen, the fluffy structure was transferred into a little bit tight structure with a slightly greenish color.

Simultaneously, with the cultivation of *Chlorella* sp. A1 and A2, a continuous growth of protozoa and diatoms was observed, in agreement with the finding by Beun *et al.* (1999). According to the proposal, the filamentous fungi or protozoa can enhance the granulation process when the AGS system is initially developed. After inoculating *Chlorella* sp. A1 and A2 for 14 days (day 46, Fig. 4-7b), different biological structures were observed in the SBRs: i) microalgae were attached to the surface of the activated sludge, ii) aggregates formed by the self-aggregation of microalgae, and iii) large particles with elliptical shaped structure and green

color appeared, indicating the growth of *Chlorella* sp. A1 and A2 in the formed aggregates. This kind of elliptical shaped structures with diatoms were maintained under the designed operation conditions till the end of experiment on day 60 (Fig. 4-7c).

Jiang *et al.* (2006) examined the formation of AGS through inoculating mono culture of *Propioniferax*-like PG-02 and *Comamonas* sp. PG-08 and the coculture of 2 strains PG-02 and PG-08 into SBRs. They found that AGS appeared on days 7 and 21 in the reactors inoculated with the coculture (PG-02 and PG-08) and single strain PG-08, respectively. These researchers proposed that not only hydraulic selection pressure but also the selection of microbial strain inoculated into the reactor were crucial for the improvement of aerobic granulation process. Ivanov *et al.* (2008) found that the high aggregation ability of the strain *Pseudomonas veronii* played an important role in AGS formation (AGS appeared after 3 days' operation), which seems much faster than the current work. However, when considering the detailed cultivation conditions like much higher influent organic concentration (2.0 g-glucose/L vs 300 mg COD/L (NaAc) in this study) and higher air uplifting velocity applied in the previous study (2.1 cm/s vs 0.4 cm/s in this study), which are beneficial for granulation process, the results from this study are more meaningful to the practical application regarding energy/chemical consumption and real domestic wastewater treatment.

In addition, this work attempted a different inoculation strategy in comparison to previous researches in which the specific algal or bacterial strain(s) was usually inoculated into the reactor from the very beginning of the operation. In this study, the inoculation of the isolated strain was performed after 4 weeks' operation of the reactor in which no apparent bacterial AGS was observed (Stage I), possibly attributable to that few slowly growing bacteria were contained in the sampled seed activated sludge. After inoculation with the isolated strain *Chlorella* sp. A1, in comparison to A2, the granulation process was obviously enhanced and accelerated due to high aggregation ability of the strain *Chlorella* sp. A1 (Stage II). This finding also agrees with Jiang *et al.* (2006) and Ivanov *et al.* (2008) and confirms the importance of microbial strain selection for the granulation process.

4.3.4 Biological community diversity

During the whole operation, totally 9 samples were collected from the two SBRs, which were sampled on days 0 (Initial activated sludge), 21 (R1-21, R2-21), 31 (R1-31, R2-31), 38 (R1-38, R2-38) and 60 (R1-60, R2-60), respectively.

After quality control, 805,119 sequences could be converted into 1739 operational taxonomic units (OTUs), and 109,605 sequences into 56 OTUs bacterial and algal profiles, for

the all samples collected, respectively. The OTUs were identified with 97% identity and were assigned to 16 classes and 45 genera in the bacterial profile. In addition, algal profile was assigned to 2 classes and 3 genera. Taxa with relative abundance < 1% and unclassified groups were named as 'others'.

The five indexes are summarized in Table 4-1 to evaluate the differences of alpha-diversity in the bacterial and algal communities. The coverage value is to evaluate the presenters of the sampled gene sequences, and high coverage estimators (≥ 99%) are the evidence of the well-generated gene sequences.

Clearly seen from Table 4-1, the bacterial profiles have much higher community richness and diversity compared to the algal profiles. And all the samples have the high coverage value of 0.99.

The relative abundances of activated sludge and algal-bacterial AGS samples are shown in Figs. 4-8, 4-9, and Figs.4-10a and 4-10b at the class and genus levels of bacterial and algal profiles, respectively. As shown in Fig. 4-8, obvious differences in microbial community were observed in bacterial profile from the phase of activated sludge and mixture of activated sludge with microalgal strains along the operation. According to the relative abundance, *Actinobacteria, Bacteroidia, Anarerolineae, Alpha-, and Gamma-proteobacteria* occupied 22%, 24%, 15%, 9% and 12% in the initial activated samples. These groups of bacteria can be often found in activated sludge. On day 21, the classes of *Actinobacteria and Bacteroidia* showed decreasing tendency, while *Anarerolineae* and *Alphaproteobacteria* were increased up to 28 % and 24% (in R1) and 23% and 28% (in R2), respectively. A noticeable change was observed on day 31, after inoculation of microalgal strains into the two reactors and the relative abundance percentage of class *Anarerolineae,* which was dramatically dropped from 28% to 2% in R1 (with strain A1), however, in R2 (with strain A2) decreased from 23% to 19%. This might be the growth of filamentous bacterial group was inhibited by microalgal strains. This statement can be proven by the increase of Chl- a content (higher in R1 compared to R2), resulting in the dramatic decrease in class of *Anarerolineae*. Besides, the relative abundance percentage of class *Alphaproteobacteria* was detected to be 24 % and 28 % in R1 and R2, respectively on day 28 (the end of the Stage I). However, this percentage showed some decrease to 6% and 9% when inoculated with microalgal strains for about a week, and then after a while, dramatically increased up to 29% and 18% in R1 and R2, respectively at the end of operation. The microbes belonging to *Alphaproteobacteria* might take a longer time to adapt to a new environment such as the symbiosis system. Meanwhile, the percentage of bacteria in class of *Gammaproteobacteria* was observed to be 12% on the first day of sampling, and continued to

slightly decrease in both reactors. Afterwards, similarly with the class of *Alphaproteobacteria*, it dramatically decreased with its percentage detected as 1% and 2% in R1 and R2, respectively on day 38. After that, the microbes in the class of *Gammaproteobacteria* increased up to 13% only in R1, and a smaller percentage (1%) remained in R2. The class of *Bacilli* was detected to be 1% in the primary sample of activated sludge; however, its relative percentage was increased up to 17% in R1 and no change was observed in R2 after inoculation of microalgal strains. Furthermore, in the subsequent samples on day 38 the percentage of class *Bacilli* was counted to be 16% in R2, while slightly decreased in R1 (14%), and then this class disappeared at the end of operation.

In terms of algal community, the inoculated microalgal strains (*Chlorella* sp.) predominated at class and genus levels, and no significant changes were observed (Figs. 4-10a-b). The class of *Cyanobacteria,* with genera of *Leptolyngbya* was detected to be 3% only in R2 at the end of operation, and the development of such *Cyanobacteria* might be expected for a longer operation period.

4.4 Summary

Two reactors were operated for 60 days under the same strategies for two stages, and during the whole operation the MLSS concentrations were averagely 2.5–2.9 g/L in both reactors. The initial Chl-a was determined as 0.40 mg/g-MLSS, which increased up to 2.51 mg/g-MLSS. Both reactors exhibited similar efficiency in TN removal (~ 59%). However, the efficiency in TP removal was decreased from 78% to 56%, and then improved up to ~82% in Stage I and II, respectively. In Chapter 3, there is a discrepancy between strains A1 and A2 in terms of their biomass growth and nutrients removal efficiency, which have been evidenced during the reactor operation in this study. In both reactors the sludge appearance turned to be fluffier with irregular structure and connected to each other by small filamentous, but no apparent granules were observed in stage I. After inoculation with the isolated strains *Chlorella* sp. A1 and A2, a continuous growth of protozoa and diatoms, together with those strains were observed and the granulation process was enhanced, with elliptical shaped structures observed in the SBRs. The continuous decreases of classes *Anaerolineae, Bacteroidia, and Actinobacteria* dominating in activated sludge operation might be inhibited by the growth of two inoculated microalgae. Besides, the percentage of class *Alphaproteobacteria* increased at the end of operation, and this group of bacteria together with microalgal strains might enhance the granulation process. In terms of algal profile, the dominant genus was detected as the inoculated *Chlorella* sp. (94 –100%). Thus, it can be prospected that inoculation of some proper

algal species into the bacterial granulation system may accelerate the formation and stable operation of algal-bacterial AGS, which is promising for the rapid transformation of bacterial AGS to algal-bacterial AGS system in practice.

Table 4-1 Changes in bacterial and algal diversities in activated sludge and mixture of activated sludge with microalgal strains A1 and A2

Sample ID	Shannon [a]		Simpson [b]		ACE [c]		Chao [c]		Coverage [d]	
	Bacteria	Algae	Bacteria	Algae	Bacteria	Algae	Bacteria	Algae	Bacteria	Algae
AS-0	5.024	-	0.017	-	917	-	911	-	0.99	0.99
R1-21	4.822	-	0.020	-	950	-	932	-	0.99	0.99
R1-31	3.671	0.310	0.085	0.911	903	47	911	46	0.99	0.99
R1-38	1.833	0.033	0.463	0.992	716	25	613	22	0.99	0.99
R1-60	3.067	0.230	0.188	0.926	546	32	572	24	0.99	0.99
R2-21	4.780	-	0.019	-	900	-	875	-	0.99	0.99
R2-31	3.806	0.245	0.109	0.933	887	44	878	45	0.99	0.99
R2-38	2.155	0.011	0.368	0.997	875	11	763	10	0.99	0.99
R2-60	1.962	0.310	0.479	0.887	514	18	502	13	0.99	0.99

Note: R-21, -31, 38 and -60 denote that the sampling was conducted on day 21, 31, 38 and 60, respectively. AS-0 is initial activated sludge sample.

[a] Community diversity: A larger number denotes more diversity.

[b] Community diversity: A larger number denotes less diversity.

[c] Community richness: A larger number denotes greater richness.

[d] Sampling depth.

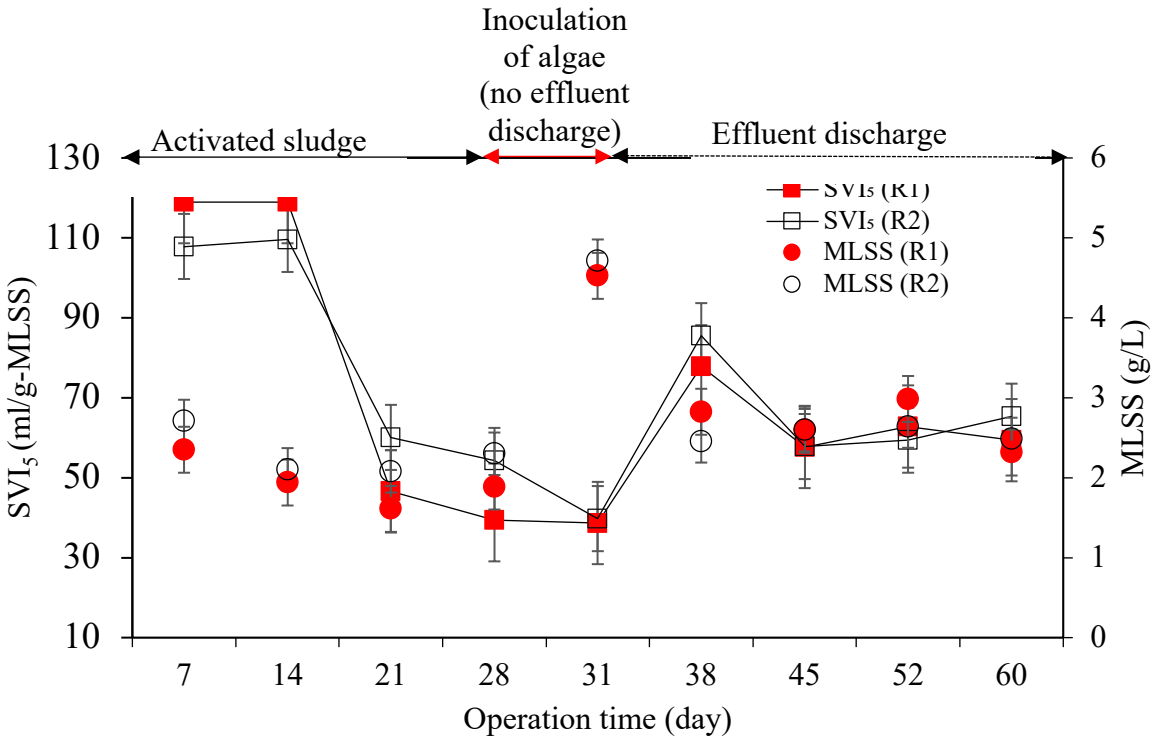

Fig 4-1 Changes in biomass concentration of activated sludge and mixture of activated sludge with microalgal strains A1 and A2 of two reactors during 60 day's operation: SVI_5 and MLSS variations.

Note: ◄⟷► Activated sludge

◄⟷► Inoculated microalgal strains into reactor with operation no effluent discharge and nutrients added manually daily

◄⟷► Activated sludge with microalgal strains

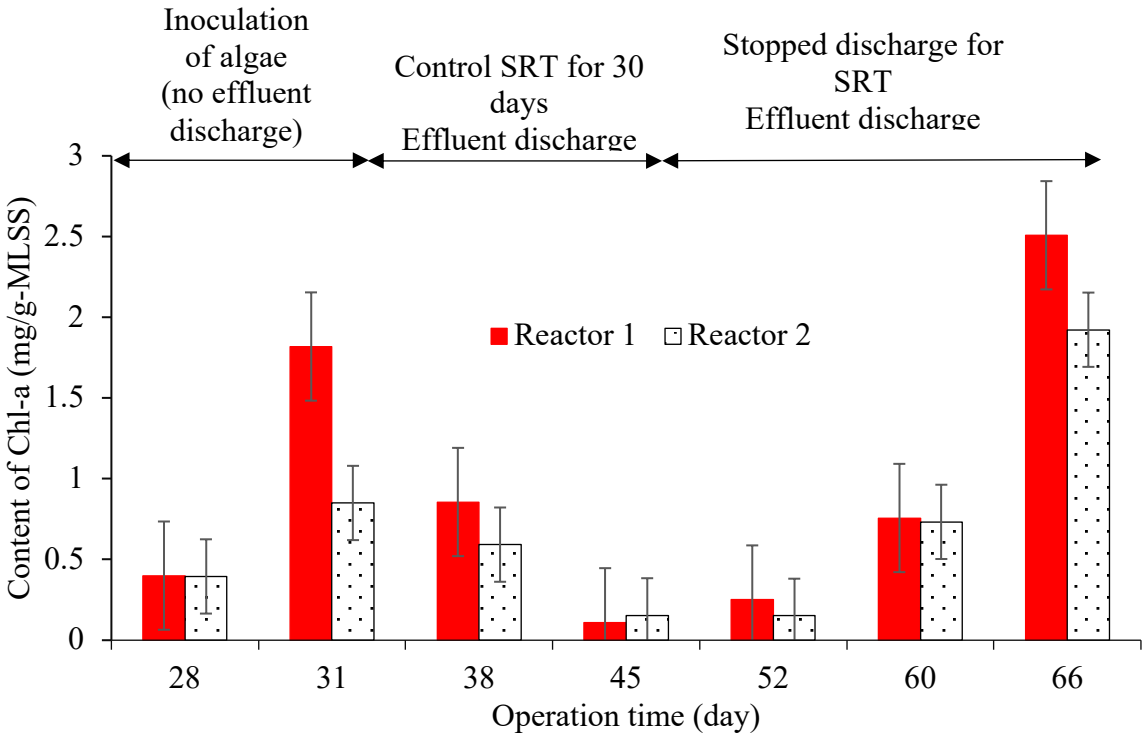

Fig 4-2 Change in Chl-a content in the biomass of two reactors (Microalgal strains were inoculated into reactor on day 28).

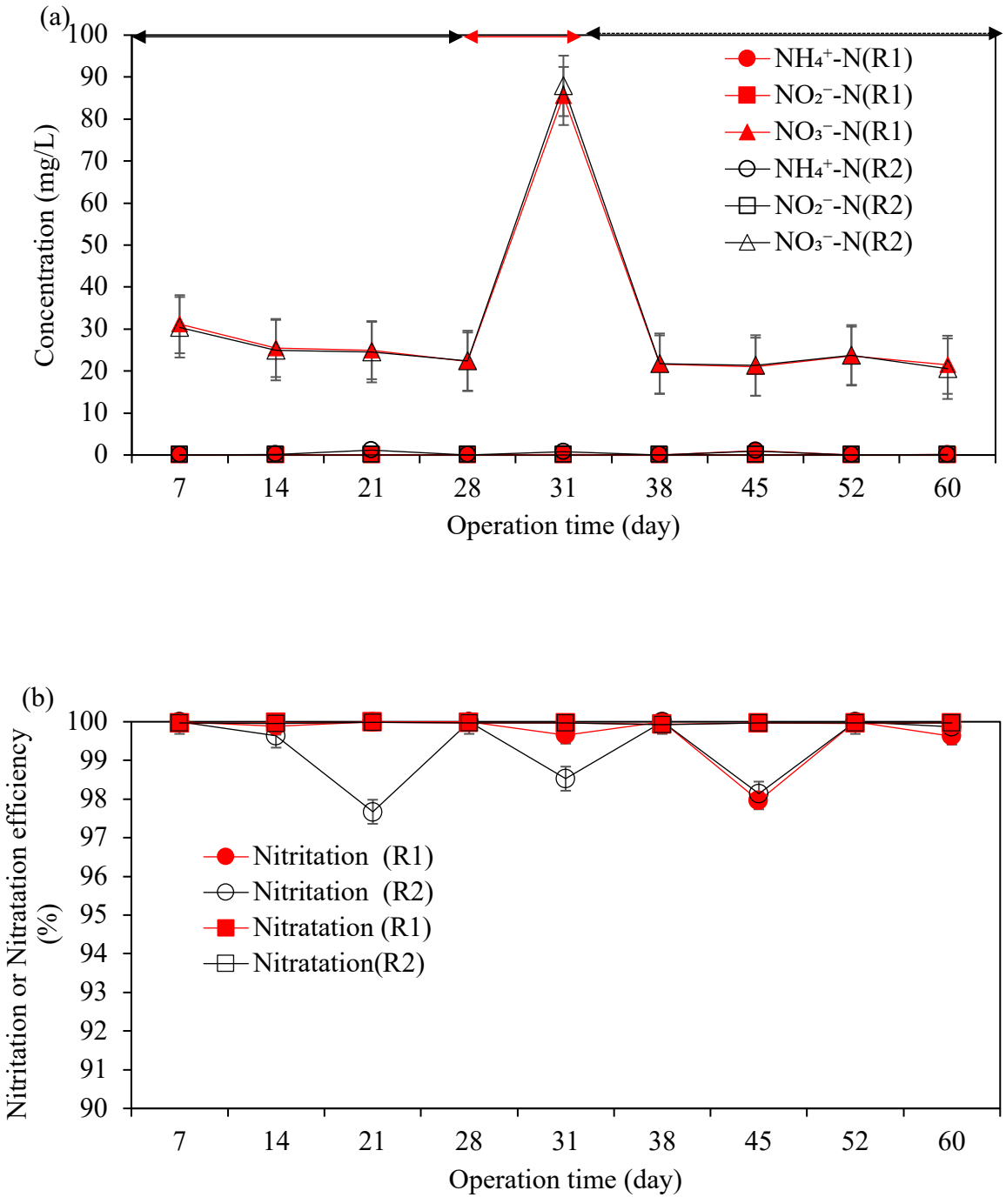

Fig. 4-3 Changes in N during 60 days' operation of the two reactors with activated sludge and mixture of activated sludge with microalgal strains A1 and A2: Concentrations of NH_4-N, NO_2-N, and NO_3-N (a), and nitritation and nitratation efficiencies (b).

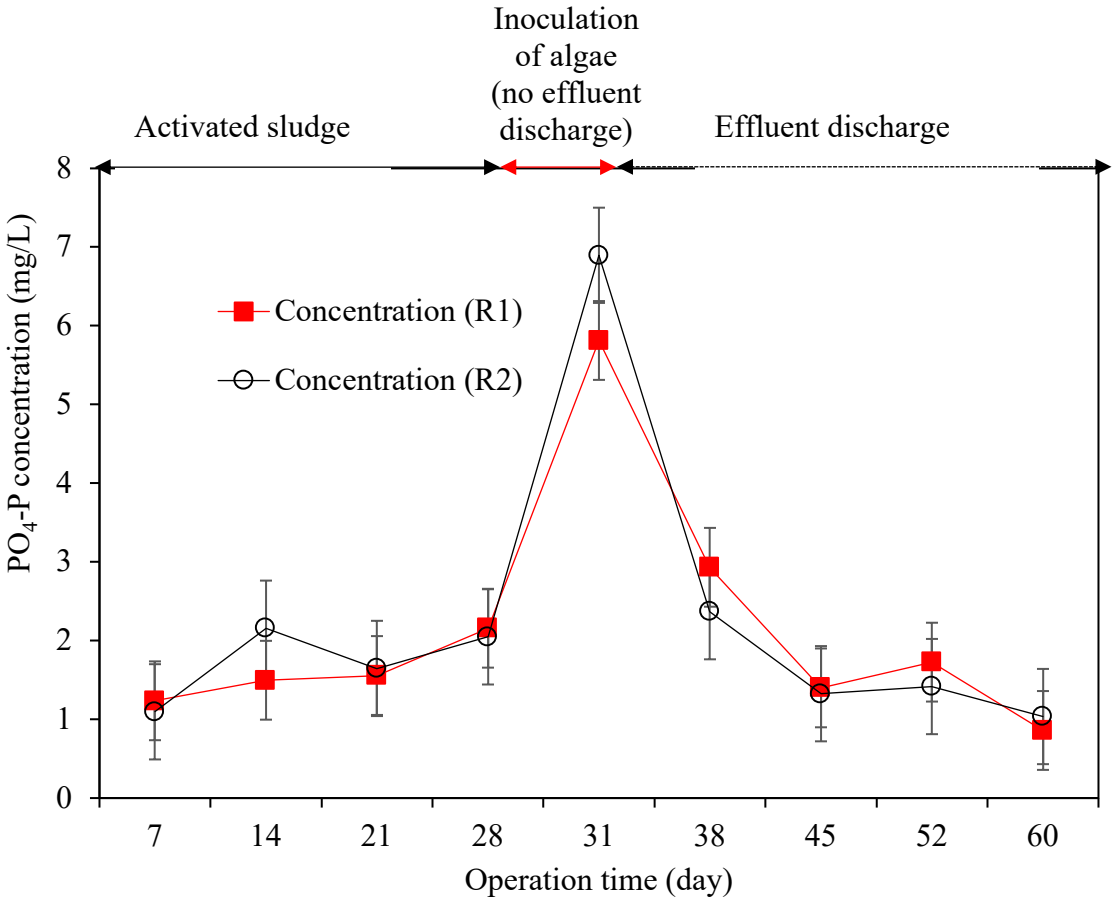

Fig. 4-4 Changes in PO_4-P concentration during 60 day's operation of two reactors with activated sludge and mixture of activated sludge with microalgal strains A1 and A2.

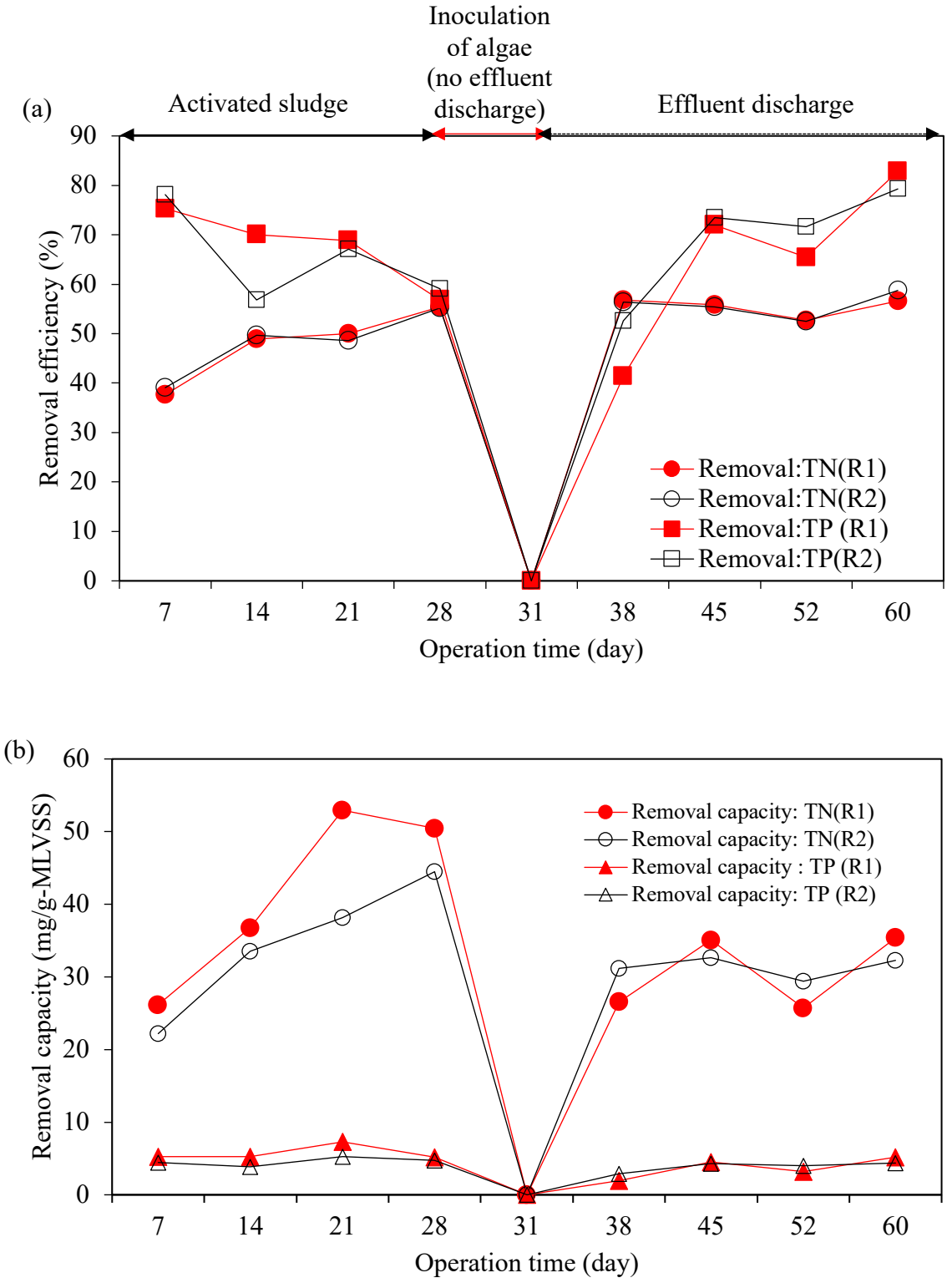

Fig. 4-5 N and P removal profiles of two reactors with activated sludge and mixture of activated sludge with microalgal strains A1 and A2 during 60 day's operation: TN and TP removal efficiency (a), and TN and TP removal capacities (b).

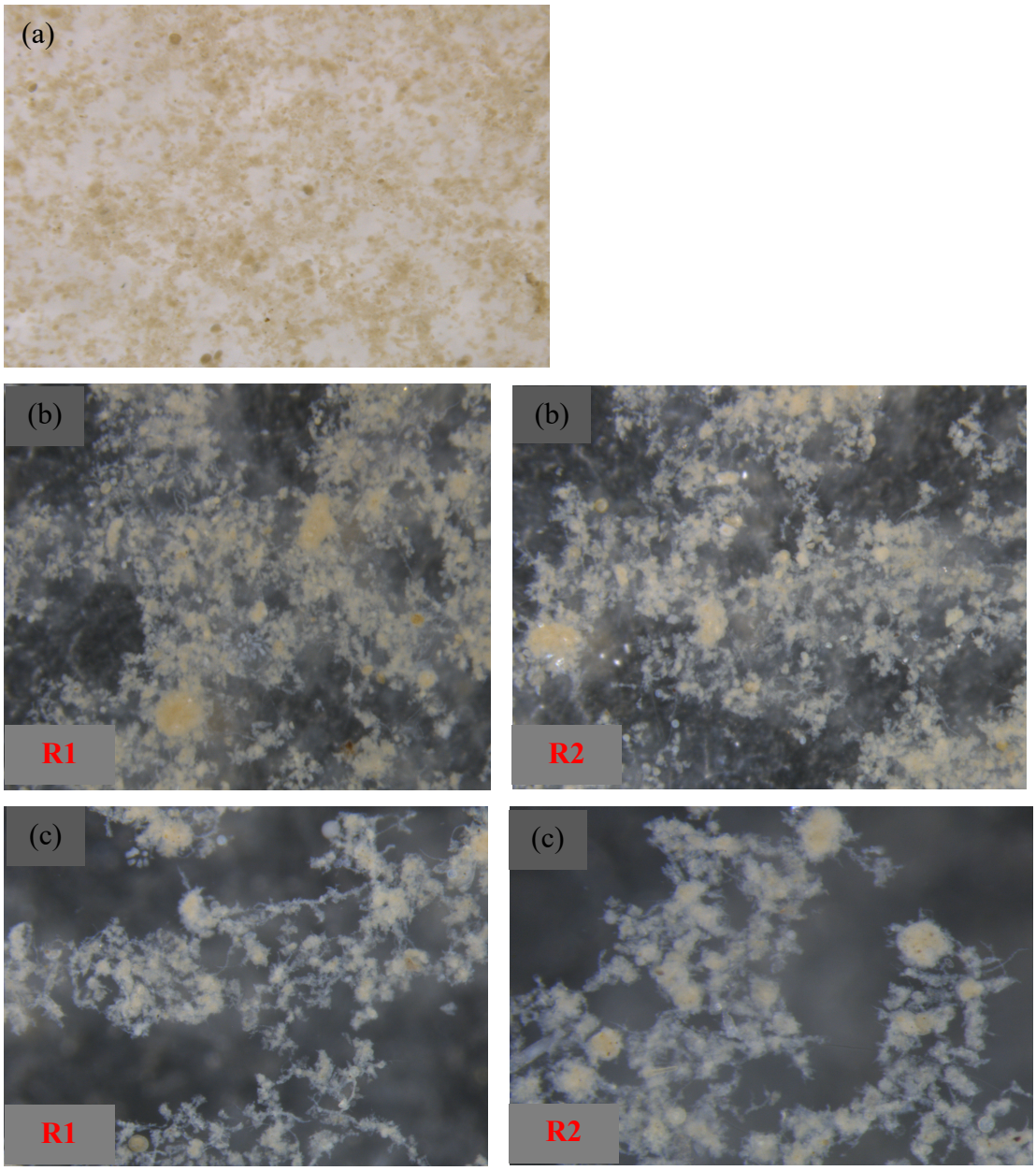

Fig. 4-6 Morphological changes of activated sludge in the two reactors during operation on day 0 (a), day 14 (b), and day 28 (c), respectively.

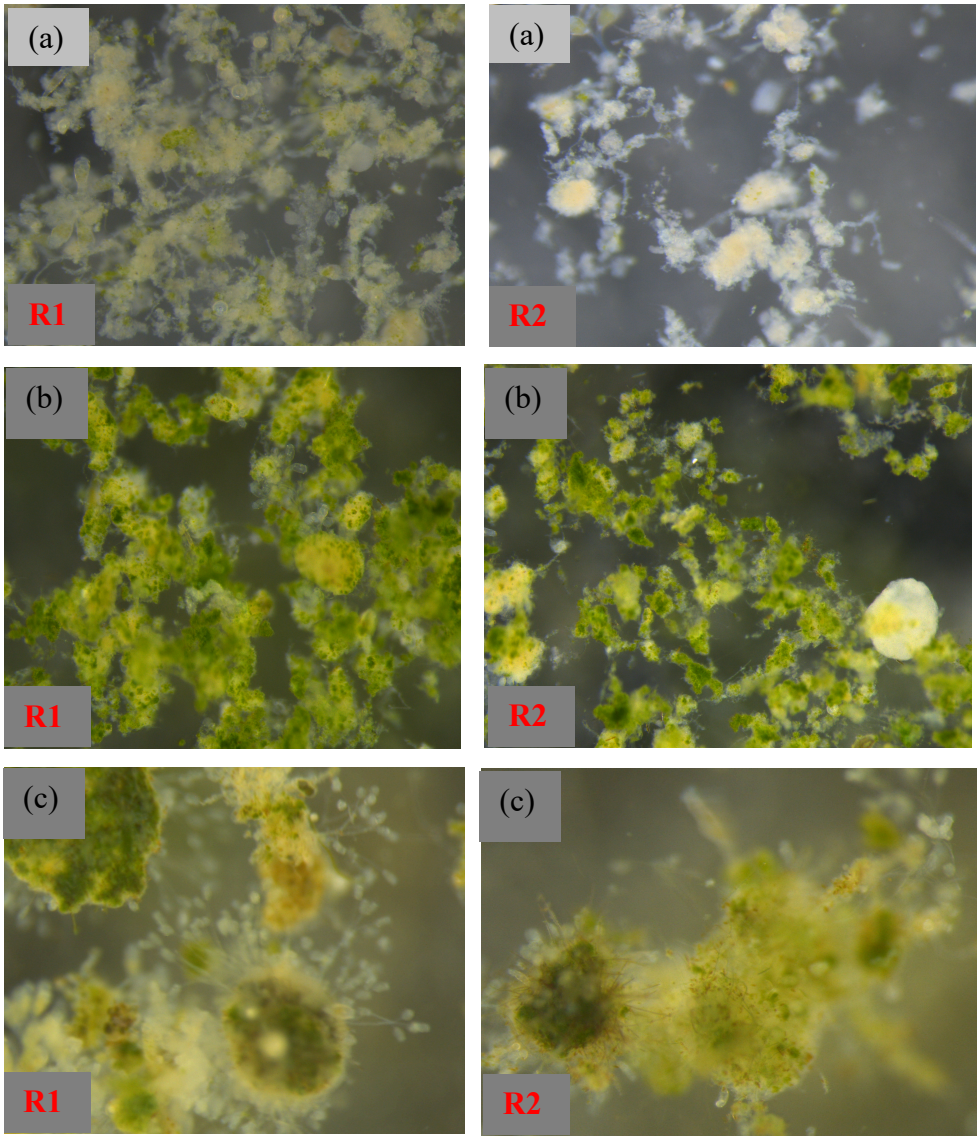

Fig. 4-7 The morphological changes of the mixture of activated sludge with microalgal strain *Chlorella* sp. A1 and A2 in the two reactors during operation on day 32 (a), day 46 (b) and day 60 (c), respectively.

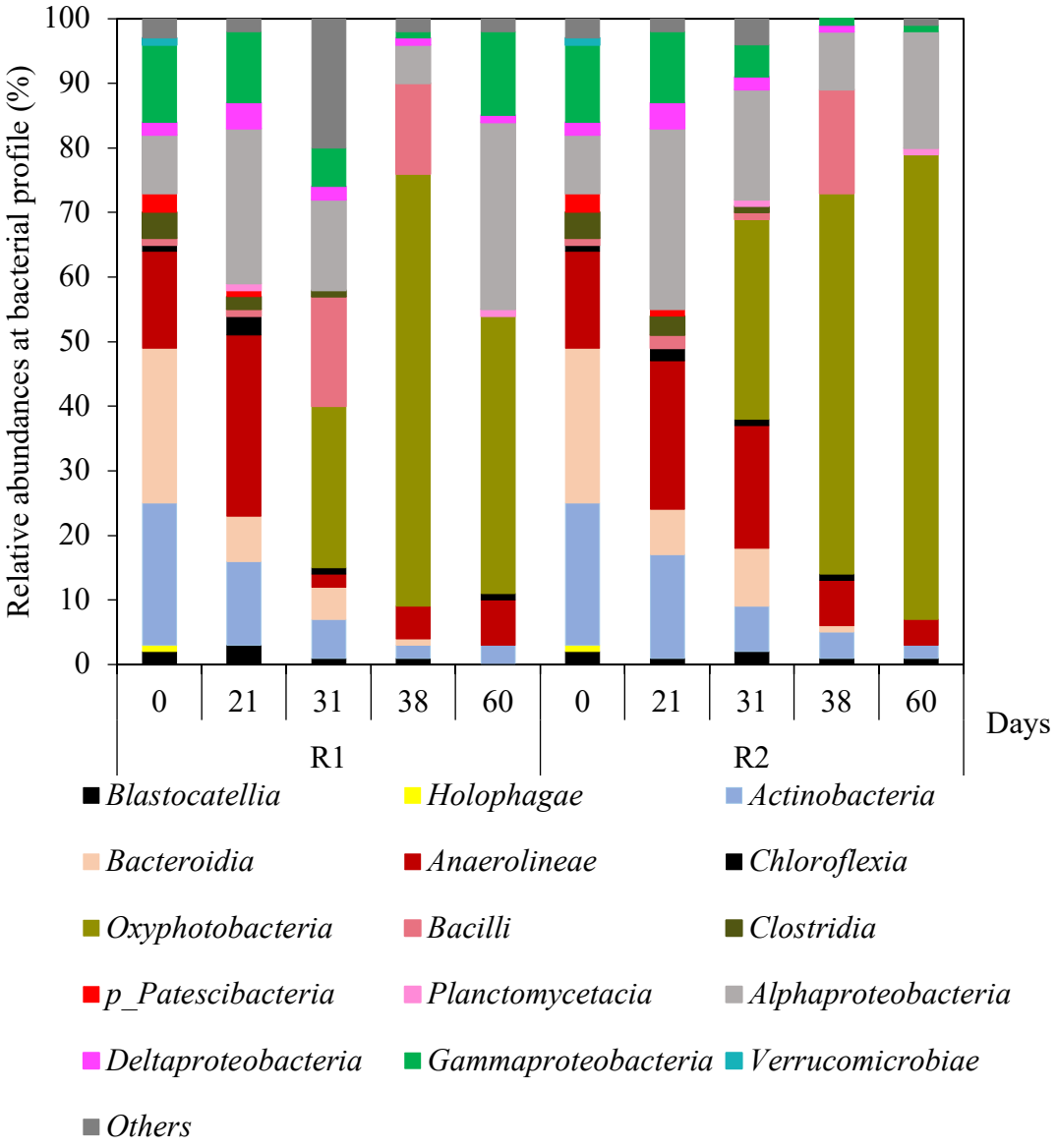

Fig. 4-8 Changes in biological community in the two reactors with activated sludge and mixture of activated sludge with microalgal strains A1 and A2 during 60 days' operation: Bacterial community profile at class level.

Note:

day 0, day 21, day of activated sludge (before microalgae inoculation), and day 31, day 38 and day 60 (after inoculation with the isolated *Chlorella* sp. A1 and A2).

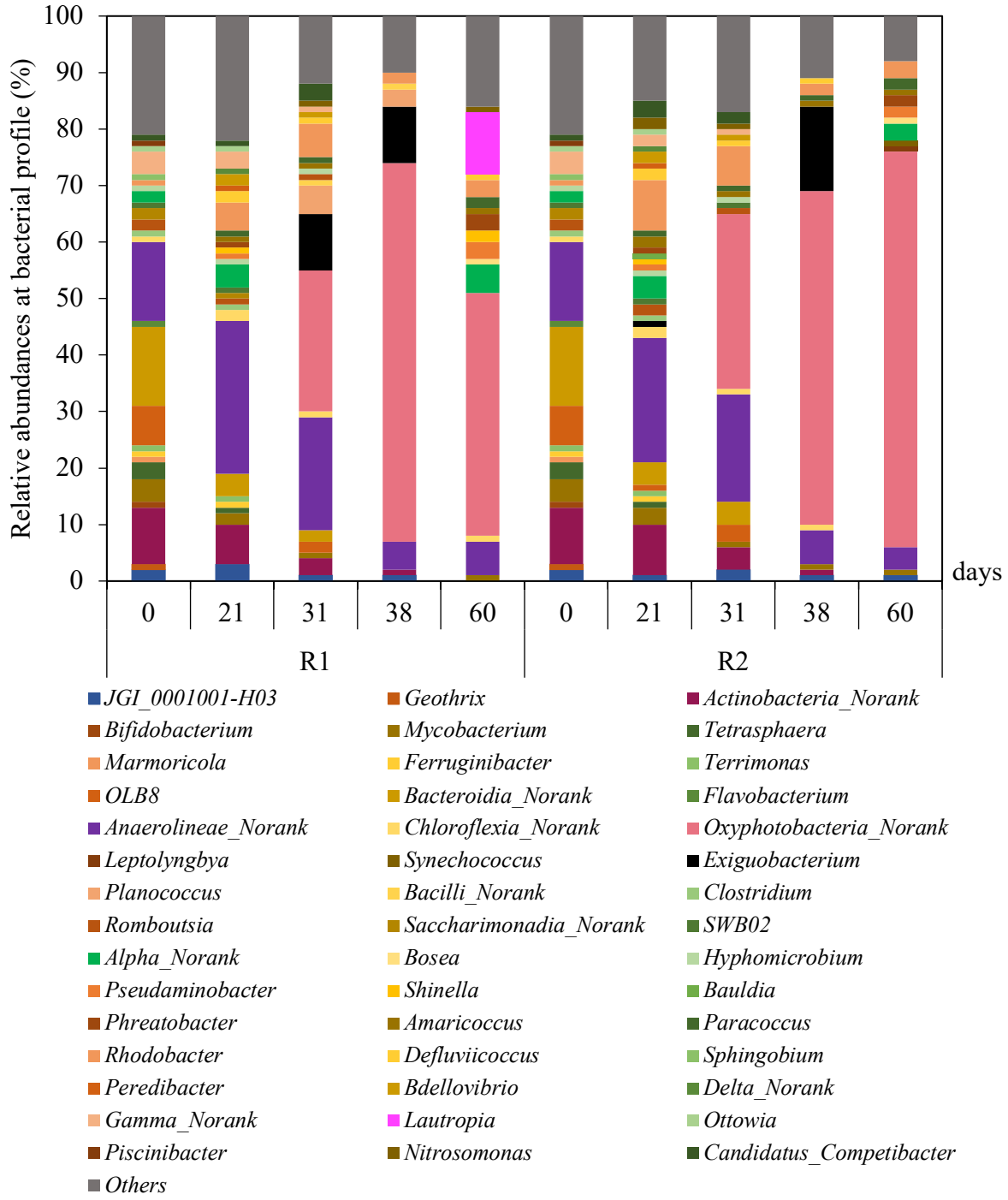

Fig. 4-9 Changes in biological community in the two reactors with activated sludge and mixture of activated sludge with microalgal strains A1 and A2 during 60 days' operation: Bacterial community profile at genus level.

Note:

day 0, day 21, day of activated sludge (before microalgae inoculation), and day 31, day 38 and day 60 (after inoculation with the isolated *Chlorella* sp. A1 and A2).

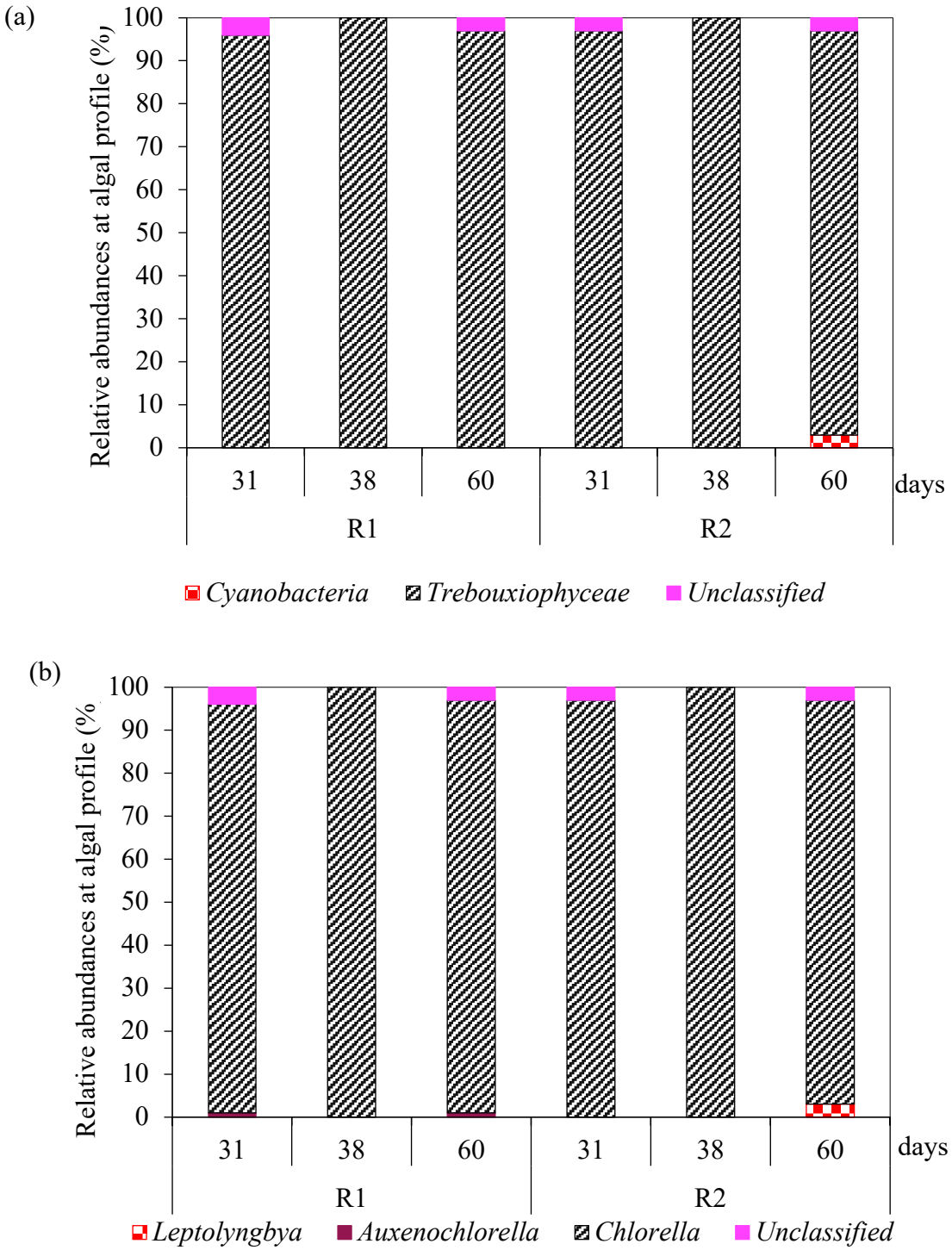

Fig. 4-10 Changes in biological community the two reactors with the mixture of activated sludge and microalgal strains A1 and A2 during operation at class level (a), and at genus level (b).

Chapter 5 Conclusions and future research perspectives

5.1 Conclusions

This study tried to figure out the biological factors that effect on the granulation of algal-bacterial symbiosis system and its nutrients removal. The main results can be summarized as follows (Tables 5-1, 5-2 and 5-3).

5.1.1 Correlation of dominant microbes with the granulation process

(1) The relative abundance percentage of the class *Alphaproteobacteria* and *Deltaproteobacteria* were detected with decreases from 27% and 23% to 3% and 2% and from 7% and 8% to 1% and 0% in R1 and R2, respectively, along with the operation. Such dramatically decreases can be an evidence that these classes are likely to be more vulnerable to the harsh environment in the most dominant phylum of the *Proteobacteria* in this study. In contrast, the number of microbes in class *Gammaproteobacteria* was lower in the reactor with the frozen granule (in R1, about 22%) in comparison to mature granule (in R2, about 40%) in the seed granule samples at the very beginning of the operation. This is possibly due to more recovery time necessary for the existing number of microbes in this class after being frozen. The sudden appearance of a large number (in R about 1-8%, and in R2 about 24%) of filamentous bacterial group of *Anaerolinea* might be one of the reasons for the disappearance of class *Flavobacteria* in both reactors at the end of the operation.

(2) The genus of *Merismopedia* in algal community was increased up to 26% and 48% in R1 and R2, respectively, when the performance of the SBRs became relatively stable. However, it disappeared at the end of the operation when breakage was observed. Thus, this genus might have some relationship with the granulation process. The genus of *Leptolyngbya* is one of the biggest representatives of algal group in this research and always tends to increase (from 18% to 44% in R1, and from 20% to 32% in R2) during the operation. And no obvious decrease was observed, indicating that these genera could be the most constant algae regardless of breakage of algal-bacterial AGS or not.

From the dynamic changes of biological communities, *Alphaproteobacteria* and *Flavobacteria* of bacterial group, and *Merismopedia* of algal group are most probably involved in the granulation process.

5.1.2 Contribution of isolated microalgae to the granulation process and nutrients removal

(1) With respect to the nutrients removal efficiency, in the two isolated microalgal strains (A1 and A2), strain A1 exhibited excellent nutrients removal, achieving 93-96% of N and P removal under the light/dark (12h/12h) cultivation when treating wastewater containing 50 mg-NH_4-N/L and 5 mg-PO_4-P/L, which were always 9-14% higher than strain A2. When these two microalgal strains *Chlorella* sp. A1 and A2 being inoculated into the SBRs, the reactor efficiency in TP removal was higher (up to ~82%) in comparison with the activated sludge process (decreased from 78% to 56%). However, the removal efficiency in TN (~ 59%) showed little difference in both reactors during the operation of the SBRs with activated sludge and the mixture of activated sludge and microalgal strains.

Inoculation of microalgal strains showed better performance in TP removal and more stable performance of nutrients removal might take a longer operation of SBRs.

(2) In both reactors, the biological community structures were changed significantly at bacterial profiles, after the inoculation of two isolated microalgal strains. The class of *Anaerolinea* (28% in R1, and 23% in R2) and *Alphaproteobacteria* (24% in R1, and 28% in R2) were the most dominant classes among the detected classes when operating with activated sludge (in Stage 1). However, after inoculation of the two microalgal strains, the relative abundance percentage was declined in both classes in the two reactors. Furthermore, the class of *Anaerolinea* showed slight increases, which could not reach to the percentage as that in the sampled activated sludge. Presumably, the class of *Anaerolinea* was not the important group of bacteria for the granulation process. Namely, when the granulation process was enhanced, the class of *Anaerolinea* continuously decreased in both reactors. The class of *Alphaproteobacteria* was increased at the end of the operation and remained as the dominant class in both reactors. The assumption is that the microbes in this class might be one of the contributors to the granulation process.

The inoculation process of two microalgal strains with activated sludge into SBRs helped to a certain extent to figure out the granulation process. Microalgal strains only itself could not be a contributor to the granulation process, instead they may be employed as an inhibitor of the unnecessary microbes which may prolong the granulation process.

5.2 Future research perspectives

In this present study, microalgal strains isolated from algal-bacterial AGS possess excellent nutrients removal efficiency. However, during the enhancement of the granulation process, the biomass growth of those strains was also associated development of protozoa and diatoms.

With the aim to figure out the exactly biological factors that effect on the granulation of algal-bacterial, further explorations are still necessary.

(1) Still, which component of PS or PN plays the key role in the aggregation of the isolated microalgae remains unclear. The detailed composition of the microalgal EPS together with the mechanisms involved in the algal aggregation is under investigation.

(2) To build in-depth concept in the contribution of these two isolated microalgal strains to the granulation process, it is also meaningful and useful to inoculate microalgal strains into SBR for operation as mono-culture system.

(3) Optimization of the operation strategy for the granulation of the isolated algal strains in the real wastewater treatment system is also necessary in the followed-up works.

Table 5-1 Summary of nutrients removal in this research

Operation mode	Strain number or reactor number	Nutrients in influent	Nutrient removal efficiency (%)	
			PO_4-P	NH_4-N
Mono-culture cultivation (Batch)	A1 (12 h/12 h)	5 mg PO_4-P/L (KH_2PO_4)	96	92
	A2 (12 h/12 h)		87	83
	A1 (0 h/ 24 h)		52	49
	A2 (0 h/ 24 h)		34	35
Activated sludge (SBR)	Reactor 1	50 mg NH_4-N/L (NH_4Cl)	75-57	37-55
	Reactor 2		78-59	39-55
Activated sludge and mixture of microalgal strains (SBR)	Reactor 1		41-82	56-56
	Reactor 2		52-79	56-59

Note: 12 h/12 h or 0 h/24 h denotes the light/dark cycle condition.

Table 5-2 Summary of most changed microbial communities in algal-bacterial AGS at bacterial and algal profiles

Sample ID	R1-5	R1-35	R1-85	R2-5	R2-35	R2-85
At class level	Relative abundance at bacterial profile (%)					
Alphaproteobacteria	27	20	3	23	20	2
Deltaproteobacteria	7	8	1	8	17	0
Flavobacteriia	11	21	0	14	20	0
Betaproteobacteria	20	18	15	10	14	24
Gammaproteobacteria	22	22	16	40	20	12
Anaerolineae	0	0	8	0	0	24
At genus level	Relative abundance at algal profile (%)					
Merismopedia	13	26	0	24	48	0
Leptolyngbya	18	17	44	20	32	32

Note: R-5, -35, and -85 denote that the granule sampling was conducted on day 5, 35 and 85, respectively.

Table 5-3 Summary of most changed microbial communities in activated sludge and its mixture with microalgal strains

Sample ID	AS-0	R1-21	R1-31	R1-38	R1-60	R2-21	R2-31	R2-38	R2-60
At class level	Relative abundance at bacterial profile (%)								
Actinobacteria	22	13	6	2	3	16	7	4	2
Bacteroidia	24	7	5	1	0	7	9	1	0
Anaerolineae	15	28	2	5	7	23	19	7	4
Bacilli	1	1	17	14	0	2	1	16	0
Gammaproteobacteria	12	11	6	1	13	11	5	2	1
Alphaproteobacteria	9	24	14	6	29	28	17	9	18
At genus level	Relative abundance at algal profile (%)								
Chlorella	0	0	95	100	96	0	97	100	94
Leptolyngbya	0	0	0	0	0	0	0	0	3

Note: R-21, -31, 38 and -60 denote that the sampling was conducted on day 21, 31, 38 and 60, respectively. AS-0 is initial activated sludge sample

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Appendix

1. Nuramkhaan M., Zhang Y., Dong X., Huang W., Lei Z., Shimizu K., Zhang Z., Utsumi M., Duu-Jong Lee., 2019. Isolation of microalgal strain from algal-bacterial aerobic granular sludge and examination on its contribution to granulation process during wastewater treatment in respect of nutrients removal, auto-aggregation capability and EPS excretion. Bioresource Technology Reports. Bioresource Technology Reports, 8, 100330
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