Effects of the exogenous N-acylhomoserine lactones on the performances of microalgal-bacterial granular consortia

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ABSTRACT
Chlorella sorokiniana, the dominant microalgal strain with fast growth rate and high nutrients' adsorption rate, was selected from the mature MBGS system. After adding C6-HSL and 3-oxo-C12-HSL to the MBGS consortia formed by combining it with mature AGS, it could also be found that C6-HSL accelerated the denitrification rate and improved the removal rate of TIN and TP effectively. Besides, the promoting effect of C6-HSL was slightly higher than that of 3-oxo-C12-HSL. By adjusting the concentration of C6-HSL (5 × 10^{-5} and 7.5 × 10^{-7} mol/L), the results revealed that adding 5 × 10^{-7} mol/L C6-HSL significantly improved the efficiency of nitrogen and phosphorus removal in MBGS consortia. However, adding higher concentration of C6-HSL (7.5 × 10^{-7} mol/L) would lead to deterioration of TIN and COD degradation. The content of EPS secreted by bacteria was regulated by different concentrations of AHLs, and the PS/PN value decreased with the increasing trend of C6-HSL concentration.

1. Introduction
Compared with the conventional flocculent activated sludge process applied in wastewater treatment, aerobic granular sludge (AGS) technology has emerged in recent decades due to their advantages of better settling property, more effective organics and nutrients removal rate, greater biomass concentration, and obvious occupied treatment plant space reduction [1,2]. However, some factors including the difficulty to keep long-term granular stability, many costs for aeration and stir as well as high energy supplied for them were also the limitations for AGS process developments [3].
Microalgae belong to single-cell eukaryotes and they are widely distributed on the land and in the ocean [4]. It presents the advantages of high photosynthetic rate, fast propagation, strong environmental adaptability and high efficiency of nitrogen and phosphorus treatment [5]. Some of them could directly use some organic matter in sewage to synthetize its own cellular material. Numerous studies had shown that microalgae could be used in wastewater to remove the nutrients without aeration, and the biomass produced through microalgal biofilm cultivation approach could also be used to make added-value products such as biofuels [2,6]. Nevertheless, microalgae are small in scale (5 ~ 50 μm), their density is close to water and microalgal cell surface is negatively charged, resulting in low possibility of natural separation between microalgae and water [7].

Considering the above factors, combine the AGS and microalgae to form the microalgal-bacteria granular sludge (MBGS) was feasible to overcome those drawbacks. Microalgae could produce a mass of oxygen (O₂) and secrete some small molecular substances as well as extracellular polymeric substance (EPS) through photosynthesis to feed bacteria metabolism, then the carbon dioxide (CO₂) produced by bacteria metabolism could also be used for photosynthesis of microalgae in turn [8,9]. Thus, the two kind of organisms would get a cooperative relationship in this new process. This symbiotic system could improve the poor sedimentation performance of microalgae and reduce the energy consumption for granular sludge’s aeration [10,11].

Last decades, there were many researchers summarized that quorum sensing was significant in microbial systems [12]. Ren revealed that specific concentrations and types of signaling molecules could benefit the cell agglomeration, then it accelerated the biofilm and granular sludge formation. Besides, it was reported that N-acylhomoserine lactone (AHL) played an important role in the formation and stability of aerobic granules [13]. Li proved that the decreasing AHLs contents led to the decreasing concentrations of polysaccharide (PS) and protein (PN) in extracellular polymeric substances (EPS), resulting in the instability of AGS [14]. Li proposed biological AHL-manipulation by dosing AHL-producing or -quenching bacteria into the activated sludge system continuously, and its granulation was investigated through various AHL concentrations addition in this system [15]. Moreover, the contents of aromatic protein were found to increase when applying the AHLs generated from activated sludge into Chlorophyta sp. and the settleability of Chlorophyta sp. cells increased by 41%, it meant AHLs secreted by bacteria could communicate with microalgal cells and promoted self-aggregation [16]. Besides, Zhang extracted AHLs when cultivating MBGS from flocculent sludge, and the results showed that AHLs helped transporting the communication between bacteria and microalgae. In this combined system, excess AHLs could restrained the initial growth of Chlorophyta sp. and caused harmful influence on the characteristic of microalgae [17,18]. However, it could be found that the researches focused on the responses of MBGS to AHLs-based quorum sensing were still limited, so how the signal molecules affect the relationships between microalgae and bacteria in this consortia need to be investigated further.

In this study, the microagal strains were isolated from the stable MBGS system, then the dominant strain was selected to combine with mature aerobic granules in the shaking photoreactors. The AHLs-based signal molecules were put into the consortia with different types and concentrations. Nutrient removal and microbial performances were detected during the whole operating cycle. Results of this work were expected to help us reveal the role of signal molecular in MBGS system and study the interaction mechanisms of microalgae and bacteria in this process.

2. Materials and methods

2.1 Establishment of microalgal-bacterial systems

In Wuhan University, one sequencing batch reactor (SBR, height was 300 mm and inner diameter was 100 mm) made of polymethyl methacrylate was established with an exchange ratio of 50% and effective volume of 2.4 L (Fig. 51). There were mature AGS treating the synthetic wastewater which contained 323.7 mg/L CH₂COONa, 14.6 mg/L KH₂PO₄, 75.6 mg/L NH₄Cl, 10 mg/L MgSO₄·7H₂O, 10 mg/L CaCl₂ as well as 1 mL/L trace element solution [19], then it was inoculated into this reactor, and the influent was the same as before. The average influent TIN, COD and TP was controlled at 19.20 ± 1.15, 225.38 ± 6.35 and 3.17 ± 0.22 mg/L, respectively. There were two light-emitting diode (LED) tubes attached on the reactor to make the illumination intensity reaching around 40μmol/m²·s (12 h every day). Moreover, the aeration was supplied through the air diffusers from the bottom of the reactor with an aeration rate of 150 mL/min. The whole cycle was 6 h including 2 min feeding, 90 min aeration, 5 min settling and 5 min discharging. During the operation, the variation of water qualities and sludge characteristics had been monitored.

2.2 Microalgal strains’ isolation and identification

After this MBGS system became stable, there were some microalgal strains isolated from it and purified through the methods according to Varshney with some modifications as shown in Fig. S2 [20]. After purification, all the selected microalgal strains were
sent to Shenggong Ltd. Shanghai, China for 18s rRNA gene sequencing. The obtained sequences were input in the National Center for Biotechnology Information (NCBI) database to identify species that were closest to these microalgal strains. The phylogenetic tree for different strains would be constructed by using MEGA 7.0 software [21], the result was shown in Fig. S3.

2.3 Comparison of nutrient removal performances and growing capacity of the isolated microalgal strains

The isolated microalgal strains were inoculated into 500 mL round-bottomed flasks containing BG11 culture medium and synthetic sewage, the composition of the latter was the same as the wastewater feeding the initial microalgal-bacterial sludge in SBR. They were placed under the illumination incubator and the light density was set as about 40μmol/m²·s (light/dark was 12 h/12 h). All the working volume of microalgal systems were 200 mL and the initial optical density of 680 nm (OD_{680}) value of microalgal cultures was controlled at 0.1. After the inoculation, the nutrient removal efficiencies and microalgal growth rates such as dry weight and Chlorophyll a (Chl-a) in those solutions were detected every day. The concentration of ammonia nitrogen (NH₄⁺-N), nitrate (NO₃⁻-N), nitrite (NO₂⁻-N), total phosphorus (TP) and chemical oxygen demand (COD) were all measured according to the standard methods [22]. OD_{680} Value in the microalgal solution in each flask was measured every day and the dry weight of microalgae could be determined by the regression equation between dry weight and the value of OD_{680} [23]. The concentration of Chl-a was determined according to the methods described in previous work [24]. Chl-a concentration was calculated through the equation:

\[ \text{Chl-a} = (A_{665} - A_{750}) \times 13.7 - A_{549} - A_{750} \times 5.76 \]

Where A_{665}, A_{750} and A_{549} represent the Optical density value at 665, 750 and 649 nm, respectively.

In addition, the microalgal strain among those isolated strains which achieved the best performances was chosen for further analysis.

2.4 Effects of AHLS concentration and type on the formation and operation of microalgal-bacterial granular sludge systems

Firstly, equivalent mature AGS was inoculated into 1000 mL round-bottomed flasks (initial MLSS was 2000 mg/L) respectively, then each flask was added in chosen microalgal fluid and OD_{680} was adjusted at 0.7. The working volume of each system was 500 mL. The strategy of AHLS addition among those flasks was shown as Table 1. All the flasks were placed on a shaker at 150rpm and set the same light condition as before. 50% of solutions in every flask would be replaced in aseptic condition every day. After the microalgae had been totally combined with the AGS in all the flasks, 10 mL MBGS solution in each system was collected at 0.5 h, 1 h, 1.5 h, 2 h, 3 h, 6 h, 12 h and 24 h in one cycle, respectively for examination on nutrient removal. The EPS concentration of MBGS would also be extracted and detected at last. EPS extraction in MBGS was operated by heating method, and the concentration of PN and PS was determined through the Bradford method and sulfuric acid-phenol colorimetric method, respectively [25].

### Table 1. The scheme of shaking flask.

| Experimental group | Number | Signal molecules species | Signal molecules concentration(mol/L) |
|--------------------|--------|--------------------------|-------------------------------------|
| I                  | S0     | Control                  | 0                                   |
|                    | S1     | C₂-HSL                   | 5×10⁻⁶                             |
|                    | S2     | 3-oxo-β-C₁₂-HSL          | 5×10⁻⁶                             |
| II                 | C0     | Control                  | 0                                   |
|                    | C₁     | C₂-HSL                   | 5×10⁻⁹                             |
|                    | C₂     | C₁-HSL                   | 2.5×10⁻⁷                           |
|                    | C₃     | C₁-HSL                   | 7.5×10⁻⁷                           |

#### 2.5 Statistical analysis

SPSS21 software was used for statistical tests. One-way analysis of variance (ANOVA) was conducted to analyze the results, and P < 0.05 was deemed to be the evidence that there are significant differences among different varieties.

3. Results and discussion

3.1 The MBGS system’s establishment and operation

3.1.1 The decontamination performances of MBGS systems

Figure 1 shows the removal performances of nutrients such as the varying effluent concentration of COD, TP and TIN which was the total concentration of NH₄⁺-N, NO₃⁻-N and NO₂⁻-N from the mature AGS systems (stage I) to MBGS systems (stage II) through artificial light effect. At the later period of stage II, the effluent concentration of different parameters had been keeping...
little variation for above 10 days, indicating the system enter the stable period relatively. The COD and TP removal rate were always above 90% in the whole operation. Effluent TP concentration showed little fluctuation from period I to period II, and the removal rate of COD in MBGS (97.1%) showed slightly improvement compared with AGS (95.6%), indicating that the combination of microalgae and AGS only had a little contribution on the COD degradation in this consortia systems. The symbiosis relationship between the bacteria and microalgae was beneficial for the gas exchange and $O_2$ transportation, which leading to some degree of superiority of removing COD in MBGS systems. Besides, the removal performances of TIN showed some fluctuation after applying the light. The TIN removal rate was 72.1% in AGS system, but it increased to 85.3% after it shifted to MBGS system. The main reason for this phenomenon was due to the decreasing accumulation of $NO_3^-$ N. The accumulated $NO_2^- N$ was maintained at a very low level (<0.5 mg/L) in both stage. However, the average $NO_3^-$ N concentration decreased from 3–4 mg/L to 1–2 mg/L from stage I to stage II. This phenomenon could be ascribed by the reason that microalgae could assimilate $NO_3^-$ N via cell membrane initiatively and those microalgae surrounding the aerobic granules increased their size, which helped increasing the resistance of mass transportation and enlarging the anoxic zone of granules, then leading to the better denitrification efficiencies.

3.1.2 The sludge characteristics of MBGS systems

3.2 The screening of the best microalgal strains

3.2.1 The characteristic of microalgal strains in culture solutions and sewage

There were three microalgae strains screened from the MBGS systems, and the results of 18S rRNA sequencing showed that they were Scenedesmus sp. (A1), Chlorella sorokiniana (A2) and Tetraselmis sp. (A3). Biomass changes of A1, A2 and A3 in culture medium and sewage were shown in Figure 2. As can be seen from Figure 2a, all the microalgae maintained a uniform growth rate in the first 5 days in BG11 culture medium, and the growth rates of them were relatively slow, indicating that all the microalgae were in the adaptation period at this time, and the minimum biomass of the three microalgae came from A2 (0.75 g/L). From the 5th day, the growth rate of A2 increased sharply, presenting an exponential growth curve, and the maximum specific growth rate $\mu_{\text{max}}$ was 0.56d$^{-1}$, indicating that A2 had entered the logarithmic multiplication stage. Although the growth rate of A1 had increased somewhat, it was not obvious, so it could be inferred that it would still take some time to enter the logarithmic stage. And A3 still maintain steady growth. In this experiment, the potential of Chlorella to achieve bioaccumulation was proved to be good. In the wastewater, those three kinds of microalgae were in the adaptation stage in the first 12 h, while the growth rate of A2 increased rapidly in the 36 h, and first reached the logarithmic growth stage, at which the biomass and maximum specific growth rate were 0.46 g/L and 0.34d$^{-1}$, respectively (Figure 2b). After that, the biomass of A2 increased slowly, and reached 0.60 g/L at 7.5d. C. sorokiniana has been reported to grow well in sewage under photoautotrophic, heterotrophic and mixed conditions [27]. Therefore, the accumulation of microalgal biomass and the synthesis of compounds such as carbohydrates, proteins, chlorophyll and lipids might be the result of photosynthesis and organic matrix assimilation. A1 and A3 entered the logarithmic growth stage after 5.5 days and then continued to increase to 1.09 and 0.83 g/L at 7.7 days, respectively. Compared with the other two microalgae strains, A2 could quickly adapt.

| Time (d) | MLSS (mg/L) | MLVSS/MLSS | SOUR (mg $O_2$/g MLVSS-h) | SVI$\_5$ (mL/g) |
|----------|-------------|-------------|---------------------------|-----------------|
| 0        | 3500        | 0.78        | 24.47                     | 42              |
| 7        | 3164        | 0.69        | 23.13                     | 57              |
| 14       | 3320        | 0.72        | 28.15                     | 50              |
| 21       | 3648        | 0.75        | 42.86                     | 44              |
| 30       | 4082        | 0.73        | 37.02                     | 35              |
| 40       | 4750        | 0.81        | 39.14                     | 31              |
to the sewage environment and absorb nutrients in the external environment for cell metabolism, indicating that it has significant advantages in sewage treatments.

The chlorophyll content of A3 in BG11 decreased from 3.81 mg/L on day 5 to 1.86 mg/L on day 7, which was significantly different from that of the other two microalgae that maintained steady upward trend (Fig. S4A). From the 4th day, the chlorophyll content of A2 increased rapidly from 7.51 mg/L to 14.00 mg/L within 2 days and exceeded that of A1 (11.89 mg/L), which was the highest among the three microalgal species. The results showed that BG11 culture medium was the most suitable for the growth of A2, which was corresponding to the trend of biomass change. According to Fig. S4B, we found that the changing trend of chlorophyll content and biomass of microalgae in artificial water distribution was roughly the same. The chlorophyll content of A2 increased rapidly to 3.03 mg/L at 36 h, and maintained steady downward trend after 3.5d, indicating that A2 began to enter in declining period. The chlorophyll content of A3 maintained a slow growing trend, but it was always lower than that of A2. However, the chlorophyll content of A1 continued to increase until the 7th day, which did not reach a stable stage, indicating its good adaptability to artificially configured sewage.

Taking the sewage without microalgae as the blank control, pH in all groups was higher than initial pH after reaction, and they showed increasing trend in the first three days (Fig. S5). It had been reported that with the rapid growth of microalgae in exponential phase, photosynthetic activity enhanced, inorganic carbon (Ci) transporters absorbed large amounts of CO$_2$ in the form of HCO$_3^-$ from chloroplasts, and protons (H$^+$) were used in the conversion of HCO$_3^-$ into CO$_2$, which was fixed by the enzyme RuBisCO and released OH$^-$ in cells during photosynthesis. This must be neutralized by H$^+$ absorption in the extracellular environment, so the decrease of H$^+$ in the mixture would inevitably lead to an increase in pH value [28]. The increase of the pH value in the medium during microalgal growing period also validated the removal way of contaminants. As can be seen from the Fig. S5, the pH value of A2 gradually decreased from the highest (10.9) from the 4th day, while the pH value of A1 and A3 still maintained an upward trend, reaching the highest value 10.6. The higher pH value of A2 in the early stage indicated that it had higher photosynthetic activity and biomass production capacity [29], which corresponds to the results of Figure 2 and Figure 3. In addition, it was speculated that when the pH value increased to a certain threshold, the growth of microalgae would be inhibited and the synthesis of chlorophyll would be affected. Therefore, the logarithmic growth period of A2 was relatively short, and the growth rate was significantly inhibited in the later period. The chlorophyll content of A3 was not high, but the increase of pH value might prevent it from entering the logarithmic growth phase rapidly, and also had a negative impact on the growth rate. It could be seen that the biomass and chlorophyll growth of A1 were not affected by pH, so it was speculated that the pH tolerance of A1 was stronger than that of A2 and A3.

### 3.2.2 The nutrient removal of microalgal strains in sewage

Scenedesmus sp., Chlorella sorokiniana and Tetraselmis sp. were screened out in this experiment, they were widely used in the field of sewage treatment [30]. Figure 3 shows the nitrogen and phosphorus removal of three strains of microalgae in artificially configured sewage after sterilization. The removal rate of NH$_4^+$-N of A2 was significantly higher than that of A1 and A3, and NH$_4^+$-N was completely removed within 5 days. Considering the excellent removal efficiencies, it was speculated that part of NH$_4^+$-N was oxidized into NO$_2^-$ N and NO$_3^-$ N. There were many studies that had shown that cells in Chlorella Sorokiniana could only absorb NH$_4^+$-N under light conditions when nitrogen sources were sufficient. However, under the condition of limited nitrogen source, NH$_4^+$-N could be absorbed at the same rate in both light and dark environment [31], which might be the reason for the significant difference in NH$_4^+$-N removal efficiency between A2 and A1 with A3 when the concentration of NH$_4^+$-N in sewage reduced from 20 mg/L to 14.5 mg/L in
1.5 h from the beginning. It was noteworthy that the NO$_2^-$ N concentration of A2 was basically stable without significant fluctuation, which might be due to the same rate of oxygen production and respiration of A2. At the same time, the TIN removal rates of A1, A2 and A3 were 91.71%, 96.27% and 95.74%, respectively, showing excellent removal efficiency. However, a small amount of TIN was not removed, which also indicated that part of nitrogen compounds in sewage could not be absorbed by microalgae, and the metabolism of microalgae needed a large number of nitrogen sources, including polypeptides, proteins, enzymes, chlorophyll, energy transfer molecules and genetic material [32]. Previous studies had found that more than 80% TIN removal rate could be achieved by using Chlorella or Cyanobacteria in municipal wastewater treatment [30], indicating that microalgae might have a great contribution to the removal of TIN in the MBGS system. Microalgae could utilize phosphate in a variety of ways. The main removal mechanisms of phosphate in microalgae include I) absorption of phosphate into RNA or phospholipids by metabolism; II) Microalgal cells crystallize through the precipitation of calcium phosphate [33,34]. The precipitation of phosphorus is related to the form of phosphorus and metal ions in sewage. When pH > 7, phosphorus mainly existed in the form of HPO$_4^{2-}$ and PO$_4^{3-}$ and tended to be precipitates with metal ions. In the first 12 hours after the addition of microalgae, the pH value was low, so it was speculated that the large reduction of TP at this stage came from the excessive uptake of phosphate by microalgae cells, and more than 90% of phosphate was completely removed in the first 3 days. When the pH value increased to 10, phosphate might be removed by reacting with metal ions to form precipitates. In addition, the rebound of phosphate concentration was found in A3 (Tetraselmis sp.) during the reaction period, which might be attributed to the decomposition of microalgal cells due to insufficient carbon sources in this system [35].

### 3.3 The effects of exogenous N-acylhomoserine lactones on the microalgal-bacterial granular consortia

#### 3.3.1 The species of N-acylhomoserine lactones

After all the microalgae in shakers were successfully coupled with AGS to form MBGS and the effluent qualities were stable, the variation of pollutant concentration over time within 24 h was shown in Figure 4. It could be seen from Figure 4a that NH$_4^+$-N concentration decreased rapidly within 30 min after the reaction started, which might be caused by microalgae adsorption. From 30 min to 1.5 h, the concentration of
NH$_4^+$-N showed a rising trend, it was speculated that the microalgae adsorption reached saturation, the oxygen concentration was low, and the nitrification didn’t achieve completely, so NH$_4^+$-N in the cell was released. With the oxygen production from microalgal photosynthesis, NH$_4^+$-N was gradually removed. The decreasing removal rate of NH$_4^+$-N in the later stage might be due to the fierce competition between AOB and NOB for electrons and O$_2$. In addition, it was found that the effluent NH$_4^+$-N in MBGS consortia supplemented with 3-oxo-C12-HSL was only 0.49 mg/L after 12 h, which was significantly lower than that of the blank control group and the experimental group supplemented with C6-HSL. Besides, the fastest NH$_4^+$-N removal efficiencies of 3-oxo-C12-HSL indicated that it had a certain promoting effect on nitrification. Li found that exogenous addition of short chain AHLs without β-position substituents (such as C6-HSL) could improve the NH$_4^+$-N degradation rate in granular sludge system compared with the long chain AHLs containing β-position substituents (such as 3-oxo-12-HSL) [36], which was contrary to the results of this experiment. It was speculated that the addition of 3-oxo-C12-HSL in this experiment might promoted the reproduction of microalgae and thus strengthened the adsorption capacities of microalgae on NH$_4^+$-N.

**Figure 4.** The nutrient removal after adding signal molecules.
Many studies had shown that adding exogenous AHLs would change the activities of enzyme participated in nitrification and denitrification, then the abundances of bacteria related to the nitrogen removal would also be affected [37]. It was speculated that adding long chain AHLs containing β-position substituents would benefit for the express of gene encoding the enzyme related to nitrification in this microalgal-bacterial consortia.

Figure 4b shows the removal of TIN after adding different species of AHLs. As can be seen from the figure, the concentration of TIN in the effluent adding AHLs in the first 12 hours (S1:6.68 mg/L, S2:5.76 mg/L) was significantly lower than that of the blank control (7.72 mg/L). However, the effluent TIN content of the two groups adding AHLs started to increase after 12 h, and the highest removal rate was 47.18% in the MBGS system adding C6-HSL, which was slightly higher than that in the blank control (43.48%). TIN in effluent mainly came from the accumulation of NO$_3^-$ N and NO$_2^-$ N, and their variation was shown in Figure 4C-D. It could be seen that the accumulation of NO$_2^-$ N and NO$_3^-$ N in the experimental group adding AHLs was smaller than that in the blank control group. The oxygen concentration was low within 4 h from the beginning, because the NOB half-velocity constant for dissolved oxygen (1.2–1.5 mg/L) was significantly higher than AOB (0.2–0.4 mg/L). NOB was more dependent on O$_2$, leading to the continuous accumulation of NO$_2^-$ N at this stage. As oxygen concentration and NOB activity increased later, the concentration of NO$_2^-$ N gradually decreased, while NO$_3^-$ N gradually increased. In the C6-HSL experimental group, the NO$_3^-$ N accumulation kept at the lowest level, which was 6.63 mg/L after 24 h. It was significantly lower than that of blank control group (7.29 mg/L) and 3-oxo-C12-HSL experimental group (8.15 mg/L). Many studies had shown that AHL-mediated quorum-sensing improved the removal rate of pollutants by regulating the activity of related enzymes and genes [38], and it also required AHL synthase proteins to create AHL signals and regulatory proteins, which were encoded by LuxI and LuxR, to respond to the actual concentration of AHL signals [39]. According to the results of this experiment, it could be speculated that the addition of C6-HSL could promote the abundance of LuxI and LuxR genes, then increasing the protein production encoding by those genes, which contributing to the improvement of the nitrate reductase (Nar) activity. The NO$_3^-$ N accumulation in the 3-oxo-C12-HSL experimental group was less than that in the blank control group before 12 h, but the former increased rapidly and exceed the blank group at later. It could be seen that adding 3-oxo-C12-HSL could inhibit the genes’ expression encoding related enzyme.

It could be seen from Figure 4E that the addition of AHLs had a certain promoting effect on COD removal. The removal rates of S1 and S2 were 82.26% and 81.95% respectively, which were higher than the blank control group (77.24%). Figure 4F showed that the addition of AHLs could improve the removal rate of TP, and its removal rate from C6-HSL was 94.88%, while S0 and S2 were 83.27% and 87.48%, respectively. In the first 3 hours, TP concentration showed an upward trend, which was speculated to be caused by the low oxygen concentration in the shaker, leading to the release of phosphorus. In the later period, when oxygen concentration increased, phosphorus uptake occurred. It had been reported that the addition of exogenous AHLs could significantly increase the phosphorus interception of biofilms and greatly improved the abundance of PAOs [40], and the promotion would be enhanced by adding major AHLs contained in biofilms. This result indicated that AHLs-based quorum sensing might regulate the microbial community behavior related to phosphate removal.

Figure 5a showed the secretion of EPS after the addition of different species of AHLs in the MBGS systems. A0 came from the inoculated AGS. It could be seen that the addition of AHLs could increase the EPS content of MBGS, and the EPS content of MBGS was also higher than that of AGS (31.05 mg/g-VSS). The EPS contents of S0, S1 and S2 were 37.87, 47.26 and 79.02 mg/g-VSS, respectively. Quorum sensing based on AHLs induced the production of PN which help to improve the Chlorophyta cell aggregation ability [16].

![Figure 5](image_url) **Figure 5.** The EPS variation of MBGS consortia after adding different species (A) and concentration (B) of signal molecules.
Bacteria could also secret AHLs, and this kind of signal transduction was one of the typical modes of interactions between microalgae and bacteria in this system. Moreover, the AHLs secreted by symbiotic bacteria would make great significance to the growth and biomass accumulation of microalgae. At the same time, it could be found that the PS/PN value in MBGS was lower than that in AGS. Many researchers have reported that PN played an important role in cationic binding in water environment, and reduced particle surface charge to form a bridge and promoted cell agglomeration [41]. Therefore, lower PS/PN value indicated that the structure of MBGS was more stable. After adding AHLs, the content of EPS increased significantly, and 3-oxo-C12-HSL promoted the content of EPS significantly (p < 0.001), which was mainly manifested in the increase of PS (S0: 34.73; S1: 43.14; S2: 72.68 mg/g-VSS). Lv pointed out that exogenous AHLs could increase EPS contents and increased organic matter removal rate and metabolic capacity [42], but too much EPS contents was not conducive to the transfer and permeability of substances in the matrix, especially in granular sludge system [43]. At the same time, PS/PN (10.48) in S1 was lower than S2 (11.48), which might be one of the reasons for the higher nitrogen and phosphorus removal efficiency in S1 than S2.

3.3.2 The concentration of N-acylhomoserine lactones

In order to further clarify the mechanism of different concentrations of C6-HSL on MBGS system and select the best adding concentration of exogenous C6-HSL, after the mature MBGS had been formed, the variation of pollutant concentration within 24 h was shown in Figure 6. It could be seen from Figure 6A that the concentration of NH$_4^+$-N showed a trend of increasing followed by decreasing, which was mainly due to the gradual increase of oxygen concentration under the dual influence of microalgae photosynthesis and continuous shaking shock, which enhanced AOB's activities after 2 h, leading to the increase of ammonia oxidation rate. The MBGS with AHLs' concentration of 5 × 10$^{-9}$ mol/L had a higher nitration rate than the blank control, but with the increasing of C6-HSL concentration, its removal efficiency gradually decreased, and the NH$_4^+$-N removal rate of C0-4 was 96.15% 96.47%, 95.98% and 94.45%, respectively. Figure 6B shows that the effluent TIN concentrations of C0-4 were 7.89, 6.70, 7.23 and 8.95 mg/L at last, respectively. The results indicated that C6-HSL within a certain range could promote the nitrogen removal efficiency of MBGS, but high concentration of it could inhibit the advantages. It was speculated that high concentration of external AHLs triggered the quorum quenching (QQ) [44], which broke the balance of microalgal-bacterial symbiosis and led to fierce competition between bacteria and microalgae, ultimately leading to the reduction of nitrogen removal rate. Due to the low concentration of NH$_4^+$-N in the effluent, TIN in the effluent mainly came from the accumulation of nitrite and nitrate. Figure 6C demonstrated that NO$_2^-$-N accumulation was less than 0.2 mg/L, so the main form of effluent TIN is NO$_3^-$N. In C0-4, effluent NO$_3^-$-N concentrations were 7.29, 6.13, 6.54 and 8.01 mg/L, respectively. This result indicated that adding exogenous C6-HSL could improve the denitrification efficiency, but when the concentration exceeded the threshold value, the denitrification efficiencies of MBGS system would be inhibited. DO gradient existed in MBGS from external to internal, so there were all kinds of organisms include the nitrifying and denitrifying bacteria found in different parts of the granules. It was reported that exogenous AHLs attributed to the abundance of mixotrophic denitrifying bacteria participating in QS, some of which played an important role in nitrite oxidation and reduction [45,46]. Some studies have reported that adding 2µmol/L AHLs into the AGS system could improve TIN removal rate, while 50–500 nmol/L AHLs would inhibit the growth of denitrifying bacteria [47,48], which was inconsistent with the results in this experiment that low concentration of C6-HSL could promote but high concentration of C6-HSL would inhibit, so the effect of exogenous AHLs concentration on biological treatment was worthy of further analysis and research.

Figure 6E-F showed the removal profile of COD and TP by microalgae-bacteria consortia with different concentrations of C6-HSL in a typical cycle. It could be found that exogenous addition of C6-HSL with different concentrations had a great influence on COD removal. When the concentration was 5 × 10$^{-9}$ mol/L, the best removal effect was 83.20%. However, when the concentration of C6-HSL was increasing, the COD removal rate decreased to 75.79% and 70.64%, which were lower than that of blank control (77.24%). It had been reported that exogenous AHLs could regulate microbial behavior and activity through QS [49], and it was speculated that the high concentration of C6-HSL in the MBGS consortia would inhibit the propagation of GAOs and reduce its biological activity. On the other hand, all kinds of concentrations of C6-HSL in this experiments could improve TP removal rate, among which higher concentration of it had the most significant promoting effect. The effluent TP concentration was only 0.17 mg/L, while the effluent TP concentration of C0, C1 and C2 was 0.39, 0.19 and 0.23 mg/L, respectively. Under the influence of AHLs-based QS, microalgae and bacteria in the MBGS consortia achieved a mutualistic relationship.

Figure 5b showed the secretion of EPS after the addition of different concentrations of C6-HSL in microalgal-bacteria consortia. With the increase of exogenous C6-HSL concentration, EPS content increased initially, then decreased, and EPS content in C0-4 was
37.87, 48.75, 57.70 and 52.95 mg/g-VSS, respectively. In general, adding AHLs could improve the secretion of EPS in MBGS. Meanwhile, the results showed that high concentration of C6-HSL had a great influence on PS/PN value. The higher the concentrations of AHLs were, the more obviously the PS/PN value decreased, and their PS/PN values were 11.04, 10.81, 8.98 and 7.08 respectively. PN was mainly hydrophobic amino acid, which could combine with polyvalent cation electrostatic to affect the hydrophobicity of microbial surface [50,51]. In addition, there was a significant positive correlation between PN and particle integrity coefficient [52], so higher PN concentration contributed to maintaining the stability of particle structure. The results showed that MBGS had the highest hydrophobicity when the concentration of exogenous C6-HSL was $7.5 \times 10^{-5}$ mol/L.

4. Conclusion

Applying artificial illumination induced the transformation from AGS to MBGS, and MBGS showed better settling abilities and denitrification efficiencies. The dominant microalgal strain screened from this mature MBGS systems was Chlorella sorokiniana. Adding exogenous C6-HSL into the MBGS consortia which was formed by combining mature AGS and Chlorella sorokiniana showed the best TIN (47.18%) and TP removal rate (94.88%). Besides, the EPS content secreted by bacteria was regulated by quorum sensing based on
AHLs. When the concentration of exogenous C6-HSL was $5 \times 10^{-9}$ mol/L, the MBGS system achieved the best nutrient removal performances.

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**Ethics declarations**

Competing interests

The authors declare that they have no competing interests

Ethics approval and consent to participate

It is not applicable to this manuscript.

Consent for publication

It is not applicable to this manuscript.

**Availability of data and materials**

All data and materials generated or analyzed during this study are included in this manuscript.

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