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Application of aerobic granules-continuous flow reactor for saline wastewater treatment: Granular stability, lipid production and symbiotic relationship between bacteria and algae

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Abstract

In this study a continuous flow reactor (CFR) was employed to compare the feasibility of bacterial aerobic granular sludge (AGS-CFR) and algal-bacterial granular sludge (ABGS-CFR) for treating 1-4% saline wastewater. High salinity was found to enhance algae growth in ABGS-CFR, which exhibited slightly higher total nitrogen and phosphorus removal efficiencies at 1-3% salinity. ABGS-CFR maintained good granular stability at 1-4% salinity, while AGS-CFR gradually disintegrated at 4% salinity with 39.3% less accumulation of alginate-like exopolysaccharides in the extracellular polymeric substances. Indole-3-acetic acid (IAA) and superoxide dismutase (SOD) analysis suggested that bacteria and algae (*Nitzschia*) in ABGS-CFR formed a good symbiotic relationship under high salinity conditions, achieving rapid algae growth and 2 times lipid production. High salinity was conducive to enriching *Halomonas* and *Nitzschia* but unfavorable for *Nitrosomonas* and *Flavobacterium*. Results from this study could provide useful information on interactions between bacteria and algae in ABGS-CFR for its future practical application.

Keywords: Saline wastewater; Algal-bacterial granules; Aerobic granular sludge; Nutrients removal; Lipid production

1. Introduction

Saline wastewaters are produced from various industrial processes like chemical, pharmaceutical, petroleum refining, textile dyeing and food processing (Wang et al., 2015; Lu et al., 2019). Physical and/or chemical processes are often adopted to treat saline wastewaters, but these processes are costly and may generate other hazardous secondary pollutants (Lefebvre and Moletta, 2006; Tan et al., 2019). Biological processes can satisfactorily compete against physical and chemical processes with good treatment performance and relatively low energy consumption and low investment/operation cost (Tan et al., 2019). Thus, biological processes have been successfully applied for saline wastewaters treatment all over the world.

Aerobic granular sludge (AGS) as a novel biotechnology has been widely studied and applied for industrial saline wastewater treatment (Wang et al., 2015; Li et al., 2017). Compared with conventional activated sludge, the dense and strong microbial aggregate structure of AGS not only enables them to tolerate high salinity, but also effectively alleviates the bulking problem of activated sludge. Recently, several authors reported the formation of algal-bacterial granular sludge (ABGS) through self-aggregation process in the sequencing batch reactor (SBR) under natural or artificial sunlight irradiation (Huang et al., 2015; Liu et al., 2017; He et al., 2018). It has been proven that algae in the granules could utilize the nutrients in wastewater (e.g. nitrogen and phosphorus) to synthesize bioactive compounds like lipids, proteins, and carbohydrates (main components of algae cell), leading to higher pollutant removal efficiencies and the production of value-added biomass products (Liu et al.,

2018; Zhang et al., 2018a; Meng et al., 2019b). Moreover, some algae species are claimed to be feasible to treat saline wastewater (0.1-5% salinity), and the lipid production and composition in algae may be influenced by salinity (Ishika et al., 2017). The lipid content in *Nitzschia* was detected to increase from 9.9% to 27.2% when the salinity was increased from 0% to 3% (Cheng et al., 2014). Up to now, however, the interactions between bacteria and algae are still unclear in this novel symbiotic granular system, especially under high salinity conditions, which are crucial for the management of saline wastewater treatment by using ABGS technology.

On the other hand, up to most recently, almost all the studies for saline wastewater treatment by AGS were carried out in SBR, the most successful cultivation and operation systems for AGS. In general, SBR is suitable for small-scale wastewater treatment. By contrast, continuous flow reactors (CFRs), the most popular and widely applied operation process in engineering, are more advantageous for pilot- or industrial-scale application due to their low installation costs, easy maintenance and control, and efficient equipment utilization (Zou et al., 2018). However, AGS is hard to form and also unstable in conventional CFR due to its lack of some important granulation factors including alternation of feast-famine period and hydraulic selection pressure (Ahmad et al., 2017). To promote the development of AGS technology in CFR, some new aerobic granular sludge-continuous flow reactors (AGS-CFRs) have been designed and applied for wastewater treatment in recent years (Ahmad et al., 2017; Zou et al., 2018; Ahmad et al., 2019). From an engineering viewpoint, the development of stable and efficient AGS-CFRs is still demanding to

tackle saline wastewater treatment. Furthermore, there are still significant gaps in the knowledge of the performance of AGS-CFR and ABGS-CFR during the treatment of saline wastewater.

This study aimed at studying the possibility of treating saline wastewater by cultivating AGS and ABGS in two identical CFRs. It was also intended to explore the interaction and symbiosis between bacteria and algae in the granules by monitoring indole-3-acetic acid (IAA) and superoxide dismutase (SOD) under the tested salinity conditions (1-4%). The physicochemical characteristics (stability and lipid production) and nutrients removal performance of the AGS-CFR and ABGS-CFR were monitored and compared. Biological community structures (prokaryote and eukaryote) of the biogranules were also comprehensively investigated in order to shed light on the mechanisms influenced by salinity. It is expected that results from this study will provide scientific data for the development of ABGS-CFR to effectively treat saline wastewater and produce value-added biomass in practice.

2. Materials and methods

2.1. Reactor design and operation

Fig. 1

Experiments were conducted in two identical lab-scale continuous flow reactors ($L \times W \times H = 30 \times 16 \times 50 \text{ cm}^3$) made of transparent acrylic plastic with an effective working volume of 20 L (Fig. 1 and Supplementary Information). The CFR was divided into three different zones to realize aeration, settling and sludge return, respectively. The settling zone ($5 \times 8 \times 50 \text{ cm}^3$) and sludge return zone ($5 \times 8 \times 50 \text{ cm}^3$)

were separated from the aeration zone by two fixed baffles ($L \times H = 8 \times 37 \text{ cm}^2$ and $L \times H = 8 \times 44 \text{ cm}^2$, respectively). An outlet port was set on the outer wall of the settling zone (about 42 cm high from the bottom) to keep the water level inside at 42 cm. A movable baffle ($L \times H = 8 \times 23 \text{ cm}^2$) was inserted in the middle of settling zone from the top of reactor in order to prevent air bubbles from disturbing sludge setting and to adjust the settling time of sludge. A 45-degree slope was arranged at the bottom of settling zone, which made it easier for the settled sludge to flow into the sludge return zone. A water hole ($L \times H = 5 \times 3 \text{ cm}^2$) was set on the bottom of the fixed baffle between the settling zone and the sludge return zone. In this study, a much higher aeration rate (1.9 cm/s) was supplied in the sludge return zone than that in the aeration zone (0.6 cm/s), in order to achieve: 1) returning of the settled sludge from the settling zone via the water hole, and 2) a strong air lifting force for the water circulation in the CFR.

The two identical CFRs were operated under similar conditions at room temperature ($23 \pm 2 \text{ }^\circ\text{C}$) with or without light illumination. The control reactor (R0) was operated under no light illumination throughout the experiment, while the test reactor, R1 was illuminated at $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$ for 12 h every day. 10 white LED tubes (Oppl, 7W, China) were evenly distributed on the two length sides of R1 at a distance of 8 cm from the outer wall. The inflow rate was controlled at 35 mL/min by a peristaltic pump, resulting in a hydraulic retention time (HRT) of about 9.5 h. During the aeration period, a DO concentration at around 7-9 mg/L was maintained by an air pump (AL-60SA, Alita, China) introducing the air from the bottom of the

reactor through 8 fine bubble diffusers. Six of them were evenly distributed in the aeration zone and the other 2 were placed in the sludge return zone. The total airflow rate for each CFR was controlled at 18 L/min, about 4.5 L/min in the sludge return zone and 13.5 L/min in the aeration zone, respectively.

2.2. Seed sludge and synthetic saline wastewater

Mature AGS harvested from the lab-scale SBR was employed as seed sludge. The seed AGS was cultivated according to the procedures described elsewhere (Meng et al., 2019b). After about 30 days' cultivation, mature AGS with a mean diameter of 0.38 mm were obtained and then inoculated into the CFRs. No external algae species were added into the two reactors. The initial mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations in the CFRs were determined to be 3.7 and 3.0 g/L, respectively. The initial MLVSS/MLSS ratio was 0.8 and the sludge volume index (SVI_{30}) was 15 mL/g. The synthetic saline wastewater used in this work consisted of 600 mg/L COD (50% of which was contributed by glucose and sodium acetate, respectively), 50 mg NH_4-N/L (NH_4Cl) and 10 mg PO_4-P/L (KH_2PO_4). Different concentrations of NaCl (10 g/L, 20 g/L, 30 g/L and 40 g/L) were added into the synthetic wastewater at different periods (0-50 d, 50-75 d, 75-100 d and 100-125 d) to gradually increase the salinity level (1-4% salinity). The wastewater pH was maintained at 7.0-8.2 by adding $NaHCO_3$. All chemicals (AR grade) were supplied by Aladdin Industrial Corporation Ltd., China.

2.3. Analytical methods

Dissolved organic carbon (DOC) in the samples was detected with a total organic

carbon (TOC) analyzer (Multi N/C 3100, Analytik Jena, Germany). Nitrate nitrogen ($\text{NO}_3\text{-N}$), nitrite nitrogen ($\text{NO}_2\text{-N}$), ammonia nitrogen ($\text{NH}_4\text{-N}$), phosphate ($\text{PO}_4\text{-P}$), ML(V)SS, and SVI_{30} were analyzed according to standard methods (APHA, 2012). Total inorganic nitrogen (TIN) was calculated as the sum of $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$. pH and dissolved oxygen (DO) in the reactors were monitored with a pH meter (AS-711, ASONE, Japan) and a DO meter (HQ40d, HACH, USA), respectively. Chlorophyll *a* content in the granules was determined according to the procedures described elsewhere (Zhang et al., 2018b). Formaldehyde-sodium hydroxide solution was used to extract extracellular polymeric substances (EPS) from the granules, in which proteins (PN), polysaccharides (PS) and alginate-like exopolysaccharides (ALE) were quantified according to a previous work (Meng et al., 2019a).

Integrity coefficient was used to indicate the stability of granular sludge using the method described in Ghangrekar et al. (2005), and a lower value of integrity coefficient denoted a better stability. Lipid content was determined according to Marella et al. (2019). The concentration of indole-3-acetic acid (IAA) in the effluents from CFRs was detected according to Sheikhian and Bina (2016). The activity of superoxide dismutase (SOD) of the granule was assessed by using a SOD ELISA kit (Meimian, Shanghai, China) after the sludge mixture being grinded in a glass homogenizer. The samples for biological community structure analysis were taken on day 50, 75, 100 and 125 day after the granules adapted to 1%, 2%, 3% and 4% salinity condition, respectively. The biological community structure was detected by

ALLWEGENE Inc. (Beijing, China) according to the method described by Meng et al. (2019b).

In addition, the granular morphology was characterized using an optical microscope (CX40, SUNNY, China) equipped with a digital camera (700D, CANON, Japan) and a scanning electron microscope (SEM, SS-550, Shimadzu, Japan). Size distribution analysis on the granules was performed with a laser diffraction particle size analyzer (Mastersizer 3000, Malvern, UK).

2.4. Calculation and statistical analysis

Sludge retention time (SRT) in this study was calculated using the following equation:

$$\text{SRT (day)} = \frac{\text{MLSS(g/L)} \times V_{\text{R(L)}}}{\text{SS}_{\text{E(g/L)}} \times V_{\text{E(L} \cdot \text{day)}}} \quad (1)$$

where MLSS is the sludge concentration in reactor, V_{R} is the reactor working volume, SS is the sludge concentration in the effluent, V_{E} is the volume of effluent discharged every day. No additional sludge was discharged daily.

All analyses were conducted in triplicate and the results are presented as mean \pm standard deviations in this work. The experimental results were also compared via one-way analysis of variance using Microsoft Office Excel 2010, and $p < 0.05$ denotes statistical significance.

3. Results and discussion

3.1. *Effect of salinity on the characteristics of granules*

Fig. 2

3.1.1. Effect of salinity on granular morphology and biomass growth

In this study, mature AGS was sampled from the SBR and used as seed sludge in the CFRs, which exhibited a light-yellow color and a smooth and compact structure. During the whole experiment period, the granules in R0 maintained this light-yellow color throughout. While after about 30 days' operation, yellow-brown granules appeared in R1 (under light illuminance, Supplementary Information). The optical microscopy and SEM images of granules demonstrated that lots of algae (like *Navicula/Nitzschia*) have covered the surface of granules in R1. This observation indicated that ABGS was successfully formed in the CFR (R1). After day 42, granules with a fluffy outer surface were observed in both CFRs, indicating the growth of filamentous bacteria. It was worth noting that the ABGS with a fluffy surface still had a clear granular structure in R1 during the whole experiment period. In contrast, the granules in R0 began to break up when the salinity was increased to 4% during day 101-125. This phenomenon suggested that 4% salinity might exert seriously negative effect on AGS stability in the CFR, while the growth of algae in the CFR could enhance ABGS stability. This was further studied in the following experiments.

MLSS increased from an initial value of 3.7 g/L to 4.4 g/L and 4.7 g/L on day 60 and then slightly decreased to 4.1 g/L and 4.5 g/L on day 100 in R0 and R1, respectively (Fig. 2a). The difference in MLSS between R0 and R1 was significant ($p = 0.003 < 0.05$). In addition, different increase trend in granular diameter was also observed in R0 and R1 (Fig. 2b). It was clear that higher biomass concentration and larger granular diameter were achieved in R1, probably attributable to the rapid

growth of algae in the CFR (Amin et al., 2015; Meng et al., 2019b). The slight decrease of biomass concentration during day 60-100 in both reactors might be contributed by the following two aspects. Firstly, the growth of some microorganisms in the granules might be inhibited with the increase of salinity (Wang et al., 2016; Li et al., 2018). Secondly, the appearance of filamentous bacteria may deteriorate sludge settleability as the SVI_{30} value of granules in both reactors increased from 15.7 mL/g on day 0 to 36.6 mL/g on day 100, leading to the wash-out of more biomass from the CFR (Supplementary Information). Meanwhile, the biomass concentration, settleability and diameter of the granules in R0 were found to significantly decrease during day 101-125, most probably due to the instability and disintegration of granules under 4% salinity condition.

3.1.2. Effect of salinity on chlorophyll and lipid synthesis

In order to quantify the amount of algae biomass in the granules, the content of chlorophyll *a* was monitored. Almost no chlorophyll *a* was measured in the granules of R0 due to the absence of light illuminance. In contrast, the chlorophyll *a* content in R1 increased steadily before day 40 (1% salinity), and stabilized at 1.1-1.2 mg/g-SS during 40-50. With the increase of salinity from 1% to 4%, the chlorophyll *a* content remarkably increased to 2.6 mg/g-SS (day 75, 2% salinity), 5.3 mg/g-SS (day 100, 3% salinity), and 10.1 mg/g-SS (day 125, 4% salinity), respectively. Obviously, the increase in salinity from 1% to 4% stimulated the growth of algae in the ABGS-CFR in this study. As reported, some algal species can adapt to a wide range of salinity and gain their highest biomass productivity under optimal saline conditions (Ishika et al.,

2017).

The lipid content and productivity were also monitored to demonstrate the influence of salinity on biosynthesis of lipid in the two CFRs (Fig. 2c), which is meaningful for the AGS and ABGS biotechnology application and the biomass recovery as high value-added feedstock when treating high saline wastewater. The lipid contents in the granules of R0 was maintained at about 39.5 mg/g-SS under 1-4% salinity condition, indicating that salinity had no obvious effect on lipid accumulation in the bacterial AGS. However, as for the ABGS, the lipid content gradually increased from 45.9 mg/g-SS to 80.0 mg/g-SS when the salinity was increased from 1% to 4%, 2 times that in AGS. In addition, the lipid productivity, calculated based on the CFR effective volume and operation time, reflected a similar variation trend with the lipid content. The increment of algae content in the ABGS might contribute to an increase of total lipid content in the granules. Restated, high salinity may increase the lipid content in some species of algae.

3.2. Effect of salinity on the stability and EPS of granules

Fig. 3

Fig. 3a shows the integrity coefficients of the granular sludge under the tested salinity conditions. The integrity coefficients of granules in R0 gradually increased to 0.13 by day 100 and then rapidly increased to 0.27 by day 125. The stability of granules in R1, on the other hand, slightly decreased with the increase of salinity, with a much lower integrity coefficient (about 0.1 on day 125) being observed. The better stability of ABGS in R1 was attributable to the growth of algae. In addition, the

increased salinity had a negative effect on the granular stability in both reactors, especially the granules in R0 which were noticed to gradually disintegrate at 4% salinity. Previous studies reported that high salinity could enhance the stability of granules in SBR (Li et al., 2017). The different observations were most probably attributable to the application of CFR in this study.

EPS as the backbone of granules plays a key role in biogranulation processes and maintenance of the granular strength (Zou et al., 2018). Proteins (PN) and polysaccharides (PS) are considered to be the main components of EPS (Cai et al., 2019). PN was found to be the dominant component of EPS in the granules from R0 and R1, and its concentration maintained at approximately 275.1 mg/g-VSS throughout the whole experimental period. However, PS was detected to decrease from an initial value of 44.7 mg/g-VSS to 34.2 mg/g-VSS in the granules from R0 and to 39.8 mg/g-VSS in those from R1 on day 125, indicating that 1-4% salinity had a negative effect on the excretion of extracellular PS from the granules in both reactors. Therefore, it could be inferred that some components of PS may be related to the stability of granules.

Alginate-like exopolysaccharide (ALE), an important component of PS, is abundant in AGS and can enhance the aerobic granulation process and stability of granules (Lin et al., 2010; Li et al., 2017). In the ALE, two uronic acid residues of mannuronic acid (M) and guluronic acid (G) could be arranged in three kinds of homopolymeric blocks (MG, MM, and GG blocks). Among them, GG blocks provide gel-forming capacity while MM and MG blocks provide flexibility to the chains (Lin

et al., 2010). The ALE contents in the granules from R0 and R1 were determined to be 17.3 and 28.5 mg/g-VSS on day 125, corresponding to a decrease by 46.1% and 10.7%, respectively in comparison to those on day 50 (Fig. 3c). In particular, the contents of GG and MM blocks in the granules of R0 decreased by 86.5% and 88.9% on day 125, respectively. In contrast, the GG block and MM block contents in the granules of R1 only decreased by 21% and 40% on day 125, respectively. These results indicated that the gel-forming capacity and chain flexibility of granules in R0 were severely suppressed by the high salinity conditions. The decreased ALE contents in the granules should be partially responsible for the low granular stability in both reactors. Taking the above results together, the growth of algae might mitigate the negative effect of salinity on ALE excretion. The in-depth mechanisms involved need further study.

3.3. Overall performance on organic and nutrients removal

3.3.1. DOC and TP removals

Fig. 4

Fig. 4a presents the effluent DOC and TP from the two CFRs. The effluent DOC concentrations from the two reactors were less than 20 mg/L before day 100 (1-3% salinity), achieving stable DOC removal efficiencies of about 91-92%. This observation suggested that AGS- and ABGS-CFR could efficiently remove the organic matters from 1-3% saline wastewater. From day 100 to 125, the effluent DOC concentration from R1 was still stable, but that from R0 significantly increased to about 52.2 mg/L with a DOC removal efficiency decreased to around 78%.

On the other hand, the effluent TP from R0 and R1 were about 6.1-6.3 mg/L at 1% salinity condition. As there was no alternating anaerobic and aerobic mode operation in the CFR, the growth of microorganism/algae and chemical precipitation were mainly responsible for P removal in the two reactors (Zhang et al., 2018a; Rey-Martínez et al., 2019). When the salinity was increased from 1% to 3%, the TP removal efficiency in R1 gradually increased to about 47.5%, about 14% higher than that (41.8%) in R0. The differences in TP removal between R0 and R1 under 1-3% salinity were statistically significant ($p = 0.004 < 0.05$). Algae growth in the ABGS-CFR was enhanced with the increase of salinity, which could result in a better P uptake and removal efficiency in R1. In addition, when the salinity was increased from 1% to 3%, more biomass was washed out, resulting in a shorter sludge retention time (SRT), which could also improved TP removal in both reactors (Supplementary Information). However, from day 100 to 125, the TP removal efficiency in R0 rapidly declined to around 14.6%, which was similar with its DOC removal trend as shown in Fig.4a. In contrast, the TP removal efficiency in R1 was found to continuously increase to approximately 49.2% at 4% salinity condition. Clearly, both AGS- and ABGS-CFR could effectively remove TP from 1-3% saline wastewater, and the growth of algae in ABGS improved TP removal performance and system stability under high salinity conditions.

3.2.2. Nitrogen profiles at different salinities

The variations of N species in the effluents and TIN removal by R0 and R1 are shown in Fig. 4b and c. The granules both reactors exhibited excellent $\text{NH}_4\text{-N}$

removal capability under 1-3% salinity conditions, achieving > 99% of $\text{NH}_4\text{-N}$ removal from day 0 to day 100 (Fig. 4b). $\text{NO}_3\text{-N}$ was the dominant N species under 1-2% salinity conditions (Fig. 4c). Since day 75, $\text{NO}_2\text{-N}$ began to accumulate and its concentration increased to about 13.2 mg/L and 12.6 mg/L on day 100 in R0 and R1, respectively, suggesting that nitrification process (from $\text{NO}_2\text{-N}$ to $\text{NO}_3\text{-N}$) was inhibited at 3% salinity. When the salinity was further increased to 4%, the effluent $\text{NH}_4\text{-N}$ concentration increased to about 22.3 mg/L and 7.6 mg/L on day 125 in R0 and R1, respectively. The low biomass concentration in R0 might contribute to the higher effluent $\text{NH}_4\text{-N}$ concentration. Furthermore, at 4% salinity condition $\text{NH}_4\text{-N}$ and $\text{NO}_2\text{-N}$ were found to be the predominant N species in the effluents from both R0 and R1, and only a very small amount of $\text{NO}_3\text{-N}$ was detected from day 105 till the end of experiment, indicating that the nitrification process (from $\text{NH}_4\text{-N}$ to $\text{NO}_2\text{-N}$) in both reactors was suppressed to some extent while the nitrification process was almost completely inhibited at 4% salinity. These results were in agreement with the statement made by Bassin et al. (2011) and Pronk et al. (2014) who claimed that $\text{NH}_4\text{-N}$ and $\text{NO}_2\text{-N}$ removals were not affected at NaCl concentration up to 3.3% and 2.2%, respectively.

In addition, when the salinity was increased from 1% to 3%, the TIN removal efficiencies (Fig. 4b) gradually increased from 45.6% to 53.1% in R0 and to 55.6% in R1 ($p = 0.269 > 0.05$). It has been reported that denitrifying bacteria exist in AGS and denitrification plays an important role in N removal in the AGS system (Akker et al., 2015; Ahmad et al., 2017; Zhang et al., 2018a). Moreover, the inner part of ABGS has

anoxic/anaerobic zones (Meng et al., 2019b). Thus, it was inferred that the increasing diameter of granules in the two reactors (Fig. 2b) could create a larger anoxic/anaerobic zone, which further enhanced the denitrification and TIN removal in the two reactors. Moreover, the growth of algae can contribute to the slightly higher TIN removal efficiency in R1 (Cai et al., 2019). When the salinity was increased to 4% during day 100-125, the TIN removal efficiency in R1 was maintained at about 55.1%, while it was noticeably decreased to 44.9% in R0 on day 115. This result suggested that the denitrifying bacteria still possessed a high denitrifying activity when treating 4% saline wastewater. The disintegration of granules and the decreased denitrifying activity in R0 at high salinity might be responsible for the deterioration of TIN removal.

Restated, the AGS- and ABGS-CFR could effectively remove about 91.5% of DOC, 41.8-49.2% of TP and 45.6-55.6% of TIN from 1-3% saline wastewater, and the growth of algae in ABGS-CFR could not only slightly improve organics and nutrients removal but also help to maintain system stability under high salinity condition.

3.4. Effect of salinity on IAA and SOD contents

Fig. 5

The indole-3-acetic acid (IAA) is a widely occurring phytohormone in plants and algae, which plays a crucial role in cell differentiation and information exchange between plants/algae and the environment (Sheikhian and Bina, 2016; Lépinay et al., 2018). It has been also reported that the IAA in bacteria has an important function for

protecting the cells from high salts environments (Noori et al., 2018). Fig. 5a shows the variation of IAA content in the effluents from R0 and R1 under different salinities. The IAA content in the effluent from R0 was determined to be 5.4 $\mu\text{g/L}$ on day 50, which increased to 8.2 $\mu\text{g/L}$ on day 125. Apparently, high salinity stimulated bacteria in the granules of R0 to secrete more IAA to protect themselves. While the IAA content in the effluent from R1 fluctuated between 2.3 $\mu\text{g/L}$ and 3.7 $\mu\text{g/L}$ during the whole experiment period, which was much lower than that from R0 ($p = 0.02 < 0.05$). The synthesized IAA by bacteria might be immediately metabolized by the symbiotic algae (Lépinay et al., 2018). Salama et al. (2014) found that phytohormones addition can effectively enhance algae growth and lipid accumulation. It was thus reasonable to infer that high salinity also promoted the IAA secretion by bacteria in the ABGS, and the synthesized IAA was quickly used by algae and promoted algae growth and lipid accumulation to some extent.

Under salinity stresses, large amounts of reactive oxygen species (ROS) produced by organisms may destroy cell membranes and macromolecules (Zeinali et al., 2015; Klein et al., 2018). Superoxide dismutase (SOD) is an important antioxidant enzyme in microorganisms, which can ameliorate the damage by ROS (Zeinali et al., 2015). Fig. 5b depicts the variation of SOD in the granules from the two reactors. The SOD activity in the granules of R0 remained stable at around 11.9-13.2 U/mg-protein, while it significantly increased from 14.6 U/mg-protein to 18.2 U/mg-protein in R1 when the salinity increased from 1% to 4% ($p = 0.003 < 0.05$). The growth of algae in granules might improve the activity of SOD, which was in agreement with Singh et al.

(2018) who stated that some algae species could improve SOD activity when the salinity was increased to a certain level. The high SOD activity in ABGS could counteract the negative impact of ROS (due to the salinity stress) on bacteria, which favored the algae and bacteria to form a good and stable symbiotic system.

Based on the above results, it can be concluded that high salinity encouraged bacteria to secrete more IAA in the ABGS system, which could be metabolized by algae and then improve algae growth and lipid production. The algae and bacteria can form a good ABGS symbiotic system under the tested salinity conditions, which was attributable to the increase of total SOD activity and thus effective elimination of ROS. These aspects could partially contribute to the better overall performance and stability of ABGS under high salinity conditions.

3.5. Effects of salinity on biological community structure

Fig. 6

Fig. 6 shows the distribution of biological community structure (Prokaryote and Eukaryote) at the genus level. Phyla Betaproteobacteria, Gammaproteobacteria and Bacteroidetes were the predominant prokaryotic microbial communities in the granules from the two reactors (Fig. 6a). Among the identified genera, *Denitromonas* dominated the sludge samples from both reactors when the salinity $\leq 2\%$. But *Halomonas* from phylum Gammaproteobacteria gained predominance at 3-4% salinity from day 100 to day 125. *Denitromonas* was reported to play an important role in denitrifying activity (Miao et al., 2017). During day 1-100, the large amount of *Denitromonas* ($> 15\%$) indicated that the granules from both reactors had higher

denitrifying activities under 1-3% salinity condition. While the percentage of *Denitromonas* in the granules from R0 decreased to 6.6% at 4% salinity, most probably attributable to the disintegration of granules which destroyed the anoxic/anaerobic zone and inhibited the growth and activity of denitrifying bacteria. *Halomonas*, one kind of salt-tolerant bacterium, was enriched and predominant under 3-4% salinity condition in the two reactors. It has been reported that *Halomonas* is closely related with organics and N removal (Berendes et al., 1994). In contrast, the genera *Nitrosomonas* and *Azoarcus* from phylum Bacteroidetes, as nitrogen removal bacteria (Lee and Wong, 2014; Sathvika et al., 2019), were gradually inhibited with the increase of salinity. Especially, the genus *Nitrosomonas* was found to significantly decrease to 1.1% and 2.1% on day 125 in the granules from R0 and R1, respectively, implying that the growth of AOB was inhibited at 4% salinity. In addition, the genera *Flavobacterium* and *Aequorivita* from phylum Bacteroidetes could not adapt to high salinity stress either, and their relative abundances decreased with the increase of salinity in the two reactors. Some members of *Flavobacterium* could remove N and P from wastewater (Bernardet and Nakagawa, 2006). *Aequorivita* has been identified from seawater and its capacity for nutrient removal is still unclear (Liu et al., 2013).

The identified Eukaryote could be mainly categorized into four phyla: Protozoa, Bacillariophyta, Chlorophyta, and Cryptomycota (Fig. 6b). In the granules from R0, the genus *Zoothamnium* from phylum Protozoa dominated the eukaryotic microbial community before day 100 (1-3% salinity). It disappeared at 4% salinity and was replaced by *Pseudocohnilembus*. In the ABGS from R1, *Zoothamnium* also dominated

the Eukaryote at the initial stage, but it rapidly decreased with the significant enrichment of algae species *Nitzschia* from Bacillariophyta. In this study, *Nitzschia* was found to increase gradually and become the dominant species (71.9-78.9%) when salinity $\geq 3\%$. *Nitzschia* is a euryhaline algae and is widely distributed in estuaries, lagoons and ocean environment, which has competitive and growth advantages in high salinity environments (Bates et al., 2018). Besides, IAA synthesized by bacteria is also known to have a promotion effect on the growth of *Nitzschia* (Amin et al., 2015). Furthermore, an elevation of salinity from 0% to 3% was reported to have a great stimulation effect on lipid production by *Nitzschia* (Cheng et al., 2014). Thus, the fast growth of *Nitzschia* in ABGS could contribute to the enhanced nutrients (N, P) removal and lipid production performance under high salinity stress. Besides, small proportions of *Chlorella* and *Neodesmus* from Chlorophyta were also observed at 4% salinity. *Chlorella* has been widely applied for wastewater treatment, and the lipid content in *Chlorella* was enhanced at an elevated salinity from 0% to 3% (Zhang et al., 2018b).

Therefore, the present results indicated that the salinity increase from 1% to 4% could significantly influence the biological communities in the conventional bacterial AGS and the algal-bacterial AGS. Some specific bacteria and algae could be responsible for nutrients removal and lipid production performances in the newly developed continuous flow reactor which exhibited good potential for large-scale wastewater treatment. More specifically, *Nitzschia* might play a critical role in the enhanced lipid accumulation and nutrients removal in the algal-bacterial granules.

4. Conclusions

ABGS and AGS were successfully cultivated in CFR with DOC removal efficiencies of 91-92% at 1-3% salinity. Due to algae growth ABGS-CFR achieved slightly enhanced nutrients removal and granular stability with significant improvement on lipid synthesis under high salinity condition. The ALE content in AGS was quickly decreased when salinity was increased from 1% to 4%, leading to the disintegration of AGS at 4% salinity. Results also show that the functional bacteria and algae were enriched and maintained a good algal-bacterial symbiotic system in the ABGS-CFR. This work suggests that ABGS-CFR is promising for high saline wastewater treatment in practice.

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Appendix A. Supplementary data

E-supplementary data of this work can be found in online version of the paper.

References

1. Ahmad, J.S.M., Cai, W., Zhao, Z., Zhang, Z., Shimizu, K., Lei, Z., Lee, D.-J., 2017. Stability of algal-bacterial granules in continuous-flow reactors to treat varying strength domestic wastewater. *Bioresour. Technol.* 244, 225-233.
2. Ahmad, J.S.M., Zhao, Z., Zhang, Z., Shimizu, K., Utsumi, M., Lei, Z., Lee, D.-J., Tay, J.H., 2019. Algal-bacterial aerobic granule based continuous-flow reactor

- with effluent recirculation instead of air bubbling: stability and energy consumption analysis. *Bioresour. Technol. Rep.* 7, 100215.
3. Akker, B., Reid, K., Middlemiss, K., Krampe, J., 2015. Evaluation of granular sludge for secondary treatment of saline municipal sewage. *J. Environ. Manage.* 157, 139-145.
 4. Amin, S.A., Hmelo, L.R., van Tol, H.M., Durham, B.P., Carlson, L.T., Heal, K.R., Morales, R.L., Berthiaume, C.T., Parker, M.S., Djunaedi, B., Ingalls, A.E., Parsek, M.R., Moran, M.A., Armbrust, E.V., 2015. Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria. *Nature* 522, 98-114.
 5. APHA, 2012. *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, American Water Work Association and Water Environment Federation, Washington, D.C., USA.
 6. Bassin, J.P., Pronk, M., Muyzer, G., Kleerebezem, R., Dezotti, M., and van Loosdrecht, M.C.M., 2011. Effect of elevated salt concentrations on the aerobic granular sludge process: linking microbial activity with microbial community structure. *App. Environ. Microb.* 77(22), 7942-7953.
 7. Bates, S.S., Hubbard, K.A., Lundholm, N., Montresor, M., Leaw, C.P., 2018. *Pseudo-nitzschia*, *Nitzschia*, and domoic acid: new research since 2011. *Harmful Algae* 79, 3-43.
 8. Berendes, F., Goitschalk, G., Heine-dobbernack, E., Moore, E.R.B., Tindal, B.J., 1994. *Halomonas desiderata* sp. nov., a new alkaliphilic, halotolerant and

- denitrifying bacterium isolated from a municipal sewage works. System. Appl. Microbiol. 19, 158-167.
9. Bernardet, J.-F., Nakagawa, Y., 2006. An introduction to the family Flavobacteriaceae. Prokaryotes 7, 455–480.
 10. Cai, W., Zhao, Z., Li, D., Lei, Z., Zhang, Z., Lee, D.-J., 2019. Algae granulation for nutrients uptake and algae harvesting during wastewater treatment. Chemosphere. 214, 55-59.
 11. Cheng, J., Feng, J., Sun, J., Huang, Y., Zhou, J., Cen, K., 2014. Enhancing the lipid content of the diatom *Nitzschia sp.* by ⁶⁰Co-γ irradiation mutation and high-salinity domestication. Energy 78, 9-15.
 12. Ghangrekar, M.M., Asolekar, S.R., Joshi, S.G., 2005. Characteristics of sludge developed under different loading conditions during UASB reactor start-up and granulation. Water Res. 39, 1123-1133.
 13. He, Q., Chen, L., Zhang, S., Chen, R., Wang, H., Zhang, W., Song, J., 2018. Natural sunlight induced rapid formation of water-born algal-bacterial granules in an aerobic bacterial granular photo-sequencing batch reactor. J. Hazard. Mater. 359, 222-230.
 14. Huang, W., Li, B., Zhang, C., Zhang, Z., Lei, Z., Lu, B., Zhou, B., 2015. Effect of algae growth on aerobic granulation and nutrients removal from synthetic wastewater by using sequencing batch reactors. Bioresour. Technol. 179, 187-192.
 15. Ishika, T., Moheimani, N.R., Bahri, P.A., 2017. Sustainable saline microalgae

- co-cultivation for biofuel production: a critical review. *Renew. Sust. Energ. Rev.* 78, 356-368.
16. Klein, A., Hüselmann, L., Keyster, M., Ludidi, N., 2018. Exogenous nitric oxide limits salt-induced oxidative damage in maize by altering superoxide dismutase activity. *S. Afr. J. Bot.* 115, 44-49.
17. Lee, D.-J., Wong, B.-T., 2014. Denitrifying sulfide removal and nitrososulfide complex: *Azoarcus* sp. NSC3 and *Pseudomonas* sp. CRS1 mix. *Bioresour. Technol.* 166, 616-619.
18. Lefebvre, O., Moletta, R., 2006. Treatment of organic pollution in industrial saline wastewater: a literature review. *Water Res.* 40, 3671–3682.
19. Lépinay, A., Turpin, V., Mondeguer, F., Grandet-Marchant, Q., Capioux, H., Baron, R., Lebeau, T., 2018. First insight on interactions between bacteria and the marine diatom *Haslea ostrearia*: algal growth and metabolomic fingerprinting. *Algal Res.* 31, 395-405.
20. Lin, Y., Kreuk, M., Loosdrecht, M.C.M., Adin, A., 2010. Characterization of algininate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant. *Water Res.* 44, 3355-3364.
21. Liu, J.-J., Zhang, X.-Q., Pan, J., Sun, C., Zhang, Y., Li, C.-Q., Zhu, X.-F., Wu, M., 2013. *Aequorivita viscosa* sp. nov., isolated from an intertidal zone, and emended descriptions of *Aequorivita antarctica* and *Aequorivita capsosiphonis*. *Int. J. Syst. Evol. Microbiol.* 63, 3192-3196.
22. Liu, L., Fan, H., Liu, Y., Liu, C., Huang, X., 2017. Development of algae-bacteria

- granular consortia in photo-sequencing batch reactor. *Bioresour. Technol.* 232, 64-71.
23. Liu, L., Hong, Y., Ye, X., Wei, L., Liao, J., Huang, X., Liu, C., 2018. Biodiesel production from microbial granules in sequencing batch reactor. *Bioresour. Technol.* 249, 908–915.
24. Li, X., Luo, J., Guo, G., Mackey, H.R., Hao, T., Chen, G., 2017. Seawater-based wastewater accelerates development of aerobic granular sludge: a laboratory proof-of-concept. *Water Res.* 115, 210-219.
25. Li, X., Yuan, Y., Yuan, Y., Bi, Z., Liu, X., Huang, Y., Liu, H., Chen, C., Xu, S., 2018. Effects of salinity on the denitrification efficiency and community structure of a combined partial nitrification- anaerobic ammonium oxidation process. *Bioresour. Technol.* 249, 550-556.
26. Lu, H., Li, Y., Shan, X., Abbas, G., Zeng, Z., Kang, D., Wang, Y., Zheng, P., Zhang, M., 2019. A holistic analysis of ANAMMOX process in response to salinity: from adaptation to collapse. *Sep. Purif. Technol.* 215, 342-350.
27. Marella, T.K., Datta, A., Patil, M.D., Dixit, S., Tiwari, A., 2019. Biodiesel production through algal cultivation in urban wastewater using algal floway. *Bioresour. Technol.* 280, 222-228.
28. Meng, F., Liu, D., Pan, Y., Xi, L., Yang, D., Huang, W., 2019a. Enhanced amount and quality of alginate-like exopolysaccharides in aerobic granular sludge for the treatment of salty wastewater. *BioResources* 14 (1), 139-165.
29. Meng, F., Xi, L., Liu, D., Huang, W., Lei, Z., Zhang, Z., Huang, W., 2019b.

- Effects of light intensity on oxygen distribution, lipid production and biological community of algal-bacterial granules in photo-sequencing batch reactors. *Bioresour. Technol.* 272, 473–481.
30. Miao, Y., Wang, Z., Liao, R., Shi, P., Li, A., 2017. Assessment of phenol effect on microbial community structure and function in an anaerobic denitrifying process treating high concentration nitrate wastewater. *Chem. Eng. J.* 330, 757-763.
31. Noori, F., Etesami, H., Zarini, H.N., Khoshkholgh-Sima, N.A., Salekdeh, G.H., Alishahi, F., 2018. Mining alfalfa (*Medicago sativa* L.) nodules for salinity tolerant non-rhizobial bacteria to improve growth of alfalfa under salinity stress. *Ecotoxicol. Environ. Saf.* 162, 129-138.
32. Pronk, M., Bassin, J.P., de Kreuk, M.K., Kleerebezem, R., van Loosdrecht, M.C.M., 2014. Evaluating the main and side effects of high salinity on aerobic granular sludge. *Appl. Microbiol. Biotechnol.* 98, 1339-1348.
33. Rey-Martínez, N., Badia-Fabregat, M., Guisasola, A., Baeza, J.A., 2019. Glutamate as sole carbon source for enhanced biological phosphorus removal. *Sci. Total Environ.* 657, 1398-1408.
34. Salama, E.-S., Kabra, A.N., Ji, M.-K., Kim, J.R., Min, B., Jeon, B.-H., 2014. Enhancement of microalgae growth and fatty acid content under the influence of phytohormones. *Bioresour. Technol.* 172, 97-103.
35. Sathvika, T., Balaji, S., Chandra, M., Soni, A., Rajesh, V., Rajesh, N., 2019. A co-operative endeavor by nitrifying bacteria *Nitrosomonas* and *Zirconium* based

- metal organic framework to remove hexavalent chromium. Chem. Eng. J. 360, 879-889.
36. Sheikhan, L., Bina, S., 2016. Simultaneous extraction and HPLC determination of 3-indole butyric acid and 3-indole acetic acid in pea plant by using ionic liquid-modified silica as sorbent. J. Chromatogr. B 1009-1010, 34-43.
37. Singh, R., Upadhyay, A.K., Chandra, P., Singh, D.P., 2018. Sodium chloride incites reactive oxygen species in green algae *Chlorococcum humicola* and *Chlorella vulgaris*: implication on lipid synthesis, mineral nutrients and antioxidant system. Bioresour. Technol. 270, 489-497.
38. Tan, X., Acquah, I., Li, W., Tan, S., 2019. A critical review on saline wastewater treatment by membrane bioreactor (MBR) from a microbial perspective. Chemosphere 220, 1150-1162.
39. Wang, R., Zheng, P., Ding, A., Zhang, M., Ghulam, A., Yang, C., Zhao, H.-P., 2016. Effects of inorganic salts on denitrifying granular sludge: the acute toxicity and working mechanisms. Bioresour. Technol. 204, 65-70.
40. Wang, Z., Gao, M., She, Z., Wang, S., Jin, C., Zhao, Y., Yang, S., Guo, L., 2015. Effects of salinity on performance, extracellular polymeric substances and microbial community of an aerobic granular sequencing batch reactor. Sep. Purif. Technol. 144, 223-231.
41. Zeinali, F., Homaei, A., Kamrani, E., 2015. Sources of marine superoxide dismutases: characteristics and applications. Int. J. Biol. Macromol. 79, 627-637.
42. Zhang, B., Lens, P.N.L., Shi, W., Zhang, R., Zhang, Z., Guo, Y., Bao, X., Cui, F.,

- 2018a. Enhancement of aerobic granulation and nutrient removal by an algal-bacterial consortium in a lab-scale photobioreactor. *Chem. Eng. J.* 334, 2373-2382.
43. Zhang, L., Pei, H., Chen, S., Jiang, L., Hou, Q., Yang, Z., Yu, Z., 2018b. Salinity-induced cellular cross-talk in carbon partitioning reveals starch-to-lipid biosynthesis switching in low-starch freshwater algae. *Bioresour. Technol.* 250, 449-456.
44. Zou, J., Tao, Y., Li, J., Wu, S., Ni, Y., 2018. Cultivating aerobic granular sludge in a developed continuous-flow reactor with two-zone sedimentation tank treating real and low-strength wastewater. *Bioresour. Technol.* 247, 776-783.

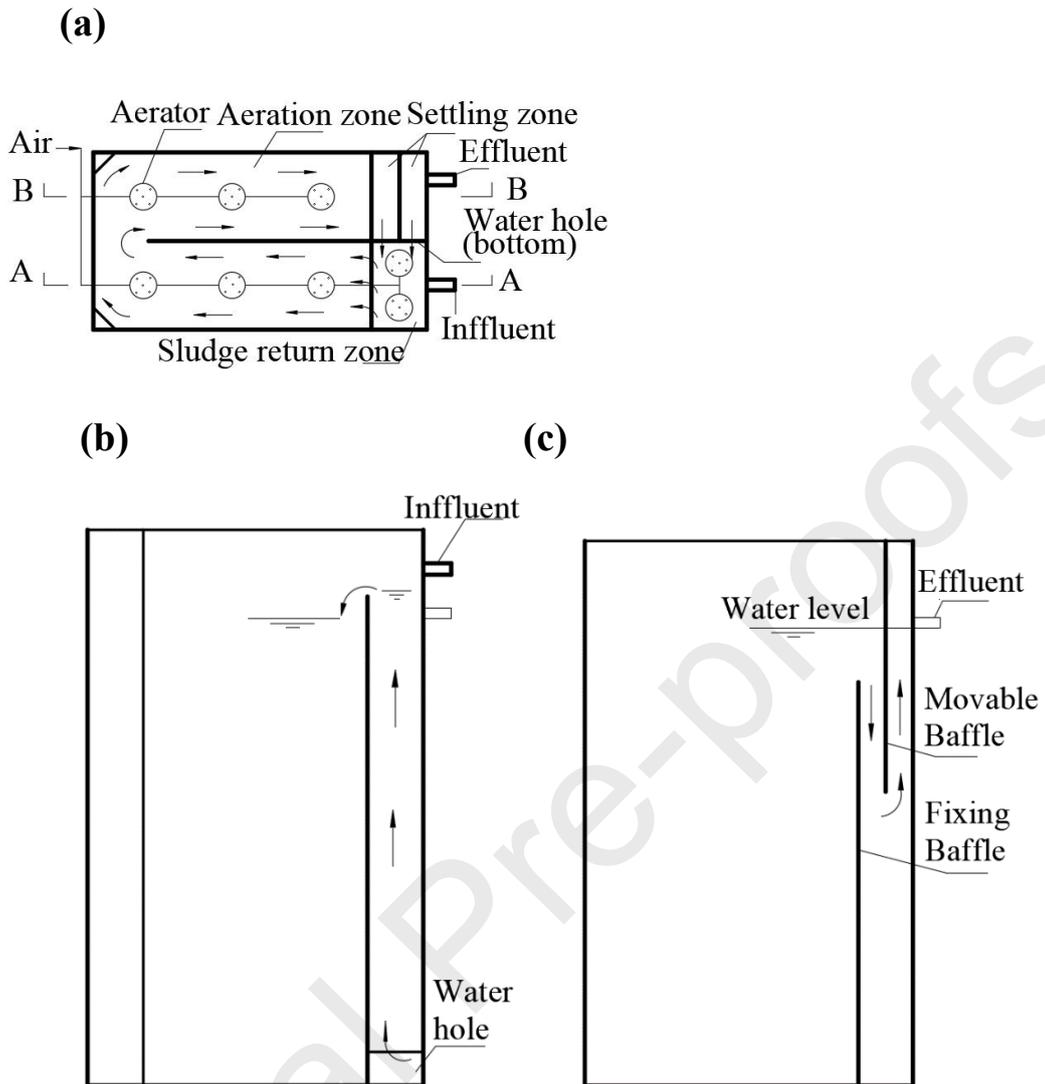


Fig. 1. Schematic diagrams of the continuous-flow reactor. (a) Plan view of the reactor, (b) Cutaway view of AA of the reactor, and (c) Cutaway view of BB of the reactor.

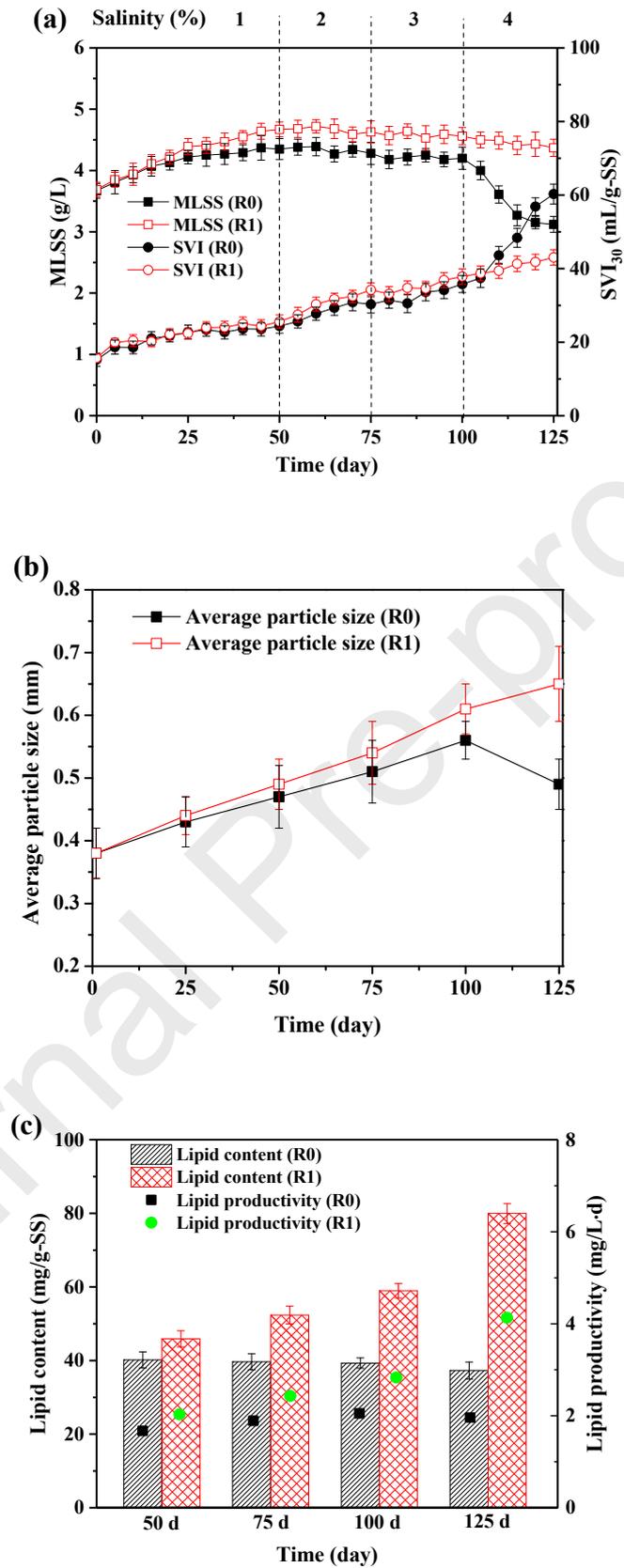


Fig. 2. Changes in MLSS, SVI₃₀ (a), average particle size (b), and lipid content and productivity (c) in the two CFRs during the 125 days' operation.

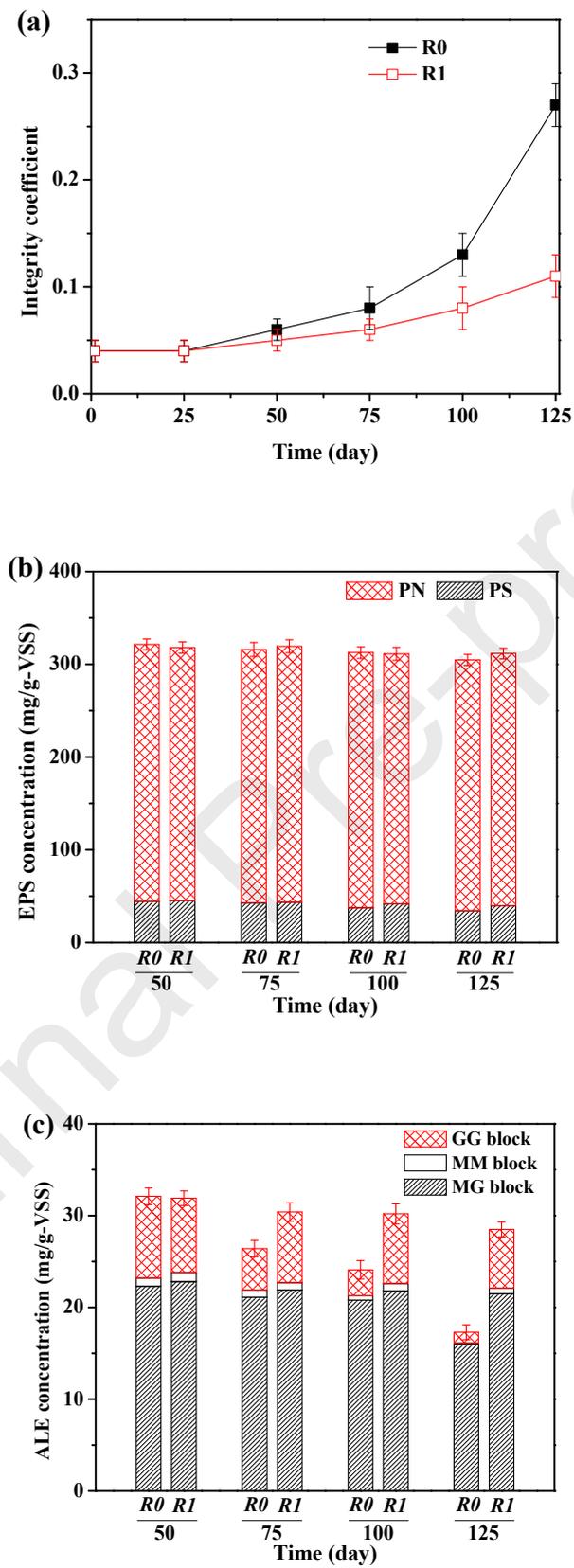


Fig. 3. Changes in integrity coefficient (a), EPS (b), and ALE content (c) in the two CFRs during the 125 days' operation.

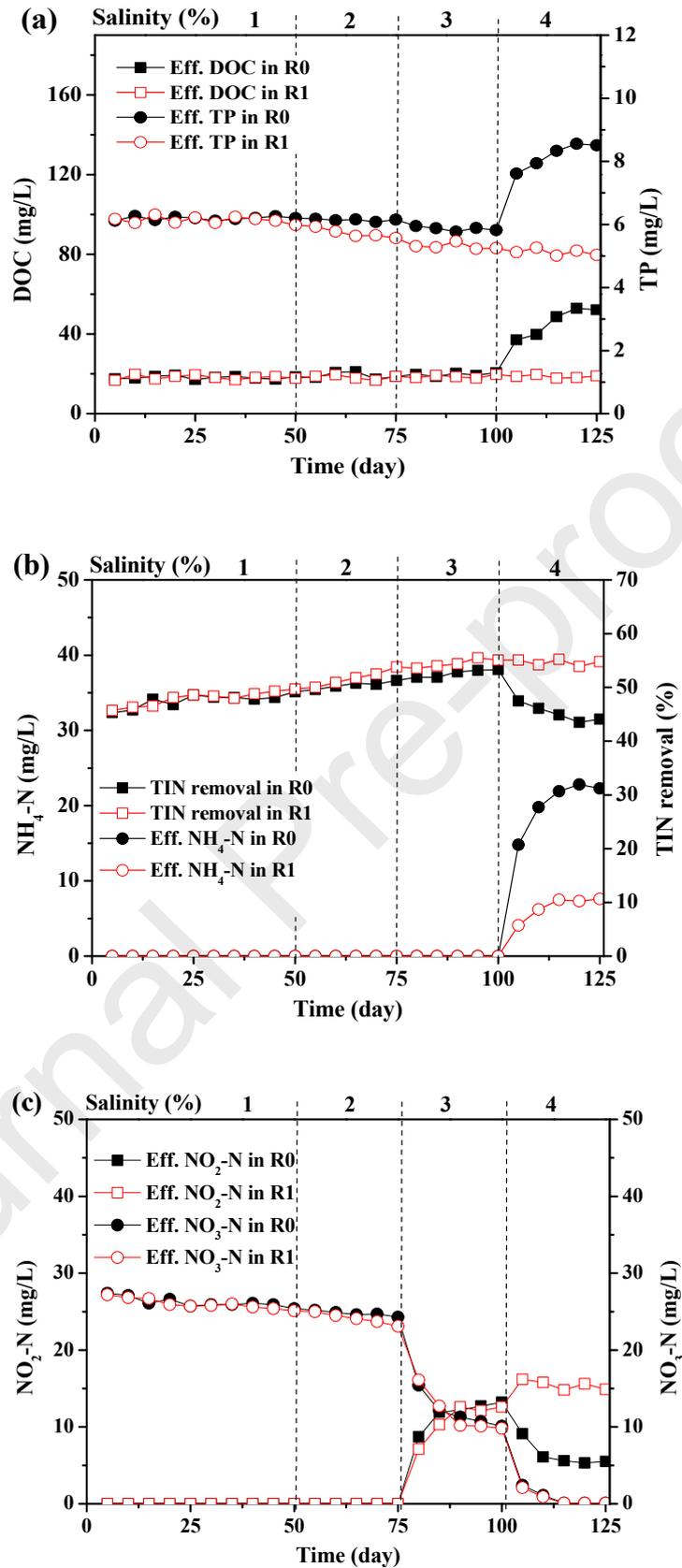


Fig. 4. Variations of effluent DOC and TP (a), $\text{NH}_4\text{-N}$ and TIN removal (b), $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ (c) from the two CFRs during the 125 days' operation.

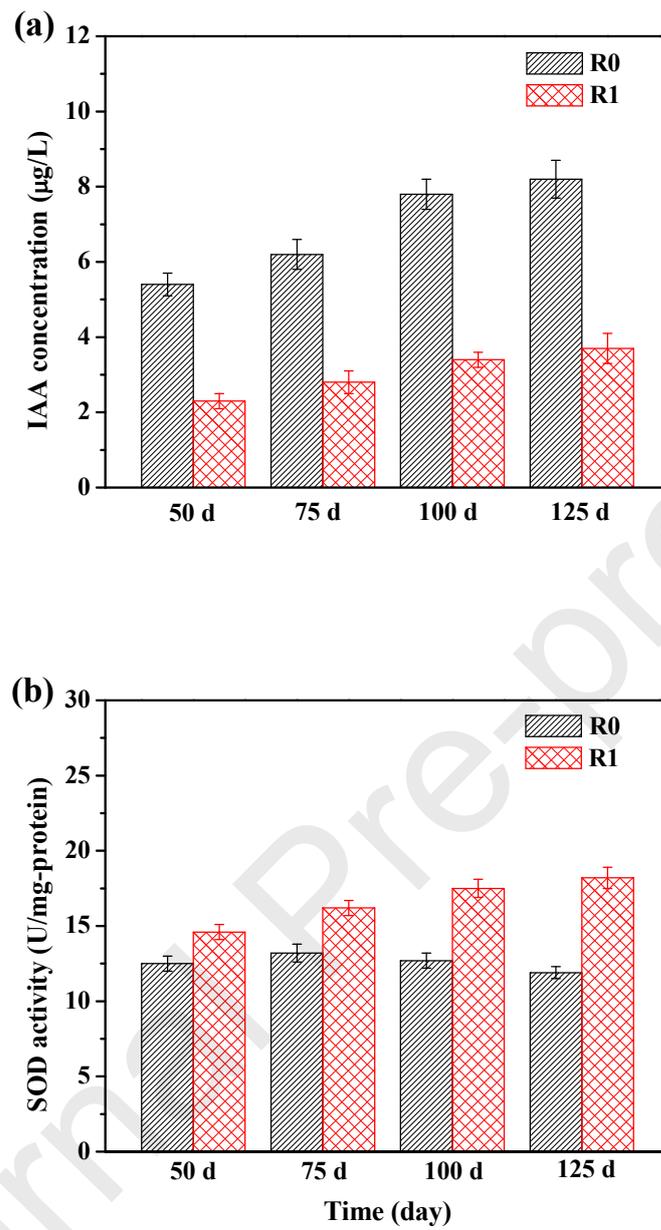


Fig. 5. Changes in IAA concentration (a), and SOD activity (b) in the two CFRs during the 125 days' operation.

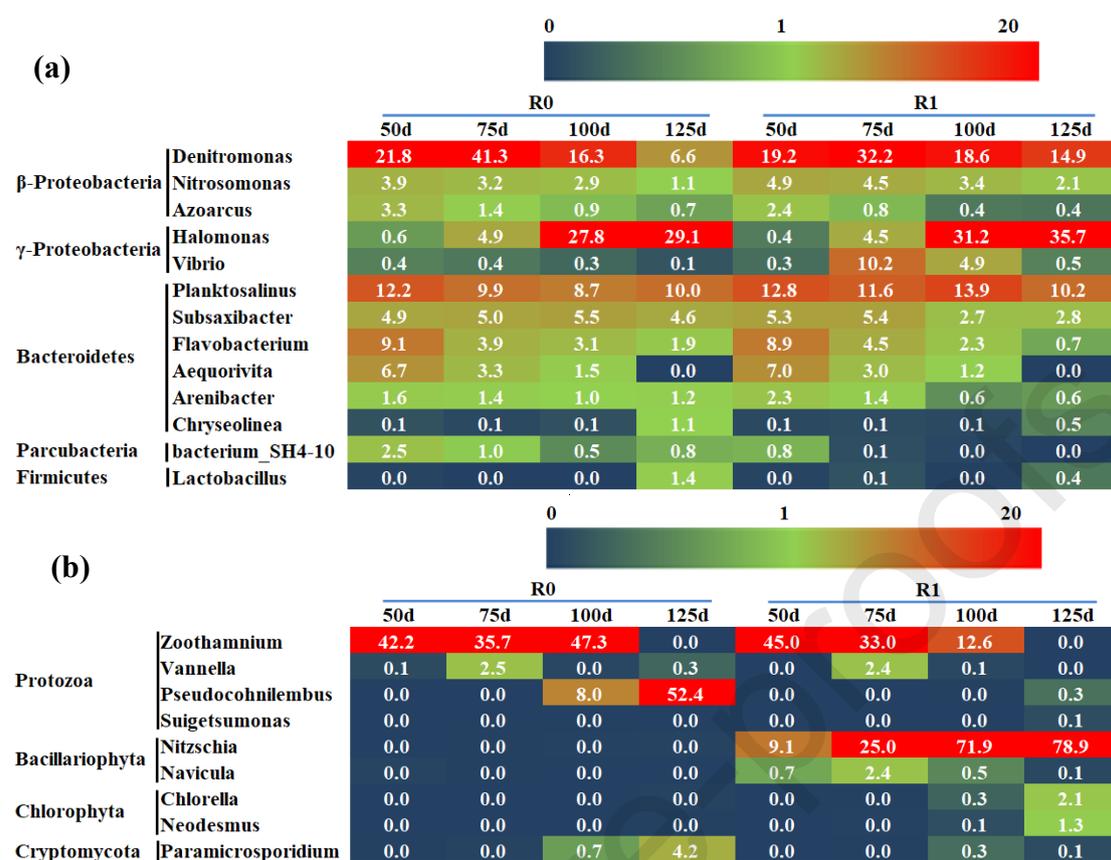


Fig. 6. Richness heat map of the biological community structure for prokaryote (a) and eukaryote (b) at the genus-level for the granules in the two CFRs. Samples were taken on day 50, 75, 100 and 125 day at 1%, 2%, 3% and 4% salinity, respectively. Only genera comprising >1% of the total OTUs in at least one sample are listed.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- Aerobic granule-continuous flow reactor was applied for saline wastewater treatment
- Algal-bacterial granule exhibited better overall performance and stability than AGS

- High salinity improved the symbiotic relationship between bacteria and algae
- Algae grew rapidly with 2 times lipid yield achieved at 4% salinity
- Salt tolerant *Halomonas* and *Nitzschia* significantly were enriched in granules

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