System performance and microbial community succession in a partial nitrification biofilm reactor in response to salinity stress

Jibin Li [a], Wei Ye [a], Dong Wei*[a], Huu Hao Ngo [b], Wenshan Guo [b], Yiming Qiao [a], Weiying Xu [a], Bin Du [a], Qin Wei [c]

[a] *School of Water Conservancy and Environment, University of Jinan, Jinan 250022, PR China*

[b] *School of Civil and Environmental Engineering, University of Technology Sydney, Broadway, NSW 2007, Australia*

[c] *Key Laboratory of Chemical Sensing and Analysis in Universities of Shandong, School of Chemistry and Chemical Engineering, University of Jinan, Jinan 250022, PR China*

Abstract

The system performance and microbial community succession in a partial nitrification biofilm reactor in response to salinity stress was conducted. It was found that the NH_4^+-N removal efficiency decreased from 98.4% to 42.0% after salinity stress increased to 20 g/L. Specific oxygen uptake rates suggested that AOB activity was more sensitive to the stress of salinity than that of NOB. Protein and polysaccharides contents showed an increasing tendency in both LB-EPS and TB-EPS after the salinity exposure. Moreover, EEM results indicated that protein-like substances were the main component in LB-EPS and TB-EPS as self-protection in response to salinity stress. Additionally, humic acid-like substances were identified as the main component in the effluent organic matter (EfOM) of partial nitrification biofilm, whereas fulvic acid-like substances were detected at 20 g/L salinity stress. Microbial

*Corresponding author. Tel: +86 531 8276 7873; fax: +86 531 8276 7873.
E-mail address: weidong506@163.com (D. Wei).

community analysis found that *Nitrosomonas* as representative species of AOB were significantly inhibited under high salinity condition.

Keywords: Partial nitrification; Salinity; Nitrite accumulation; Biofilm; Microbial community.

1. Introduction

Excessive nitrogen-containing compounds discharged from wastewater treatment plant could cause critical eutrophication for the receiving water (David et al., 2013). Compared with conventional biological nitrogen removal process, partial nitrification via nitrite has been developed as a cost-effective and energy saving microbial technology for treating both industrial and municipal wastewaters (Li et al., 2017a). It is commonly accepted that partial nitrification theoretically saves approximately 25% of oxygen consumption for nitrification and 40% of carbon source for denitrification, and achieves a lower sludge production (Gabarró et al., 2012). In order to achieve partial nitrification, a suitable management for the favorable growth of ammonia oxidizing bacteria (AOB) and inhibition of nitrite oxidizing bacteria (NOB) has been well reported, such as temperature, pH value, dissolved oxygen (DO) and free ammonia (FA), and free nitrous acid (FNA) etc (Wang et al., 2017c; Wang and Yang, 2004).

Till now, partial nitrification attached onto biofilm has also been developed to enhance the stability and shock resistance of the AOB-riched system. Nevertheless, the growth of AOB in partial nitrification process could be affected by many factors, such as antibiotics, heavy metals and salinity etc (Cortés-Lorenzo et al., 2015; Huang et al., 2016; Wang et al., 2017b). Salt is considered a common stress factor for both nitrification and denitrification processes. The ions contained in high saline organic

wastewater discharged from chemical, petroleum refinery, pharmaceuticalor dyeing factories mainly were Cl^-, SO_4^{2-}, Na^+ and Ca^{2+} etc (Wan et al., 2014). Although these ions are necessary for the growth of microorganisms and play an important role in promoting membrane balance, they will inhibit microorganisms at certain concentrations. Several studies on biological treatment of saline wastewater have certified that high osmotic pressure generated by high salt concentration promotes the dehydration of microbial cells and the separation of cellular protoplasm (Aslan and Simsek, 2012). In addition, the density of wastewater could change with increasing salt concentration, which might lead to variations in sludge sedimentation characteristics and sludge flocs architecture, seriously affecting the purification effect of the biological treatment system (Bassin et al., 2011). Therefore, high saline organic wastewater was considered as one of the major constraints to the microbial viability and species diversity of the biological wastewater treatment system (Kim et al., 2016). However, most of researches were focused on the salinity stress on the influence of activated sludge system, little information could be found in AOB-riched partial nitrification system.

Extracellular polymers substances (EPS) played an important role on the formation and maintenance of the stable structure of biofilm, which are mainly composed of proteins (PNs), polysaccharides (PSs), lipids, nucleic acids and other polymers (Zhang et al., 2011). EPS are usually divided into two categories based on its dynamic double-layered structure: loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) (Zheng et al., 2016). It also acts as gel-like hydrated matrix commonly found outside the cell surface with a purpose of keeping the cells aggregates together to against the harsh external environment (e.g. salinity) (Wang et al., 2018). The presence of salinity could lead to microbial metabolic disorders and potentially

disrupt biofilm stability through EPS (Bassin et al., 2011). Additionally, changes in species and/or dominant microorganisms will lead to different physicochemical characteristics and abnormal conditions of the biological wastewater treatment system, which will definitely affect the property of effluent organic matter (EfOM) (Xia et al., 2016). EfOM mainly consists of particulate and dissolved organic compounds that persist through biological wastewater treatment system, and its release could bring biotoxicity like anti-estrogenic activity and genotoxicity to aquatic organisms (Chen et al., 2017b). Although the production and properties of EPS and EfOM have been recognized in recent years, their evaluations in partial nitrification system under salinity stress still need to be confirmed. Moreover, the stimulation of salinity will lead to the evolution of microorganisms, which will further affect the production and release of EPS and EfOM. Therefore, it is crucial to conduct comprehensive to evaluate the performance of partial nitrification in response to salinity stress.

Thereby, the objective of this study was to evaluate the nitrite accumulation elimination and microbial community succession in a partial nitrification biofilm system under salinity stress. Three-dimensional excitation-emission matrix (3D-EEM) was applied to characterize the structures and compositions of EPS and EfOM. The high-throughput sequencing technology was conducted for the detection of bacterial community structure. The obtained result could provide new insights about the behaviors of microbial community and microbial products under high salinity environment in wastewater treatment system.

2. Materials and Methods

2.1. Experimental set-up and synthetic wastewater

A plexiglass sequencing batch biofilm reactor (SBBR) with an effective volume

of 5.7 L was conducted in the present experiment. The height and inner diameter of SBBR were 50 and 12 cm, respectively. Cylindrical fillers (K3, plastic media) with 40% (v/v) packing rate were added into the reactor as biomass support. The diameter and height of fillers were 25 and 15 mm, respectively. The specific surface area and the specific gravity of fillers were 500 m^2/m^3 and 110 kg/m^3, respectively.

The 8h cycle operation of SBBR was achieved by a time controller, and each cycle consisted of 5 min of influent filling, 85 min of anoxic stage, 360 min of aeration, 15 min of settling and 15 min of effluent. Influent wastewater was fed into the biofilm system by using a submersible pump from a water storage tank. A magnetic controlled stirrer for SBBR was applied to guarantee liquid adequate mixing during each operating cycle. The volume exchange ratio of SBBR was controlled at 50%. Air was introduced into the bottom of the reactor by using an air pump and the aeration rate was kept at 0.4 L/min.

The compositions of influent synthetic wastewater were as follows (mg/L): COD (as $C_6H_{12}O_6$), 600; NH_4^+-N (as NH_4Cl), 200; P (as K_2HPO_4), 15; $MgSO_4 \cdot 2H_2O$, 20; $CaCl_2$, 40; $FeSO_4 \cdot 2H_2O$, 20 and trace element solution 1.0 mg/L. Sodium chloride (NaCl) was added to synthetic wastewater to create stressful condition of high salinity. NaCl concentration was gradually increased from 5.0 to 20.0 g/L and then kept constant at 20.0 g/L for the duration of the study. Mixed liquid suspended solids (MLSS) of seed sludge obtained from a lab-scale sequencing batch reactor (SBR) was controlled at 3.0 g/L.

2.2. EPS extraction

A modified heating extraction method was used to extract LB-EPS and TB-EPS from biofilm samples (Li and Yang, 2007). Both LB-EPS and TB-EPS supernatants were filtered through the 0.45 μm glass fiber filter membrane for further analysis. PS

content was measured by means of throne-sulfuric acid method using glucose as the standard (Dubois et al., 1956). PN content was measured by means of modified Lowry method using bovine serum albumin (BSA) as the standard (FrØlund et al., 1995).

2.3. Microbial community analysis

Four biofilm samples corresponding to each test stage were subsequently collected from the surface of the carriers and then sent to Novogene Co., LTD (Beijing, China) for high-throughput sequencing analysis. RS1-RS4 represented for biofilm samples corresponding to different salinity concentrations (0, 5.0, 10.0 and 20.0 mg/L). Total genome DNA of the sample was extracted by using cetyltrimethyl ammonium bromide/sodium dodecyl sulfate (CTAB/SDS) method. DNA concentration and purity were monitored on 1% agarose gels. DNA was diluted to 1 ng/μL by using sterile water according to the concentration in centrifuge tube. The diluted genomic DNA was used as a template on the 16S V4 region. Efficient high-fidelity enzyme and bacterial diversity primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with the barcode were used to amplify the V4 region of 16S rDNA gene. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs) to ensure amplification efficiency and accuracy.

2.4. Analytical methods

Measurement of NH_4^+-N, NO_2^--N and NO_3^--N was undertaken according to their respective standard methods (APHA, 2005). Specific oxygen uptake rates ($(SOUR)_{NH_4}$ by AOB and $(SOUR)_{NO_2}$ by NOB) were determined by using the method

reported by Liu et al. (2004). A luminescence spectrometer (LS-55, Perkin-Elmer Co., USA) was used for the detection of fluorescence spectra of samples. Weighted unifrac distance based on Unweighted Pair-group Method with Arithmetic Mean (UPGMA) was performed for hierarchical clustering analysis in phylum level (Lozupone et al., 2011). According to the species annotation and abundance information of all samples at the genus level, the top 35 genera of abundance were selected and clustered to form a heatmap from the species and sample levels.

3. Results and discussion

3.1. Effect of salinity stress on partial nitrification SBBR

The experiment was carried out at two stages based on the types of influent synthetic wastewater: start-up stage I (days 1-70) and salinity stress stage II (days 71-130). The variations of nitrogen compound in partial nitrification SBBR system were shown in Fig. 1. In the first 7 days, the seed sludge in reactor was quickly adapted to the influent, in which the effluent NH_4^+-N and NO_2^--N concentrations decreased from 86.4 and 29.5 mg/L to 1.3 and 2.7 mg/L, respectively. The effluent NO_3^--N concentration was high of 144.3 mg/L and as the main form of nitrogen compounds. In the following operational days (8-26), the effluent NH_4^+-N removal efficiency was average at 99.5%, whereas the effluent NO_3^--N concentration was average at 120.1 mg/L (n=19), suggesting that the system operated at full nitrification mode. To establishment of nitrite accumulation, the aeration rate of reactor was reduced from 0.7 to 0.4 L/min. It was found from days 27 to 70 that NO_2^--N concentration increased from 2.1 mg/L to 93.6 mg/L and NO_3^--N concentration decreased from 153.7 mg/L to 6.7 mg/L, resulting in nitrite accumulation ratio (NAR, NO_2^--N/(NO_2^--N+NO_3^--N)) increased from 1.3% to 95.8%. Additionally, NH_4^+-N

concentration was always less than 6.0 mg/L during the achievement of partial nitrification biofilm. It was noticed that the TN removal efficiency in partial nitrification mode was significant higher than that of full nitrification mode (57.6% and 37.0%), suggesting the better N removal ability by partial nitrification biofilm than by full nitrification sludge. The main reason may be attributed to the anoxic microenvironment caused by the biofilm thickness that suitable for heterotrophic denitrification by using limited carbon sources. Moreover, nitrite reduction pathway requires much less carbon source than that of nitrate (saves 40%). This finding is of particular interest in biotechnology as it reduces the COD requirements per nitrogen denitrified by partial nitrification biofilm when treating low COD wastewater.

From days 71 to 82 (Stage II, salinity stress stage), the effluent NH_4^+-N concentration from partial nitrification biofilm gradually increased from 11.7 to 40.3 mg/L (day 76) and then decreased to 4.2 mg/L in response to 5.0 g/L NaCl. In contrary, NO_2^--N concentration exhibited an opposite trend that decreased first from 93.6 to 36.7 mg/L and then recovered to 89.6 mg/L. At the same time, the concentration of NO_3^--N was almost negligible, suggesting the activity of NOB was inhibited regardless of the presence of low salinity (5.0 g/L NaCl). In the following days (83-102), the similar variation of nitrogen compounds was observed in response to salinity exposure at 10.0 g/L but expressed different detail. The effluent NH_4^+-N removal efficiency was reduced to 52.7% at day 92, suggesting that there was a significant reduction in AOB activity in partial nitrification biofilm that widely depended on different levels of salinity. This value recovered to 98.7% at day 97 with only 5 days, implying the relative strong shock resistance of partial nitrification biofilm. The temporary fluctuations in inorganic nitrogen concentration might be attributed to the present of NaCl exhibited a significant negative impact on partial

nitrification, especially for the inhibition of AOB. However, the inhibition of microorganisms by salinity stress occurred primarily via osmotic stress, and partial nitrification system could recover to the original level (or even higher levels) as the microorganisms gradually adapt to the current osmotic stress (Zhao et al., 2016). Therefore, the results revealed that AOB do not have an intrinsic resistance to salinity stress, but they would adapt to higher salinity stress by gradually increasing the salt concentration. Windey et al. (2005) also reported that bioreactors could tolerate high-salinity stress with gradually increasing NaCl concentration. From days 103 to 130, the partial nitrification system was completely destroyed at NaCl exposure concentration of 20.0 g/L. It was found that NH_4^+-N concentration gradually increased from 3.1 to 116.0 mg/L, resulting in the removal efficiency of NH_4^+-N decreased from 98.4% to 42.0%, while NO_2^--N concentration decreased from 91.7 to 7.9 mg/L with no obvious accumulation of NO_3^--N. It could be drawn that salinity had a great influence on the choice of bacterial life strategies. Although bacteria could survive in high salinity environments by regulating the osmotic pressure of the cytoplasm to the surrounding environment, it could be predicted that the damage to microorganisms appeared to be irreversible when the concentration of NaCl reached to a certain threshold (Oren, 1999).

Although DO was relatively at high level (3 mg/L), partial nitrification biofilm was still successfully achieved due to the combined inhibition effects of FA and FNA, for selective inhibiting of NOB activity, as similarly reported in our previous study (Wei et al., 2018). Nevertheless, the salinity exposure had obviously changed the biological nitrogen removal efficiency, and therefore transformed the equilibrium between pH, NH_4^+-N and NO_2^--N in liquid phase that further affected the FA and FNA values. The fact that nitrite accumulation was continuous existed during the whole

period in the presence of salinity (stage II, days 71-132). The NAR values were average at 94.0%, 93.9% and 90.6% at salinity exposure of 5.0, 10.0 and 20.0 g/L, respectively, suggesting that the increasing salinity did not destroy the inhibitory relationship between AOB and NOB.

3.2. Activities of autotrophic bacteria in biofilm under salinity stress

SOUR is considered as a reliable mean to characterize biological activity in nitrogen removal process. The effect of high salinity on the activity of autotrophic bacteria in biofilm samples before and after salinity exposure was presented in Fig. 2. It was clearly shown that the initial values of $(SOUR)_{NH_4}$ for AOB, and $(SOUR)_{NO_2}$ for NOB were 3.8 ± 0.2 and 1.7 ± 0.1 mg O_2/g MLSS·h, respectively. The results reflected that AOB and NOB were co-existed in the partial nitrification system, but the activity of AOB was significant higher than that of NOB. With increasing NaCl concentration to 20.0 g/L, the values of $(SOUR)_{NH_4}$ and $(SOUR)_{NO_2}$ showed an obvious decreased tendency and maintained at 1.5 ± 0.1 and 1.0 ± 0.1 mg O_2/g MLSS·h, respectively. The ratio of $(SOUR)_{NH_4}/(SOUR)_{NO_2}$ declined from 2.2 to 1.5, revealing that AOB activity was more sensitive to the stress of salinity than that of NOB. Thereby, the increasing salinity exposure to partial nitrification biofilm seems to be more possible to reduce the microbial metabolism of AOB than that of NOB, which was consistent with the observation of effluent analysis (Section 3.1). Due to the long-term selectivity of biofilm, NOB was more preferred to be in the inner layer of biofilm because of its weaker ability to compete for DO than heterotrophic bacteria and AOB. This reason increased the survival of NOB in the co-existed microenvironment. Theoretically speaking, nitrite accumulation could be achieved by inhibition of NOB activity (e.g. FA and FNA), but not completely wash out of NOB

from the system (Peng and Zhu., 2006). Moussa et al. (2006) also reported that *Nitrobacter* was the only species of NOB as NaCl concentration was lower than 16.5 g/L, and it completely disappeared above that concentration.

3.3. Characteristics of LB-EPS and TB-EPS in biofilm in response to salinity stress

The presence of EPS might have an impact on adhesion phenomena, matrix structure formation and microbial physiology (Wang et al., 2015). In present study, the main components of LB-EPS and TB-EPS were measured and compared in the control biofilm (day 70) and salinity stress biofilm (20.0 g/L, day 130). As shown in Fig.3, the contents of PN and PS showed an increasing tendency both in LB-EPS and TB-EPS after the salinity reached to 20.0 g/L, implying that the addition of NaCl might stimulate the microorganisms to secrete EPS due to the self-protection strategy against external pressure (Li et al., 2017b). Nevertheless, Zhang et al. (2014) insisted that the increase of PN and PS contents might be attributed to the acceleration of endogenous respiration of microorganisms and the release of organic cell components owing to salinity addition. The changes in PS content appeared to be more sensitive to salinity stress than that of PN content, resulting in a decrease in the PN/PS ratio in LB-EPS and TB-EPS from 0.6 and 3.4 to 0.5 and 2.6, respectively. PS contained a large number of polar groups and had a strong ability to bind water, which could thicken the hydration layer around the cells, thereby slowing down the stress of high salinity (Grossutti and Dutcher, 2016; Wang and Zhang, 2010).

3D-EEM was applied to evaluate the changes in chemical compositions of two kinds of EPS before and after the exposure of NaCl (Supplementary materials). It was found that two main peaks (Peak A and Peak B) were identified in the LB-EPS of biofilm regardless of the addition of salinity. Peak A and Peak B were located at excitation/emission wavelengths (Ex/Em) of 260/347.5-348.5 nm and 240/438 nm,

which were attributed to tryptophan protein-like substances and fulvic acid-like substances, respectively (Chen et al., 2003). The fluorescence intensity of Peak A showed a more obvious increased tendency than that of Peak B. Additionally, a new peak C (220/361-364.5 nm) was identified in the TB-EPS spectra of partial nitrification biofilm under salinity of 0 and 20.0 g/L, which was assigned to aromatic protein-like substances (Wei et al. 2017). The fluorescence intensities of Peak A and Peak C significantly increased from 376.4 and 602.0 a.u. to 513.0 and 994.1 a.u., respectively. The results showed that protein-like substances was the main component both in LB-EPS and TB-EPS, implying protein-like substances might play an important role in the case of bacterial cells subjected to osmotic pressure caused by high salinity. Zhou et al. (2017) reported that tryptophan protein-like substances (α-amino acid) were commonly used for proteins biosynthesis and played an important role in bacterial metabolism and protection system. Moreover, a slight blue-shift in terms of emission wavelength was observed in both Peak A and Peak C, suggesting the chemical composition changes in two kinds of EPS. The reason might be attributed to the fact that the alteration of certain enzyme activities in the biosynthesis and metabolic pathways of organic matter, and the redistribution of metabolic flux with increasing salinity (Wang et al., 2013).

3.4. Fluorescent property of effluent organic matter in response to salinity stress

The composition and distribution of organic matter discharged into water bodies could provide some information related to the changes in microbial physiological structure under high salinity stress. Therefore, 3D-EEM fluorescence spectra of EfOM at different salinity concentrations were evaluated and compared, as shown in Supplementary materials. Two main peaks (Peak A and Peak B) were identified from the spectra, whereas peak C was only identified at NaCl concentration of 20.0 g/L.

Peak A and Peak B were located at Ex/Em of 340/410.5-423 nm and 260/423-451 nm, which were both assigned to humic acid-like substances. Peak C was located at Ex/Em of 230/408 nm, which represented fulvic acid-like substances. The intensities of Peak A and Peak B exhibited a consistent growth trend from 162.9 and 198.4 a.u. to 627.0 and 327.0 a.u. with the addition of NaCl. Previous literature reported that aggregation of individual cells and acceleration of endogenous respiration were direct responses of microorganisms to salinity stress, releasing organic celler components (such as SMP) through secretion and autolysis of cells (Reid et al., 2006). The production of humic acid-like substances might be attributed to the decomposition of dead cells and macromolecular organic substances such as PN and PS (Qu et al., 2012). EfOM are significantly produced in response to environmental stress, such as extreme temperature changes and osmotic shocks. Wang and Zhang (2010) reported that microorganisms subjected to stress conditions (such as starvation, salinity stress and low pH) are induced to produce more low-molecular-weight components during the utilization of the substrate.

3.5. Microbial community analysis and functional profiles of partial nitrification

UPGMA as a commonly used hierarchical clustering method was carried out to analyze the similarity between different samples. The results based on weighted unifrac distance illustrated in Fig. 4 revealed that the RS1 treatment was distinctly grouped from the RS2, RS3 and RS4 treatments, while the RS2 and RS3 treatments were grouped together, but separated from the RS4 treatment. To further evaluate the bacterial diversity among four samples, the sequences of ten known phyla and candidate divisions were also illustrated. The results revealed that the microbial species and their relative abundance had changed significantly with increasing NaCl dosage. In detailed, *Proteobacteria* whose proportion showed a growing tendency was

dominant population, followed by the sequences affiliated with *Bacteroidetes*. *Proteobacteria* and *Bacteroidetes* was the most common phylum in wastewater treatment bioreactors, which might form the core microorganisms in wastewater treatment bioreactors, no matter under what conditions (saline or normal) (Liu et al., 2018). Numerous studies have shown that most of the nitrogen-removing microorganisms (such as AOB, NOB) belonged to phylum *Proteobacteria* (Kong et al., 2017). *Bacteroidetes* was known for its metabolism of EPS generated by nitrifying bacteria and secondary metabolites produced by decayed biomass, and the decrease in its proportion seemed to contribute to the increase of EPS production (Yang et al., 2017). The relative abundance of *Firmicutes* showed a slightly increase with increasing salt concentration. *Firmicutes* is known for its ability of producing endospores to resist extreme environmental threats, and its existence is beneficial to biofilm systems (Chen et al., 2017a). *Actinobacteria* possesses a more stable structure and higher adaptability than others, which allows microorganisms to better maintain biofilm structures (Yan et al., 2015). It was reported that *Nitrospirae* as representative flora of the NOB population played an important role in biological nitrogen removal process and was found in large quantities in partial nitrification system (Ye et al., 2012). Further comparison of the microbial species down to the class, family and genus level was conducted to give more information on the community structure in the four biofilm samples.

As shown in Fig. 5, the phylogenetic characteristics of the bacterial communities were studied by comparing the relative abundances of the top 10 classes and families, and top 35 genera at different NaCl concentrations. At the class level (Fig. 5A), the relative abundances of *Betaproteobacteria*, *Alphaproteobacteria*, *Flavobacteriia*, *Verrucomicrobiae*, and *Sphingobacteriia* declined from 50.99%, 5.78%, 6.95%,

4.74%, and 4.21% at 0 g/L NaCl to 35.97%, 5.19%, 4.07%, 0.69% and 2.56% at 20.0 g/L NaCl, respectively, suggesting that these classes might be not well adapted to survive and counteract in high salinity conditions. Furthermore, *Betaproteobacteria*, composed of AOB, NOB, organic-decomposing bacteria, and denitrifying bacteria, was significantly reduced, confirming that significant inhibition on partial nitrification have been occurred at high salinity conditions (Hoang et al., 2014). In addition, the relative abundances of *Gammaproteobacteria*, *Deltaproteobacteria*, *Cytophagia* and *Bacilli* elevated from 8.74%, 1.95%, 2.04% and 1.64% at 0 g/L NaCl to 27.51%, 3.13%, 3.53% and 2.43% at 20.0 g/L NaCl, respectively. *Gammaproteobacteria* was well known for producing EPS and its increase might be the main factor in the variations of EPS. The microbial community structure before and after NaCl supplement at family level was displayed in Fig. 5B. The relative abundances of *Rhodocyclaceae*, *Nitrosomonadaceae*, *Comamonadaceae*, *NS9_marine_group*, *DEV007* and *Saprospiraceae* were observed to clearly decrease from 36.36%, 5.33%, 7.73%, 6.78%, 4.64% and 3.31% at 0 g/L NaCl to 24.41%, 3.38%, 5.13%, 3.42%, 0.60% and 2.19% at 20.0 g/L NaCl, respectively. On the contrary, the relative abundance of other families rose from 2.11% to 17.15% for *Moraxellaceae*, from 1.3% to 1.83% for *Xanthomonadaceae*, from 2.72% to 4.43% for *Enterobacteriaceae* and from 2.04% to 3.49% for *Cytophagaceae*.

The differences in bacteria genera among the four biofilm samples were displayed in Fig. 5C. A total of 35 genera (belonging to eight taxonomic phylam) were identified before and after the application of NaCl to characterize the composition of bacterial community. The results of the heatmap revealed that bacterial communities had undergone significant migration and transformation with increasing the concentrations of NaCl. In detailed, *Phaeodactylibacter*, *Blastocatella*, *Haliangium*,

Hydrogenophaga, *Thauera*, *Truepera* and *woodsholea* were the predominant genera at 5.0 g/L NaCl. *Anoxybacillus*, *Flavisolibacter*, *Sphingomonas*, *Bacillus*, *Massilia*, *Arthrobacter*, *Hymenobacter*, *Pseudomonas*, *Lewinella* and *Nitrosomonas* were the dominant microorganisms after the concentration of NaCl increased to 10.0 g/L. However, high-dose NaCl concentration (20.0 g/L) showed a significant inhibitory effect on the richness of the above species at genera level. Additionally, the relative abundances of *Chryseolinea*, *Planctomyces*, *SM1A02*, *Lactobacillus*, *Aeromonas*, *Stappia*, *Enterococcus*, *Escherichia-Shigella*, *Halomonas*, *Acinetobacter* and *Paracoccus* were clearly increased, suggesting that these genera could tolerate the inhibitory effect of high-dose NaCl. The results also appeared that different level of NaCl concentration altered the microbial community composition in different degrees. Furthermore, the abundances of some genera changed significantly under the corresponding NaCl concentration, out of which *Nitrosomonas* as autotrophic AOB could oxidize ammonia to nitrite using inorganic carbon as carbon source, and its occupation ratio decreased from 3.51% to 2.58% at genera level (Wang et al., 2017a). The abundance of NOB at the genus level decreased from 0.25% to 0.19%, which is consistent with the results of SOUR. The presence of *Arthrobacter* as the predominant heterotrophic nitrifies might be attributed to the rich NH_4^+-N and organic carbon in the influent (He et al., 2018). The genera *Halomonas* as halophilic microorganism could reduce nitrate and nitrite under the hyperhaline environment (Hoang et al., 2014). In general, the microbial community in the partial nitrification biofilm system had undergone significant evolution with increasing salt concentration. Not only the diversity and richness of the bacteria showed significant differences in the four samples, but the proportion of dominant microorganisms within individual samples also changed.

4. Conclusions

The performance of partial nitrification SBBR under the stress of salinity was characterized in this study. After 130 days operation, partial nitrification biofilm was significantly inhibited under high salinity stress. The decreased $(SOUR)_{NH_4}/(SOUR)_{NO_2}$ ratio suggested that AOB was more sensitive to high salinity than NOB. Protein-like substances were the main component both in LB-EPS and TB-EPS. Microbial community analysis revealed that *Proteobacteria* and *Bacteroidetes* were the dominant phylum and composed the core microorganisms in biofilm system. The result is of great significance for evaluating the performance of AOB-riched partial nitrification under salinity stress.

Acknowledgments

This study was supported by the Key Research and Development Plan of Shandong Province (2018GSF117027), Natural Science Foundation of Shandong Province (ZR201702070162), National Natural Science Foundation of China (21777056, 51508226), and QW thanks the Special Foundation for the Taishan Scholar Professorship of Shandong Province and UJN (No. ts20130937).

References

1. APHA, AWWA, WEF. 2005. Standards methods for the examination of water and wastewater. 21st ed. Washington, D.C.
2. Aslan, S., Simsek, E. 2012. Influence of salinity on partial nitrification in a submerged biofilter. *Bioresource Technology*, **118**(4), 24-29.
3. Bassin, J.P., Dezotti, M., Jr, G.L.S.A. 2011. Nitrification of industrial and domestic saline wastewaters in moving bed biofilm reactor and sequencing batch reactor. *Journal of Hazardous Materials*, **185**(1), 242-248.
4. Chen, W., Westerhoff, P., Leenheer, J.A., Booksh, K. 2003. Fluorescence excitation-emission matrix regional integration to quantify spectra for dissolved

organic matter. *Environmental Science and Technology*, **37**(24), 5701-5710.

5. Chen, Y., He, H., Liu, H., Li, H., Zeng, G., Xia, X., Yang, C. 2017a. Effect of salinity on removal performance and activated sludge characteristics in sequencing batch reactors. *Bioresource Technology*, **249**, 890.
6. Chen, Z., Mo, L., Wen, Q., Ren, N. 2017b. Evolution of molecular weight and fluorescence of effluent organic matter (EfOM) during oxidation processes revealed by advanced spectrographic and chromatographic tools. *Water Research*, **124**, 566.
7. Cortés-Lorenzo, C., Rodríguez-Díaz, M., Sipkema, D., Juárez-Jiménez, B., Rodelas, B., Smidt, H., González-López, J. 2015. Effect of salinity on nitrification efficiency and structure of ammonia-oxidizing bacterial communities in a submerged fixed bed bioreactor. *Chemical Engineering Journal*, **266**(6), 233-240.
8. David, C.G., Marco, D.E., Germán, T., David, B., Laurent, P., Bedmar, E.J. 2013. Spatial distribution of N-cycling microbial communities showed complex patterns in constructed wetland sediments. *Fems Microbiology Ecology*, **83**(2), 340-351.
9. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, **28**(3), 350-356.
10. FrØlund, B., Griebe, T., Nielsen, P.H. 1995. Enzymatic activity in the activated-sludge floc matrix. *Applied Microbiology and Biotechnology*, **43**(4), 755-761.
11. Gabarró, J., Ganigué, R., Gich, F., Ruscalleda, M., Balaguer, M.D., Colprim, J. 2012. Effect of temperature on AOB activity of a partial nitritation SBR treating landfill leachate with extremely high nitrogen concentration. *Bioresource Technology*, **126**(6), 283-289.
12. Grossutti, M., Dutcher, J.R. 2016. Correlation Between Chain Architecture and Hydration Water Structure in Polysaccharides. *Biomacromolecules*, 17(3), 1198-204.
13. He, S., Wang, Y., Li, C., Li, Y., Zhou, J. 2018. The nitrogen removal performance and microbial communities in a two-stage deep sequencing constructed wetland for advanced treatment of secondary effluent. *Bioresource Technology*,**248**, 82-88.
14. Hoang, V., Delatolla, R., Abujamel, T., Mottawea, W., Gadbois, A., Laflamme, E., Stintzi, A. 2014. Nitrifying moving bed biofilm reactor (MBBR) biofilm and biomass response to long term exposure to 1°C. *Water Research*, **49**(49C), 215-224.
15. Huang, X., Feng, Y., Hu, C., Xiao, X., Yu, D., Zou, X. 2016. Mechanistic model

for interpreting the toxic effects of sulfonamides on nitrification. *Journal of Hazardous Materials*, **305**, 123-129.

16. Kim, H.C., Choi, W.J., Chae, A.N., Park, J., Kim, H.J., Song, K.G. 2016. Evaluating integrated strategies for robust treatment of high saline piggery wastewater. *Water Research*, **89**, 222-231.
17. Kong, Q., He, X., Feng, Y., Miao, M.S., Wang, Q., Du, Y.D., Xu, F. 2017. Pollutant removal and microorganism evolution of activated sludge under ofloxacin selection pressure. *Bioresource Technology*, **241**, 849-856.
18. Li, J., Wei, J., Ngo, H.H., Guo, W., Liu, H., Du, B., Wei, Q., Wei, D. 2017a. Characterization of soluble microbial products in a partial nitrification sequencing batch biofilm reactor treating high ammonia nitrogen wastewater. *Bioresource Technology*, **249**.
19. Li, X.Y., Yang, S.F. 2007. Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. *Water Research*, **41**(5), 1022-1030.
20. Li, Z., Chang, Q., Li, S., Gao, M., She, Z., Guo, L., Zhao, Y., Jin, C., Zheng, D., Xu, Q. 2017b. Impact of sulfadiazine on performance and microbial community of a sequencing batch biofilm reactor treating synthetic mariculture wastewater. *Bioresource Technology*, **235**, 122-130.
21. Liu, M., Li, Q., Sun, H., Jia, S., He, X., Li, M., Zhang, X.X., Ye, L. 2018. Impact of salinity on antibiotic resistance genes in wastewater treatment bioreactors. *Chemical Engineering Journal*, **338**.
22. Liu, Y., Yang, S.F., Tay, J.H. 2004. Improved stability of aerobic granules by selecting slow-growing nitrifying bacteria. *Journal of Biotechnology*, **108**(2), 161.
23. Lozupone, C., Lladser, M.E., Knights, D., Stombaugh, J., Knight, R. 2011. UniFrac: an effective distance metric for microbial community comparison. *Isme Journal*, **5**(2), 169-172.
24. Moussa, M.S., Sumanasekera, D.U., Ibrahim, S.H., Lubberding, H.J., Hooijmans, C.M., Gijzen, H.J., van Loosdrecht, M.C. 2006. Long term effects of salt on activity, population structure and floc characteristics in enriched bacterial cultures of nitrifiers. *Water Research*, 40(7), 1377-1388.
25. Oren, A. 1999. Bioenergetic aspects of halophilism. *Microbiol Mol Biol Rev*, 63(2), 334-348.
26. Peng, Y., Zhu, G. 2006. Biological nitrogen removal with nitrification and denitrification via nitrite pathway. *Applied Microbiology and Biotechnology*, 73(1), 15-26.

27. Qu, F., Liang, H., He, J., Ma, J., Wang, Z., Yu, H., Li, G. 2012. Characterization of dissolved extracellular organic matter (dEOM) and bound extracellular organic matter (bEOM) of Microcystis aeruginosa and their impacts on UF membrane fouling. *Water Research*, **46**(9), 2881-2890.
28. Reid, E., Liu, X., Judd, S.J. 2006. Effect of high salinity on activated sludge characteristics and membrane permeability in an immersed membrane bioreactor. *Journal of Membrane Science*, 283(1), 164-171.
29. Wan, C., Yang, X., Lee, D.J., Liu, X., Sun, S., Chen, C. 2014. Partial nitrification of wastewaters with high NaCl concentrations by aerobic granules in continuous-flow reactor. *Bioresource Technology*, **152**(1), 1-6.
30. Wang, J., Yang, N. 2004. Partial nitrification under limited dissolved oxygen conditions. *Process Biochemistry*, **39**(10), 1223-1229.
31. Wang, L., Li, Y., Wang, L., Zhu, M., Zhu, X., Qian, C., Li, W. 2018. Responses of biofilm microorganisms from moving bed biofilm reactor to antibiotics exposure: Protective role of extracellular polymeric substances. *Bioresource Technology*, **254**, 268-277.
32. Wang, S., Wang, L., Deng, L., Zheng, D., Zhang, Y., Jiang, Y., Yang, H., Lei, Y. 2017a. Performance of Autotrophic Nitrogen Removal from Digested Piggery Wastewater. *Bioresource Technology*, 241, 465.
33. Wang, Y., Zhang, X., Zhang, X., Meng, Q., Gao, F., Zhang, Y. 2017b. Characterization of spectral responses of dissolved organic matter (DOM) for atrazine binding during the sorption process onto black soil. *Chemosphere*, 180, 531-539.
34. 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. *Separation and Purification Technology*, **144**(39), 223-231.
35. Wang, Z., Gao, M., Wang, Z., She, Z., Chang, Q., Sun, C., Zhang, J., Ren, Y., Yang, N. 2013. Effect of salinity on extracellular polymeric substances of activated sludge from an anoxic–aerobic sequencing batch reactor. *Chemosphere*, **93**(11), 2789-2795.
36. Wang, Z., Liu, X., Ni, S.Q., Zhang, J., Zhang, X., Ahmad, H.A., Gao, B. 2017c. Weak magnetic field: A powerful strategy to enhance partial nitrification. *Water Research*, **120**, 190-198.
37. Wang, Z.P., Zhang, T. 2010. Characterization of soluble microbial products (SMP) under stressful conditions. *Water Research*, **44**(18), 5499.

38. Wei, D., Ngo, H.H., Guo, W., Xu, W., Du, B., Wei, Q. 2018. Partial nitrification granular sludge reactor as a pretreatment for anaerobic ammonium oxidation (Anammox): Achievement, performance and microbial community. *Bioresource Technology*, 269, 25-31.
39. Wei, D., Zhang, K., Ngo, H.H., Guo, W., Wang, S., Li, J., Han, F., Du, B., Wei, Q. 2017. Nitrogen removal via nitrite in a partial nitrification sequencing batch biofilm reactor treating high strength ammonia wastewater and its greenhouse gas emission. *Bioresource Technology*, **230**, 49-55.
40. Windey, K., De, B.I., Verstraete, W. 2005. Oxygen-limited autotrophic nitrification-denitrification (OLAND) in a rotating biological contactor treating high-salinity wastewater. *Water Research*, **39**(18), 4512-20.
41. Xia, C., Ma, D., Gao, B., Hu, X., Yue, Q., Meng, Y., Kang, S., Zhang, B., Qi, Y. 2016. Characteristics and trihalomethane formation reactivity of dissolved organic matter in effluents from membrane bioreactors with and without filamentous bulking. *Bioresource Technology*, **211**, 183.
42. Yan, Z., Yan, Z., Hong-Qiang, R., Jin-Ju, G., Ke, X., Hui, H., Li-Li, D. 2015. Physicochemical characteristics and microbial community evolution of biofilms during the start-up period in a moving bed biofilm reactor. *Bioresource Technology*, **180**, 345-351.
43. Yang, Y., Chen, Z., Wang, X., Zheng, L., Gu, X. 2017. Partial nitrification performance and mechanism of zeolite biological aerated filter for ammonium wastewater treatment. *Bioresource Technology*, **241**, 473-481.
44. Ye, L., Zhang, T., Wang, T., Fang, Z. 2012. Microbial structures, functions, and metabolic pathways in wastewater treatment bioreactors revealed using high-throughput sequencing. *Environmental Science and Technology*, **46**(24), 13244-52.
45. Zhang, H., Gao, Z., Zhang, L., Song, L. 2014. Performance enhancement and fouling mitigation by organic flocculant addition in membrane bioreactor at high salt shock. *Bioresource Technology*, **164**(7), 34-40.
46. Zhang, Z.J., Chen, S.H., Wang, S.M., Luo, H.Y. 2011. Characterization of extracellular polymeric substances from biofilm in the process of starting-up a partial nitrification process under salt stress. *Applied Microbiology and Biotechnology*, **89**(5), 1563-1571.
47. Zhao, L., She, Z., Jin, C., Yang, S., Guo, L., Zhao, Y., Gao, M. 2016. Characteristics of extracellular polymeric substances from sludge and biofilm in a simultaneous nitrification and denitrification system under high salinity stress.

Bioprocess and Biosystems Engineering, **39**(9), 1375-1389.

48. Zheng, D., Chang, Q., Li, Z., Gao, M., She, Z., Wang, X., Guo, L., Zhao, Y., Jin, C., Gao, F. 2016. Performance and microbial community of a sequencing batch biofilm reactor treating synthetic mariculture wastewater under long-term exposure to norfloxacin. *Bioresource Technology*, **222**, 139.
49. Zhou, L., Zhuang, W., Wang, X., Yu, K., Yang, S., Xia, S. 2017. Potential acute effects of suspended aluminum nitride (AlN) nanoparticles on soluble microbial products (SMP) of activated sludge. *Journal of Environmental Sciences*, **57**(7), 284-292.

Figure capations

Fig. 1 Variations of effluent nitrogen compounds in partial nitrification biofilm system under salinity stress.

Fig. 2 Evaluation of autotrophic nitrifying bacteria activities in biofilm before (day 70) and after salinity exposure (day 130).

Fig. 3 Changes in PN and PS contents in LB-EPS and TB-EPS under salinity stress.

Fig. 4 Weighted unifrac distance among four biofilm samples on the left and the relative microbial species abundance in phylum level on the right.

Fig. 5 Taxonomic classification of the bacterial communities: (A) class level; (B) family level. (C) Heatmap revealing the significant enrichment and decrease of bacterial communities in four sludge samples at genus level with different NaCl application.

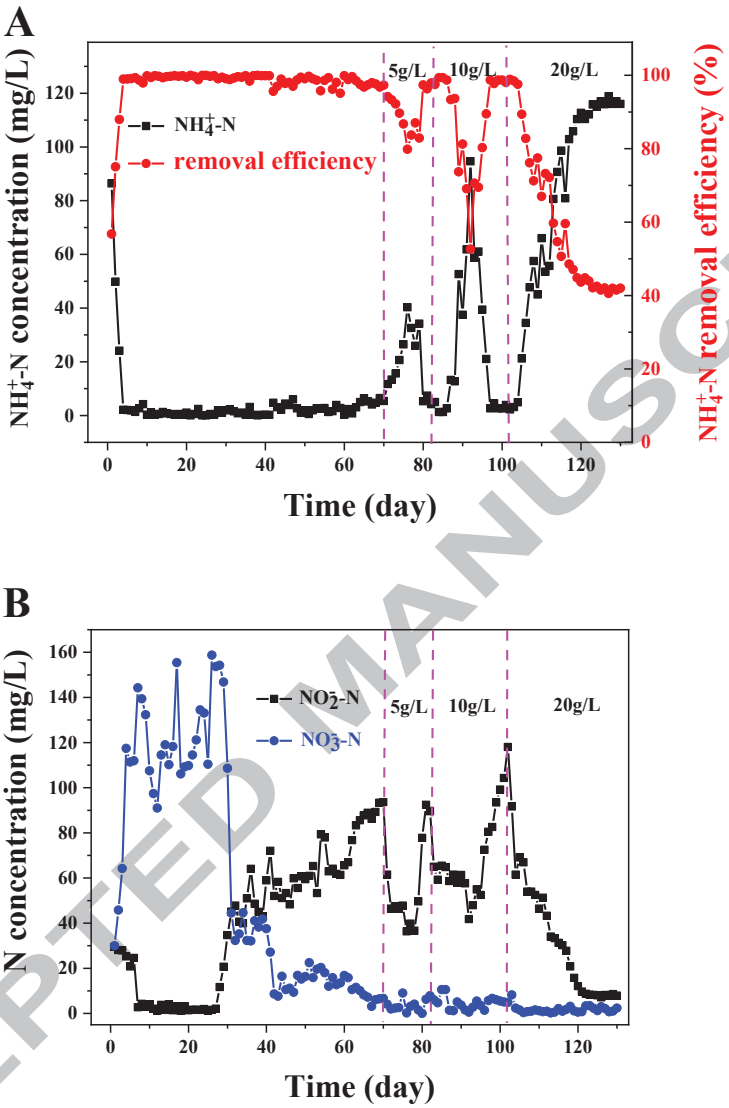

Fig. 1. Variations of effluent nitrogen compounds in partial nitrification biofilm system under salinity stress.

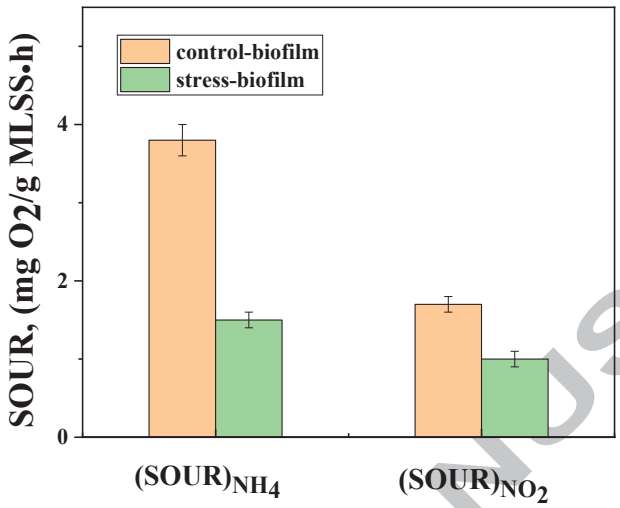

Fig.2. Evaluation of autotrophic nitrifying bacteria activities in biofilm before (day 70) and after salinity exposure (day 130).

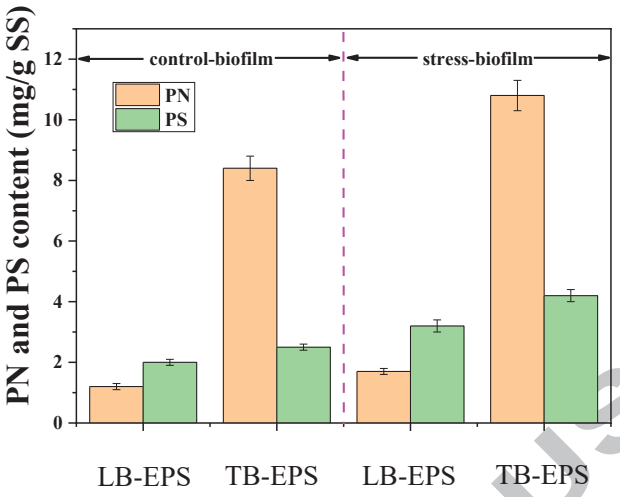

Fig.3 Changes in PN and PS contents in LB-EPS and TB-EPS under salinity stress.

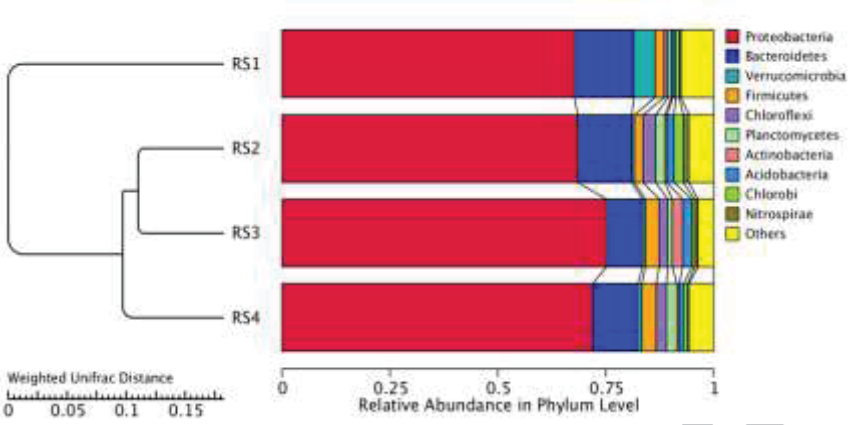

Fig. 4. Weighted unifrac distance among four biofilm samples on the left and the relative microbial species abundance in phylum level on the right.

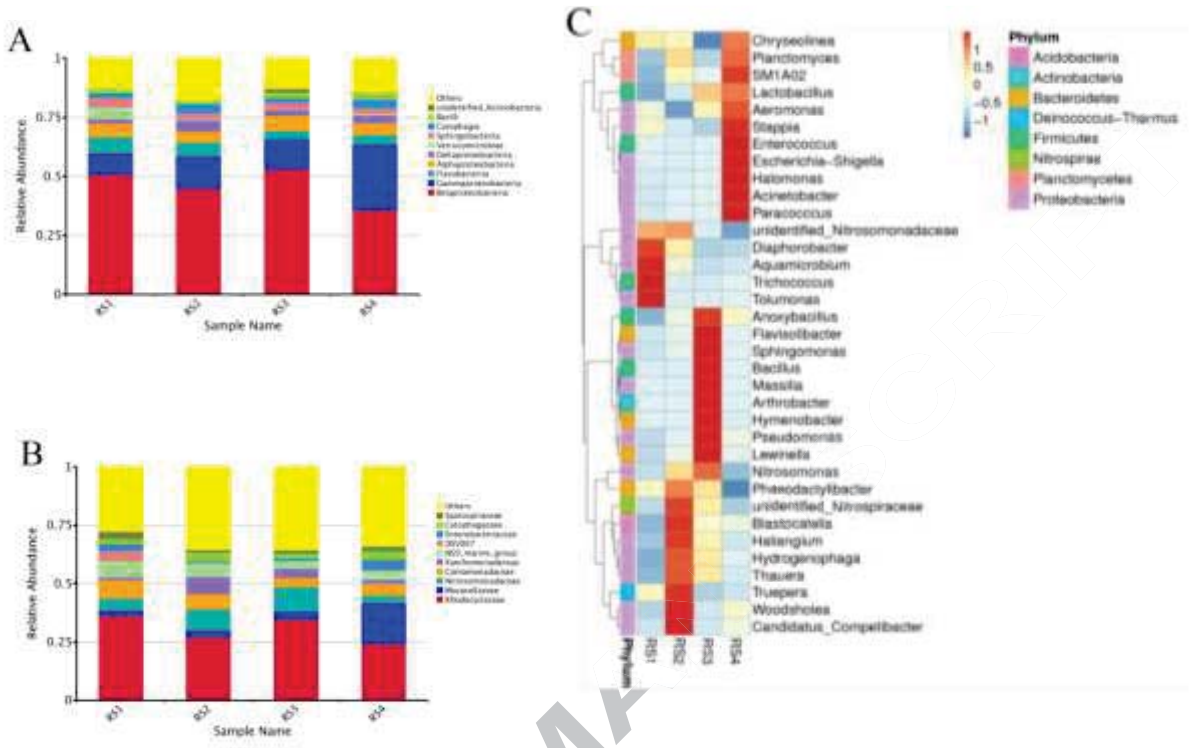

Fig. 5. Taxonomic classification of the bacterial communities: (A) class level; (B) family level. (C) Heatmap revealing the significant enrichment and decrease of bacterial communities in four sludge samples at genus level with different NaCl application.