Global NEST Journal, Vol 19, No 3, pp 377-388

Impact of salinity on the performance and microbial community of anaerobic ammonia oxidation (Anammox) using 16S rRNA High-throughput Sequencing technology

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Received: 06/12/2016, Accepted: 14/02/2017, Available online: 09/10/2017

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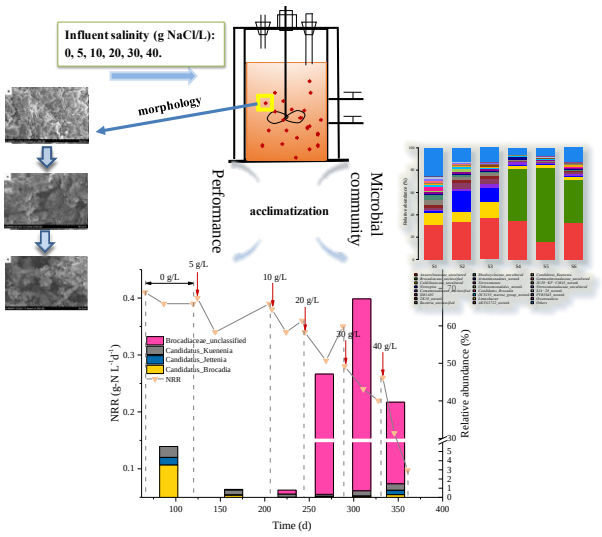
Abstract

Salinity is a key environmental factor for the successful application of anammox technology in wastewater treatment, because it impacts the activity and the community structure of anammox bacteria. In this study, the changes in activity and population shifts of an anammox system response to the elevated salt stress (0, 5, 10, 20, 30 and 40 g NaCl/L) were studied.



The results show that the anammox reactor performed effectively even at 30 g NaCl/L salinity after an appropriate acclimatization. The nitrogen removal rate maintained at 0.28 g N $L^{-1}d^{-1}$ with the nitrogen removal efficiency of 76%, though the high environmental salinity might inhibit the anammox growth in the long-term operation. 16S rRNA high-throughput sequencing results revealed that *Ca. Brocadia*, *Ca. Jettenia* and *Ca. Kuenenia* were the dominant anammox bacteria at all salinities. *Ca. Brocadia* and *Ca. Jettenia* were quite sensitive to salinity, and 5 g NaCl/L dosing could cause a sharp decline in their abundance. Nevertheless, these three anammox genus finally survived in the system with a steady specific anammox activity of 0.13 g N g $VSS^{-1}d^{-1}$. Specially, a novel cluster, *Brocadiaceae_unclassified*, which possibly belongs to anammox bacteria, became the dominant genus at the salinity over 20 g NaCl/L and likely contributed partially to the nitrogen removals. Our findings elucidated the inherent link between community dynamics and anammox system performance and stability under salty environment, and proved that anammox technologies can be an effective technology for treatment of saline ammonia-rich wastewater.

Keywords: Salinity; anammox bacteria; nitrogen removal; 16S rRNA high-throughput

1. Introduction

The discharge of wastewater containing high nitrogen can lead to eutrophication which causes the excessive growth of algal, and thus posing a great threat to the ecological health of water resources (Van Hulle *et al.*, 2010). In most modern wastewater treatment plants, nitrogen in

wastewater is biologically removed by nitrification and denitrification processes, which requires much electric energy to create aerobic conditions for bacterial nitrification as well as organic carbons like methanol or ethanol to remove nitrate by bacterial denitrification (Kartal *et al.,* 2010). Anaerobic ammonia oxidation (anammox), termed as autotrophic nitrogen removal process, is a cost effective and environment friendly nitrogen removal technology, which offers a sustainable alternative to remove nitrogen with less demand of oxygen supply and no extra carbon sources (Kartal *et al.,* 2006). In anammox processes, ammonium is oxidized using nitrite as electron acceptors and conserves energy for CO_2 fixation (Strous *et al.,* 1999). Although anammox technology possesses significant advantages compared to conventional nitrogen removal technologies, the widespread implementation of anammox may be hindered by long term start-up because of the low growth rate (doubling time of approximately 15 d), high temperature requirement, and high sensitivities to inhibitory matters presented in wastewater such as organic matters and salt (Jin *et al.,* 2012; Van Hulle *et al.,* 2010; Xing *et al.,* 2015).

From a practical perspective, salinity is an important parameter for wastewater treatment as the high salt concentration could inhibit microorganism growth by destroying the cell-wall and enzymes of bacteria, resulting in cell plasmolysis and losing activity of the cells (Uygur, 2006). Actually, many industrial wastewaters, such as petroleum and pharmaceutical industries, seafood processing, landfill leachate and leather industry, which are rich in ammonium, are also characterized by high salt concentrations (total salinity excesses 10 g NaCl/L) (Yi *et al.,* 2011). Moreover, due to the shortage of fresh water resources, many coastal cities generally use sea water as municipal water flushing lavatories and sprinkling street, which would engender a large volume of saline sewage (Chen *et al.,* 2003). Salt impacts on N-removals have been previously investigated, and salt stress could be commonly alleviated by two strategies: i) utilization of halophilic microbial consortia from brackish sediments (Din Er and Kargi, 2001) and ii) gradual acclimatization of freshwater microorganism to saline conditions (Panswad and Anan, 1999). These strategies may also be suitable for anammox processes to resist salty environment.

Until now, five genera of candidate anammox bacteria have been provisionally proposed in *planctomycets,* i.e. *Candidatus Brocadia*, *Ca. Kuenenia*, *Ca*. *Scalindua*, *Ca*. *Anammoxoglobus*, and *Ca*. *Jettenia* (Awata *et al.,* 2015). Among them, only "*Scalindua*" genus has been detected in natural saline ecosystems (Thamdrup and Dalsgaard, 2002). As for the first salt stress alleviation strategy, Nakajima *et al.,* (2008) enriched *Ca. Scalindua* from Ago Bay in Japan using a continuous culture system, providing a possibility of the salty wastewater treatment by anammox technologies. In most cases, the second strategy that gradual adaptation of fresh anammox species to saline condition is applied to obtain anammox bacteria that could endure salty conditions (Bassin *et al.* 2012; Kartal *et al.,* 2006). For example, Kartal *et al.,* (2006) seeded anammox bacteria from a freshwater system, and successfully adapted the microorganisms to high salinity level of 30 g/L; the seed sludge consisted of 50% of *Candidatus "Kuenenia stuttgartiensis"* and 50% of *Candidatus "Scalindua wagneri"*, and in a salt adapted sludge, the freshwater anammox species *Candidatus "Kuenenia stuttgartiensis"* was the dominant population. Likewise, in a Sharon–anammox system, a nitrogen loading rate (NLR) of 600 g N $m^{-3}d^{-1}$ was maintained even at a salinity of 15 g NaCl/L without a remarkable change in anammox activities (Dapena-Mora *et al.,* 2006). Later, Liu *et al.,* (2009) reported that, in an anammox fixed-bed reactor with a non-woven biomass carrier, freshwater anammox sludge can be applied in the treatment of saline wastewater, and the nitrogen removal rate (NRR) remained at 1.7 kg N $m^{-3}d^{-1}$ with the salinity up to 30 g NaCl/L after extended acclimatization. They found the survived anammox bacteria was a freshwater anammox bacterium KU2. However, due to the different seed sludges in different studies, the information on microbial structure shift along with increasing salinity is still limited, which however is most important to anammox reaction performance. In order to develop environmentally conscious operational practices and get a better understanding of biological N-removal performance while treating saline wastewater by anammox processes, studies about the dynamics of the microbial community structure in anammox systems is urgently required.

In this study, a sequential batch reactor (SBR) was operated for 361 days to investigate the effects of increasing salinity

(0, 5, 10, 20, 30 and 40 g NaCl/L) on anammox performance and sludge physical morphology. In particular, the microbial community dynamic of anammox bacteria response to the increasing salt stress was comprehensively evaluated using 16S rRNA high-throughput sequencing technique that has overwhelming superiority on profiling complex bacteria community for its unprecedented sequencing depth (GLENN, 2011). We aim to elucidate the relationship between anammox performance and evolution of microbial community structure for the stable and effective application of anammox processes in the industrial field.

2. Materials and Methods

2.1 Experimental set-up and operation strategy

The experiment was conducted in a sealed SBR with a total volume of 2.5 L and an effective volume of 2 L. The experimental temperature was maintained at 35 °C using a thermostatic water bath, and the complete mixture was achieved using a mechanical stirrer. The SBR was operated with a cycle of 8 h which consisted of 5 minutes of feeding, 395 minutes of mixing, 55 minutes of settling, 5 minutes of drainage and 20 minutes of idle time. The ratio of effluent volume and reactor effective volume was kept at 0.5, and the hydraulic retention time (HRT) was 16 h.

Experimental data were collected over a period of 361 days and the entire experimental period was separated to seven phases according to the different salt concentrations (Table 1). At the start-up phase (day 1-60), the anammox sludge was acclimated for 60 days with a stepwise increased nitrogen loading rate (NLR) to get a stable nitrogen removal rate. Then, six-phase experiment was conducted with gradually increasing salt concentration.

Table 1. Operational phases and the corresponding influent characteristics of the lab-scale anammox reactor

Phases	Time (d)	Salinity (g/L)	NH_4^+-N (mg-N L^{-1})	NO_2-N (mg-N L^{-1})	NLR (g N L^{-1} d^{-1})
start up	1-60	0	40→140±20	50→190±20	-
Ⅰ	61-123	0	138±28	186±17	0.49±0.05
Ⅱ	124-207	5	131±17	174±17	0.46±0.06
Ⅲ	208-244	10	140±5	179±8	0.48±0.02
Ⅳ	245-289	20	132±13	165±24	0.45±0.03
Ⅴ	290-332	30	111±17	138±25	0.37±0.06
Ⅵ	333-361	40	95±25	116±16	0.32±0.07

2.2 Seed anammox sludge and feeding media

The seeding sludge was taken from another anammox reactor in our laboratory that had run steadily for over one year, keeping a maximum specific anammox activity (SAA) of 0.31±0.01 g N/ (g VSS·d). Fluorescence *in situ* hybridization analysis revealed that the freshwater anammox species Candidatus *"Kuenenia stuttgartiensis"* dominated in the seeding sludge (probes EUB338 mix and KST-1275 were used).

The experimental reactor was fed synthetic medium with a ratio around 1.32 of nitrite to ammonium. The composition of the synthetic mediums was as follows: 40~140±20 mg NH_4^+-N/L, 50~190±20 mg NO_2^-/L (range at different phases), 1.05 g/L $NaHCO_3$, 0.02 g/L KH_2PO_4, 0.014 g/L $CaCl_2$, 0.08 g/L $MgSO_4·7H_2O$, 0.015 g/L $FeSO_4·7H_2O$, 0.02 g/L Na_2EDTA and 1 ml/L trace element solution (0.018 g/L H_3BO_3, 0.262 g/L $CoCl_2·2H_2O$, 0.312 g/L $CuSO_4·5H_2O$, 0.538 g/L $ZnSO_4·7H_2O$, 1.238 g/L $MnCl_2·4H_2O$, 0.238 g/L $NiCl_2·6H_2O$, 0.275 g/L $NaMoO_4·2H_2O$, 7.5 g/L EDTA).

2.3 Scanning electron microscopy observation

Morphological studies of the anammox flocs and granules were performed with scanning electron microscopy (SEM). The sludge samples were centrifuged for 5 min at a speed of 4000 r/min and subsequently fixed with a solution of 2.5% glutaraldehyde over night. Next, the sludge samples were washed in the phosphate buffer solution three times for 15 min each and fixed the samples for 60 to 120 min in a phosphate buffer solution with 1% OsO_4. After being washed in the phosphate buffer solution three times for 15 min each, the sludge samples were dehydrated for 15 min each in serially graded ethanol solutions of 50%, 70%, 80%, 90% and 95%. Then, the samples were shaded with gold

and observed under the scanning electron microscopy (SUPERSCAN SSX-550).

2.4 Analytical methods

Ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N) concentrations and VSS were measured according to standard methods (APHA, 1998). The maximum SAA was conducted according to Dapena-Mora et al (Dapena-Mora *et al.,* 2004). The pH and ORP were measured by online probes (WTW pH3310 SenTix41 and WTW pH3310 SenTixORP respectively). The extracellular polymeric substance (EPS) was extracted using the heating method according to ref. (Adav and Lee, 2008). Carbohydrates in the EPS were taken by the anthrone method with a glucose standard. Proteins and Humus were determined with the Folin-Lowry method using bovine serum albumin as a standard (Liu and Fang, 2002).

2.5 DNA extraction and PCR amplification

The granular sludge sample was stored at -80 °C for subsequent experiments. Total genomic DNA was extracted in duplicate from each sample using Power Soil DNA Isolation Kit (Sangon, China) according to the manufacture's instructions. Following extraction, the quality of the DNA was examined by 1% (w/v) agarose gel electrophoresis and concentration measured with NanoDrop spectrophotometer 2000 (Thermo Scientific, USA).

Polymerase chain reaction (PCR) of the V3-V4 region of the 16S rRNA gene was amplified using bacterial primers 338F (5'-ACT CCT ACG GGA GGC AGC AG-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3') (Claesson *et al.,* 2010), with the reverse primer containing a 6 bp barcode used to tag each sample. PCR amplifications were carried out in triplicate for each sample using 20 µL reaction mixtures, containing 4 µL of 5× PCR buffer, 10 ng of template DNA, 0.2 M of each primer, 0.25 mM of each dNTP, and 1 U FastPfu polymerase (TransGen, China). The PCR conditions involved an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and ended with an extension step at 72 °C for 5 min in a GeneAmp 9700 thermocycler (ABI, USA). The triplicate amplicons were pooled together, electrophoresed on 2% (w/v) agarose gel, and recovered using an AxyPrep DNA Gel Extraction Kit (AXYGEN, China).

2.6 16S rRNA-based illumina library preparation, sequencing and data analysis

The purified amplicons were quantified by QuantiFluor-ST Fluorometer (Promega, USA), and then a composite sequencing library was constructed by combining quimolar ratios of amplicons from all samples. The resulting library was sent for paired-end sequencing (2×250 bp) on an Illumina Miseq platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Raw sequence data of this study have been deposited to the NCBI Sequence Read Archive, compared with sequences in the GenBank database.

Sequence data was then analyzed by the software of Trimmomatic and FLASH. To facilitate the comparison between different samples, the numbers of sequences were normalized to the same sequencing depths of 21,169 by MOTHUR program (Schloss *et al.,* 2009). The effective sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity using Uearch program, and a representative sequence was then picked for each OUT by selecting the most abundant sequence in that OUT.

These representative sequences were assigned to taxonomic classifications by Ribosomal Database Project (RDP) Classifier with a confidence threshold of 70% (Wang *et al.,* 2007). Additionally, the alpha diversity including rarefaction curves, Shannon and Simpson diversity index, species richness estimators of Chao, Ace and diversity coverage were calculated in MOTHUR for each sample.

3. Results and discussion

3.1 Performance of anammox reactor in response to increasing salinity

The performance of the anammox system over a period of 361 days is shown in Fig. 1. During phase I (day 61–123) when without salt addition, the anammox system reached a steady state with the nitrogen removal rate (NRR) of 0.40 g N $L^{-1}d^{-1}$ and a nitrogen loading rate (NLR) of 0.49 g N $L^{-1}d^{-1}$; also, both the ammonium and nitrite removal efficiencies achieved over 96%.

After running for 123 days, 5 g NaCl/L was added into the influent in phase II (day 124–207). During the initial 20 days after the living environment changed from freshwater to salty water, the anammox bacteria activity was negatively impacted, leading to a slight decline in the NRR.

Specifically, the NRR declined by 15% from 0.40 g $NL^{-1}d^{-1}$ (day 124) to 0.34 g $NL^{-1}d^{-1}$ (day 138), while the ammonium and nitrite removal efficiencies decreased to 83% and 86% respectively. Thereafter, the anammox system started to recover gradually. On day 155, the reactor regained the stable nitrogen removal capacity; the NRR stabilized at 0.39 g $NL^{-1}d^{-1}$ with a constant NLR of 0.46 g $NL^{-1}d^{-1}$, indicating that anammox bacteria had adapted to the salt stress.

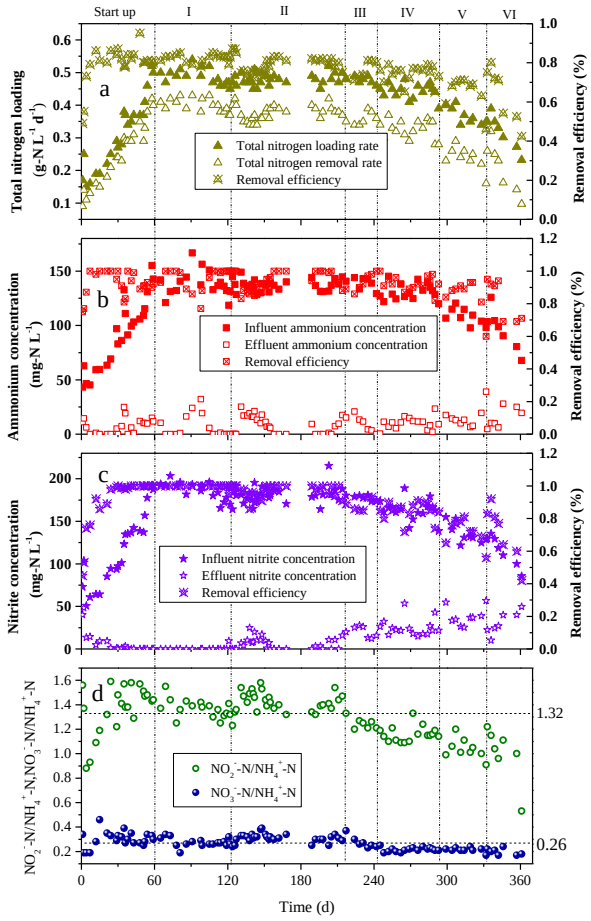


Figure 1. The long-term performance of the anammox reactor at different salinity levels (a, b, c and d represent the changes in the nitrogen load, ammonia concentration in the influent and effluent, nitrite concentration in the influent and effluent, and the stoichiometric parameters under different salinity levels, respectively. I (0 g NaCl/L), II (5 g NaCl/L), III (10 g NaCl/L), IV(20 g NaCl/L), V (30 g NaCl/L), VI (40 g NaCl/L))

During phase Ⅲ (day 208–244), the anammox reactor was fed with 10 g NaCl/L. The nitrogen removal performance was decreased initially and then increased gradually. Similar to the case of 5 g NaCl/L, the performance of nitrogen removal decreased greatly during the first 20 days that the ammonium removal efficiency declined from 96% (day 208) to 86% (day 224), and nitrite removal efficiency decreased from nearly 100% to 86%. Nevertheless, after 20 days of acclimatization, the NRR was gradually stable at 0.38 g N $L^{-1}d^{-1}$ under the NLR of 0.48 g N $L^{-1}d^{-1}$.

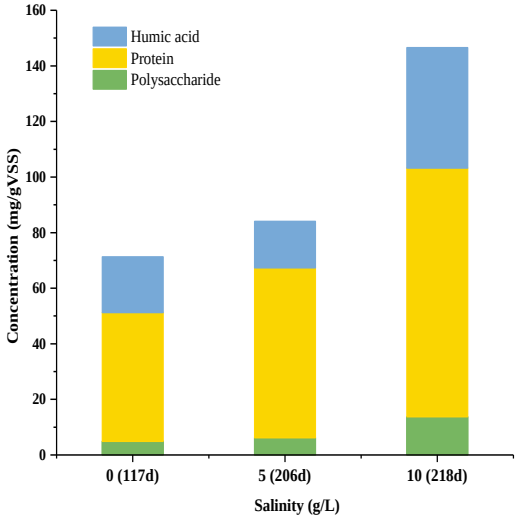


Figure S1 (supplementary). Variations in the EPS content and composition of the anammox sludge at different salinity levels (second row represent the number of days)

When the influent salinity increased to 20 g NaCl/L during phase Ⅳ (day 245–289), the NLR was 0.45 g N $L^{-1}d^{-1}$, but the NRR decreased to 0.29 g N $L^{-1}d^{-1}$ with the ammonium removal efficiency of 89% and the nitrite removal efficiency of 83% on day 269. At the end of phase Ⅳ, the TN removal efficiency improved with the NRR of 0.35 g $NL^{-1}d^{-1}$ upon the acclimatization to the salt stress. Then, the salt concentration was further increased to 30 g NaCl/L in phase Ⅴ (day 290–332). The nitrogen removal capacity of the anammox reactor became poor. The NRR was 0.28 g $NL^{-1}d^{-1}$ on day 290, but decreased to 0.16 g $NL^{-1}d^{-1}$ on day 332, confirming the high salt concentration had posed a negative effect on the anammox performance at a long-term operation.

After the salt concentration continued to increase to 40 g NaCl during phase Ⅵ (day 333–361), the reactor performance became worse. The NRR continuously decrease from 0.16 g $NL^{-1}d^{-1}$ to 0.10 g $NL^{-1}d^{-1}$ during a 29–day span at the NLR of 0.32 g $NL^{-1}d^{-1}$. In this phase, the water density with high salinity was too high to maintain a good retention of the biomass, leading to a significant loss

of the anammox bacteria inside the system. Consequently, the anammox reactor tended to collapse.

3.2 Evolution of microbial community structure with elevated salinity

3.2.1 Bacterial richness and biodiversity variation

Six sludge samples at different salinities, i.e., S1 (0 g NaCl/L), S2 (0 g NaCl/L), S3 (10 g NaCl/L), S4 (20 g NaCl/L), S5 (30 g NaCl/L) and S6 (40 g NaCl/L), were collected under a steady state of different phases. 16S rRNA high-throughput sequencing was conducted to elucidate the microbial community structure and dynamics of the studied anammox reactor with gradually elevated salinity.

The Shannon-Wiener curves obtained for each sludge sample are shown in Fig. 2. All the curves reached a plateau, suggesting that taxonomic diversity was almost fully exploited by the Miseq sequencing. Also, the coverage of each sample reached saturation over 0.99 (Table 2), indicating that the coverage degree is high enough to reflect the whole information about the bio-system.

The multiple α-diversity indices are shown in Table 2. When the salinity increased from 0 to 20 g NaCl/L, Shannon and Simpson indices showed a decrease trend, corresponding well to the species richness indices (Ace and Chao), implying concomitant disappearance of some species. These results revealed that the selective pressure, including the osmotic environment and the settling properties, would wash out the bacterial species who could not survive in the salt stressful condition. Then, the species richness and diversity showed an increasing trend as the anammox system adapted to the elevated salinity from 20 to 40 g NaCl/L.

Table 2. Alpha-diversity of the anammox sludge samples at different salinities

Sample	Salinity (g/L)	0.97*					
		OTU	Ace Index	Chao Index	Coverage	Shannon Index	Simpson Index
S1	0	525	584	600	0.995796	4.53	0.0316
S2	5	426	499	497	0.995843	3.81	0.0634
S3	10	382	421	425	0.997166	3.96	0.0462
S4	20	285	326	327	0.997355	2.61	0.2428
S5	30	322	399	398	0.995937	2.03	0.4462
S6	40	321	378	384	0.996788	3.19	0.1614

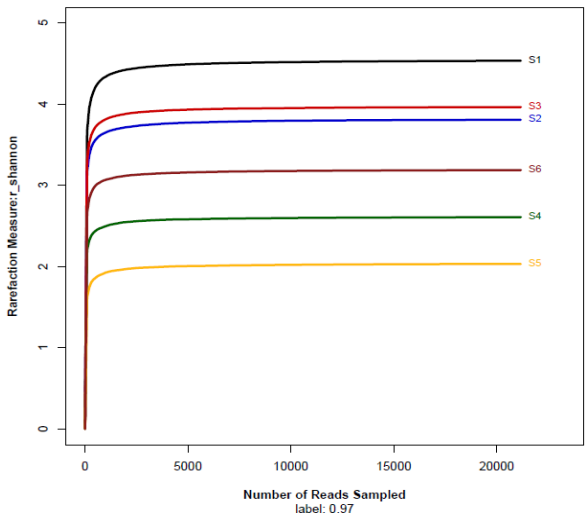


Figure 2. Shannon-Wiener curve of the anammox sludge sample S1 (0 g NaCl/L), S2 (5 g NaCl/L), S3 (10 g NaCl/L), S4 (20 g NaCl/L), S5 (30 g NaCl/L), S6 (40 g NaCl/L)

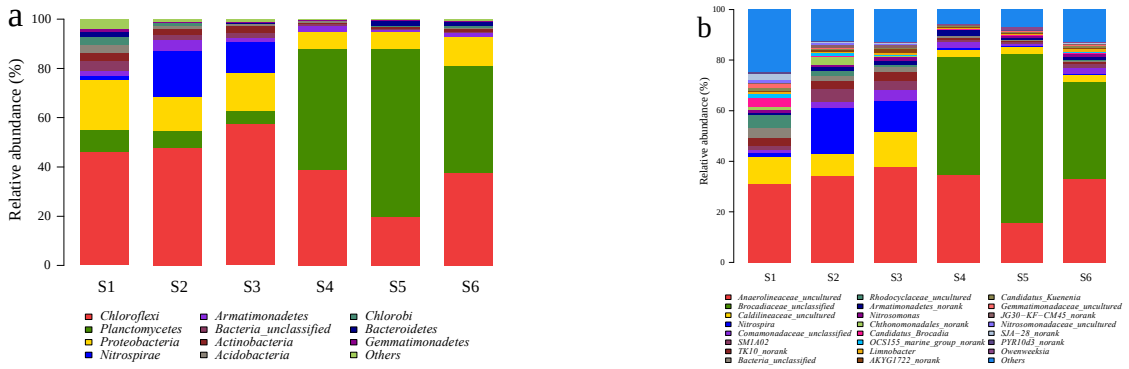


Figure 3. The bacterial flora distribution histogram at the level of "phylum" (a) and "genus" (b) of the anammox sludge samples S1 (0 g NaCl/L), S2 (5 g NaCl/L), S3 (10 g NaCl/L), S4 (20 g NaCl/L), S5 (30 g NaCl/L), S6 (40 g NaCl/L)

3.2.2 Microbial community composition and dynamics

The phylogenetic classification of effective bacterial sequences at the phylum level is summarized in Fig. 3a. Clearly, the bacterial community composition at different salinities was distinct. The dominant phyla across samples S1, S4, S5 and S6 were *Chloroflexi*, *Planctomycetes* and *Proteobacteria.* While, the dominant phyla in both samples of S2 and S3 were *Chloroflexi*, *Planctomycetes*, *Proteobacteria* and *Nitrospira.* It has been reported that *Proteobacteria* is the predominant phylum in various of full-scale A/A/O systems, MBR systems, and the oxidation ditch processes (Hu *et al.,* 2012). Venter *et al.,* (2004) also found that *Proteobacteria* was dominant in the marine microbial community of the surface/sub-euphotic layers, and they concluded that salinity was closely related to *Proteobacteria*. Their findings can partially explain our findings that the relative abundance of *Proteobacteria* slightly decreased from 20.29% to 14.05% when the salinity addition increased from 0 to 5 g NaCl/L, and remained at 12.09% with 40 g NaCl/L addition (Fig. 3a).

Notably, *Chloroflexi,* as a common sort of filamentous bacteria, was the most dominant phylum in the anammox system at 0, 5 and 10 g NaCl/L. They maintained at a relatively high abundance following to *Planctomycetes* at 20, 30 and 40 g NaCl/L salinities. However, the high amounts of *Chloroflexi* did not lead to the sludge bulking but promoted the granular process for the anammox flocs (Fig. 5b and c). Bossier and Verstraete, (1996) addressed that the filamentous organisms would provide a stabilizing backbone for the three-dimensional microbial aggregates called flocs. *Chloroflexi* is also ecologically significant and responsible for degradation of carbohydrates and cellular materials (Miura *et al.,* 2007). Kindaichi *et al.,* (2012) found that *Chloroflexi* was frequently detected in an anammox reactor, and they can scavenge organic matter derived from anammox bacterial cells to survive with the medium without external organic carbon compounds. The existence of *Chloroflexi*, being heterotrophic bacteria, in anammox autotrophic nitrogen removal reactor, would utilize the soluble microbial products (SMP) and EPS released by the autotrophs, for metabolism (Chu *et al.,* 2015). These results supported our findings that the inhibition of *Chloroflexi* by the high salinity (≥ 20 g NaCl/L) as the decline of the relative abundance in this study deteriorated the sludge morphological and physical properties.

Planctomycetes, which contains all known anammox genera (Strous *et al.,* 1999), became the most dominant phylum after the acclimatization of elevated salinity to 20 g NaCl/L. There was a slight decline in the relative abundance of *Planctomycetes* when the salinity rose to 5 g NaCl/L and 10 g NaCl/L. Surprisingly, the relative abundance of this phylum at the salinity of 20, 30 and 40 g NaCl/L were sharply increased to 49.3%, 68.48% and 43.13%, respectively. It seems that the inhibition of the high salinity (≥ 20 g NaCl/L) was more severe to the other phyla than to *Planctomycetes.*

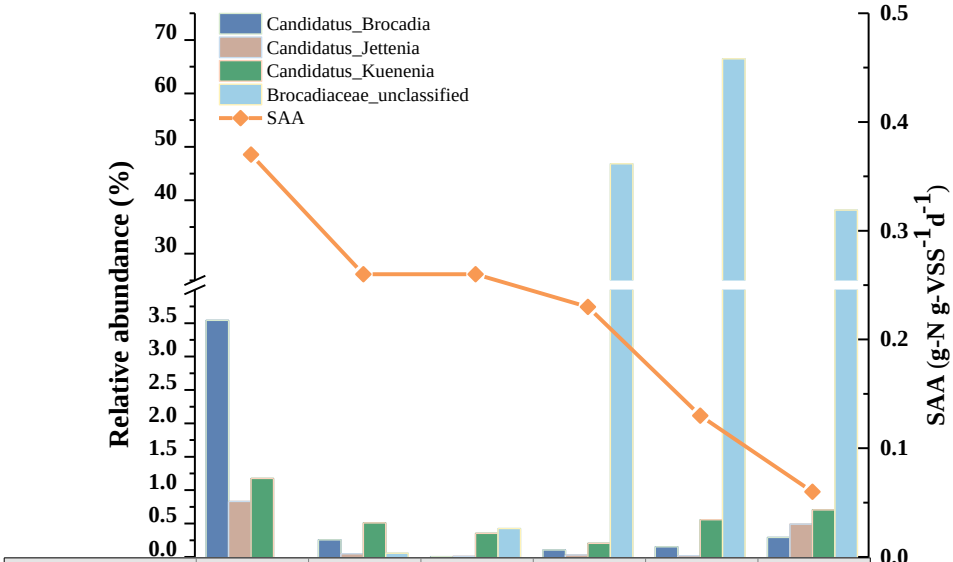
The phylum *Nitrospira,* affiliated to nitrite oxidizing bacteria (NOB) (Moussa *et al.,* 2006), was detected as a predominant phylum in samples of S2 and S3. Relative to the other samples, the relative abundance of *Nitrospira* accounted for the highest proportion in S2. Correspondingly, at 5 g NaCl/L salinity, the stoichiometric ratios of NO_2^--N conversion to NH_4^+-N consumption (R_S) was higher than the theoretical ratio of 1.32 (Fig. 1d),

indicating the occurrence of the nitrite oxidization by NOB in the studied anammox system. At 10 g NaCl/L, a slight decline occurred in the relative abundance of *Nitrospira,* and the corresponding R_S was maintained at 1.32. Then, *Nitrospira* displayed a dramatic decline from 12.65% to 0.39% when the salinity reached 20 g NaCl/L, even being absent from the system with the salinity over 20 g NaCl/L. Therefore, it could be inferred that Nitrospira could easily survive at 5 g NaCl/L, but were inhibited while the salinity elevated over 10 g NaCl/L. Indeed, it has been reported that the specific nitrification rate was significantly inhibited and the nitrite accumulation occurred while the salinity exceeded 10 g/L Cl (equal to16.5 g NaCl/L) (Chen *et al.* 2003).

3.2.3 Anammox bacteria genus variations

Analyzing microorganisms at the genus level allows a deeper understanding of the community function in the microbial ecosystem (Fig. 3b). The dominant genera as well as the distinct bacterial community were *Brocadiaceae_unclassfied*, *Caldilineaceae_uncultured* and *Nitrospira*. *Ca. Brocadia*, *Ca. Kuenenia* and *Ca Jettenia,* which affiliated with anammox bacteria, were detected at all salinities, though with a disparity of the distribution and the relative abundance. The relative abundance of *Ca. Brocadia*, *Ca. Jettenia* and *Ca. Kuenenia* in S1 were at the maximum values of 3.543%, 0.831% and 1.176%, respectively, among the all samples (Fig. 4).

After acclimatization to 5 g NaCl/L salinity for 62 days, the relative abundance of *Ca. Kuenenia* encountered a slight decline by 56.7%. Similarly, the relative abundance of *Ca. Brocadia* and *Ca Jettenia* greatly declined from 3.543% to 0.255%, and from 0.831% to 0.047% with decrease percentages of 93% and 94%, respectively (Fig. 4). It seems that *Ca. Brocadia* and *Ca. Jettenia* were extremely sensitive to salinity. Indeed, Sonthiphand *et al.,* (2014) explored global distributions and diversity of anammox bacteria to identify factors that influence their biogeography, and found that neither *Ca. Brocadia* nor *Ca. Jettenia* were associated with saline-related environment. Also, the 50% inhibitory concentration (IC_{50}) of *Ca. Jettenia* was not high, being only 68 mM for sodium chloride (equal to 4 g NaCl/L) (Ali *et al.,* 2015).



	S1	S2	S3	S4	S5	S6
Candidatus_Brocadia	3.543%	0.255%	0.005%	0.104%	0.151%	0.293%
Candidatus_Jettenia	0.831%	0.047%	0.009%	0.028%	0.019%	0.491%
Candidatus_Kuenenia	1.176%	0.510%	0.354%	0.203%	0.557%	0.704%
Brocadiaceae_unclassified	0%	0.057%	0.425%	46.828%	66.475%	38.169%

Figure 4. Changes in the relative abundance of total anammox bacteria and corresponding specific anammox activities (SAA) of sample S1 (0 g NaCl/L), S2 (5 g NaCl/L), S3 (10 g NaCl/L), S4 (20 g NaCl/L), S5 (30 g NaCl/L), S6 (40 g NaCl/L)

After the salinity increased from 5 to 10 g NaCl/L, the relative abundance of these three genera of anammox bacteria continued to decline (Fig. 4). *Ca. Brocadia* and *Ca. Jettenia* almost disappeared from the system with the

relative abundance of only 0.005% and 0.009%, respectively. The relative abundance of *Ca. Kuenenia* also reduced from 0.510% (5 g NaCl/L) to 0.354% (10 g NaCl/L). Fortunately, after the salinity increased above 20 g NaCl/L, these three genera of anammox bacteria began to adapt to the saline environment that a rising trend of their relative abundance occurred, though they were still lower than the initial value when without NaCl addition. The relative abundance of *Ca. Kuenenia* increased from the minimum value of 0.203% at 20 g NaCl/L to 0.557% when the salinity up to 30 g NaCl/L and continued to increase to 0.704% under 40 g NaCl/L. This result was consistent with the previous study that the freshwater anammox bacteria species *Ca. Kuenenia* could adapt to the salt concentration as high as 30 g NaCl/L and sustain proper anammox activity (Kartal *et al.* 2006). Likely, the relative abundance of *Ca. Brocadia* and *Ca. Jettenia* increased slightly to 0.293% from 0.104% and 0.491% from 0.028% respectively, when the salinity increased from 20 to 40 g NaCl/L. Similar results were also observed by Liu *et al.* (2009), who found that anammox bacteria affiliated with *Ca. Jettenia* could adapted to high salinity and the anammox activity was maintained constant after the salinity elevated to 30 g NaCl/L.

Interestingly, after the salinity increased from 5 to 40 g NaCl/L, *Brocadiaceae_unclassified*, which likely belong to anammox bacteria, as all of the detected anammox bacteria are affiliated with the family of *Brocadiaceae*, was the only dominant species in the sludge samples. As shown in Fig. 4, the relative abundance of this genus increased stepwise with the elevated salinity. Particularly, when the salinity increased to 20 g NaCl/L, *Brocadiaceae_unclassified* increased remarkably and eventually occupied a high portion of 46.828%, becoming the most dominant genus in the anammox system even though it was not detected in the initial sludge sample S1. The relative abundance of *Brocadiaceae_unclassified* continued to increase to a peak value of 66.475% at 30 g NaCl/L, and declined slightly to 38.169% at 40 g NaCl/L salinity. It is possible that *Brocadiaceae_unclassified* is a novel clusters affiliated with anammox.

3.3 Specific anammox activity of biomass

The SAA of the biomass at the elevated salinity was evaluated using anaerobic batch tests. As shown in Fig. 4, the maximum SAA was 0.37 g N g $VSS^{-1}d^{-1}$ at 0 NaCl g/L. A stepwise decline in the SAA occurred along the increasing influent salinity, indicating that salinity had a negative impact on the anammox activity even at a low level. Compared to the initial state that without salt addition, the salinity of 5 g NaCl/L caused a 29.7% loss in SAA with the value of 0.26 g N g $VSS^{-1}d^{-1}$. Luckily, the SAA maintained around 0.26 g N g $VSS^{-1}d^{-1}$ at 20 g NaCl/L salinity through 10 g NaCl/L acclimation. When the salinity increased to 30 g NaCl/L, there was a notable decline in the SAA with the value of 0.13 g N g $VSS^{-1}d^{-1}$, being 64.9% lower than that of 0 g NaCl/L. Then the SAA continued to decline to 0.06 g N g $VSS^{-1}d^{-1}$ at 40 g NaCl/L, indicating that the anammox activity was almost totally inhibited. The great decrease in the SAA at 40 g NaCl/L suggests that 30 g NaCl/L might be the threshold for anammox bacteria in the studied system. A similar threshold value was also reported by a previous study that anammox bacteria could retain activity at the salt concentration as high as 30 g NaCl/L (Kartal *et al.* 2006).

3.4 The morphology of anammox sludge with increasing salinity

Fig. 5 shows the morphology of anammox sludge (by SEM) along the increasing salinities (0, 5, 10, 30 and 40 g NaCl/L). When without salt addition, there were many cavities inside the anammox sludge, indicating that the produced N_2 would be attached to the inner layers of the sludge through these cavities, which caused a decrease in the sludge density and enhanced the buoyancy as well. Strous *et al.,* (1997) demonstrated that the nitrogen gas bubbles would become entrapped in the flocs, lifting up clusters of agglomerated beads due to a combination of clogging and intense N_2 production. These factors resulted in the flotation of anammox sludge with a high wash-out of biomass. Indeed, anammox bacteria tend to being washed out of the reactor by intense gas production attached to the outer and inner layers of the sludge (Yang *et al.,* 2011).

When the salt concentration increased to 5 and 10 g NaCl/L, the inner layers of the granules became much densely populated, and there were fewer cavities for the transfer of nitrogen gas (Fig. 5b and c), indicating that there formatted more granules with well-defined structure under a low salinity. The granule flotation may be effectively prevented by either the capture and degradation to the intense gas bubbles of dissolved salt in

the substance by the anammox biomass or friction and collision between the granules and the dissolved salt (Yang *et al.,* 2011). Fernández *et al.,* (2008) also reported that the presence of NaCl (5-10 g NaCl/L) can promote the granulation of anammox sludge. By changing the sludge physical properties from flocculent to granular, the settling property of the biomass was sharply enhanced, contributed to a better effluent quality of the studied anammox system. After the salinity increased from 30 to 40 g NaCl/L, the surface of the sludge became loose and rough, promoting the decaying of anammox bacterial cellular materials (Fig. 5d and e). Meanwhile, the water density with high salinity was too high to maintain a good retention of the biomass inside the anammox system and a considerable washout of solids was observed by the increased turbidity of the supernatant. Bassin *et al.,* (2012) also found an increased supernatant turbidity resulted in higher washout of biomass with the elevated salinity. The findings demonstrated that the high salt concentration (≥ 30 g NaCl/L) would inhibit the growth and metabolism of microbes, and regulated the sludge morphology and the microbial structure as well.

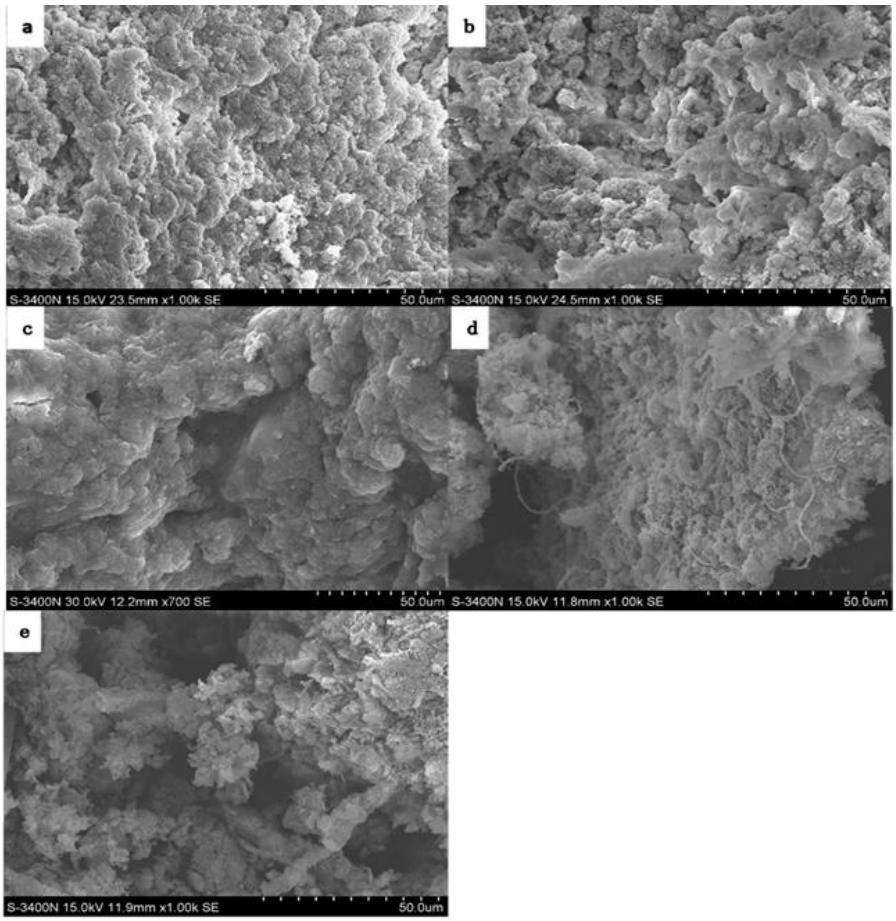


Figure 5. The morphology of the cultivated anammox sludge at different salinities (by scanning electron micrographs) (a, b, c and d represent the sludge samples conducted at NaCl salinities of at 0, 5, 10, 30, 40 g/L respectively)

3.5 EPS variations with increasing salinity

EPS producing favors the survival of microorganisms under various circumstances (Liu *et al.,* 2004). At high osmotic environment, EPS are necessary for anammox bacteria to survive in the harmful high salinity conditions. In general, EPS are composed of polysaccharide, proteins and humus. With the successful acclimatization under the relatively low salinity (≤10 g NaCl/L), anammox bacteria generated more EPS to resist the increasing salt stress (Fig. S1). Abbasi and Amiri, (2008) also illustrated that producing EPS is a bacterial reaction to stressful conditions of the environment (e.g. osmotic pressure). Thus, the secretion of more EPS can be a protection response for anammox bacteria under the different salt stress, ensuring the stable NRR of the anammox bioreactor.

4. Conclusions

The main results are summarized as follows:

1) Robust anammox performance was achieved at salinity of 0–20 g NaCl/L with the steady nitrogen removal rate approximately 0.40 g N $L^{-1}d^{-1}$ and nitrogen removal efficiency over 80%. 30 g NaCl/L could be the threshold value of the studied anammox bioreactor.

2) Salinity is a key environmental factor regulating the anammox bacterial community. 16S rRNA high-throughput sequencing results showed that although both *Ca. Brocadia* and *Ca. Jettenia* were sensitive to salt, they still adapted to the high salinity environment and survived in the anammox system. *Ca. Kuenenia* can even adapt to the salinity up to 30 g NaCl/L. Whereas, *Brocadiaceae_unclassified* which possibly belong to anammox bacteria, became a novel cluster coexisted with anammox bacteria at high salinities ≥ 20 g NaCl/L, and likely contributed a part of the nitrogen removals.

3) The anammox granules became dense at the low salinity (≤ 10 g NaCl/L) but loose and tough at high salinities (≥ 30 g NaCl/L). Our findings confirmed that a low level salinity would favor anammox sludge to form the well-defined granular structure while the high salinity would collapse granules.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (NSFC) (nos. 51522809 and 51378370). The Fundamental Research Funds for the Central University (Tongji University) (0400219238) and the foundation of State Key Laboratory of Pollution Control and Resource Reuse (Tongji University), China (PCRRY 0400231010), are also acknowledged.

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