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Impact of elevated salinity on anammox-UASB systems: Performance and microbial community analysis

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Abstract. Anaerobic ammonia oxidation (Anammox) faces challenges in high salinity environments due to inhibited microbial activity, while upflow anaerobic sludge bed (UASB) reactors maintain a higher biomass concentration. To explore how the Anammox-USAB system responds to the high salinity (NaCl) environment, a UASB reactor seeded with heterotrophic nitrification sludge. The salinity was gradually increased from 0 to 40 g NaCl/L. The results show that, when salinity increased from 0 to 15 g NaCl/L, the conversion rate of ammonia nitrogen ($NH4^+$-N) and total nitrogen (TN) decreased by about 25% and 22 %, respectively. At the same time, the ammonium removal load of unit sludge gradually stabilized at about 3.65 mg $NH4^+$-N/g VSS over 10 g NaCl /L. When the salinity gradient increased to 30 g NaCl/L, microorganisms preferentially increased their polysaccharide (PS) content from 5.50 to 8.26 mg/g VSS to resist the high osmotic pressure environment. Notably, extracellular protein increased significantly, from 5.78 to 29.01 mg/g VSS to stabilize the cell structure and maintain metabolic activities at 40 g NaCl/L. With the increase of salinity, some salt-intolerant bacteria were inhibited or killed, resulting in a continuous decline in abundance while the abundance of salt-tolerant bacteria increased. The abundance of dominant species Candidatus Kuenenia and Halomonas increased from 5.97% and 0.53% to 12.29% and 12.17%, respectively. It could be seen that the Anammox-USAB system used the structural adjustment of the microbial itself and community as an adaptive strategy in response to the changes of the high-salinity environment.

Keywords: anammox; denitrification; high ammonia nitrogen wastewater; high salinity; UASB

1. Introduction

Anammox, a novel biological deamination process [1], presents distinct advantages over traditional biological denitrification methods. It obviates the need for additional aeration and an organic carbon source, boasts a low sludge yield, and exhibits high denitrification efficiency [2,3]. Consequently, the Anammox process not only saves operating cost and energy consumption but also enables efficient biological denitrification [4]. However, anaerobic ammonia oxidation bacteria (AnAOB) have strict requirements on external conditions. Their effect of treating high salt and high $NH4^+$-N wastewater is easily affected by salinity fluctuations, which hinders the

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application of anaerobic ammonia oxidation process in actual engineering [5]. Hence, enhancing AnAOB enrichment and ensuring the stability of the Anammox process have become significant areas of research.

Salinity impacts the treatment efficiency of the Anammox system through osmotic pressure, enzyme activity, and sludge composition [6]. It can severely affect microbial morphology and intracellular enzyme reactions, inhibiting growth and metabolism, and ultimately influencing denitrification [7]. Meng et al. discovered that at a salinity of 20 g NaCl/L, the heme proteins associated with the color and activity of ammonia - oxidizing bacteria (AOB) declined sharply [8]. This finding provides one possible explanation for the salinity-induced inhibition of AOB activity. Moreover, several studies have indicated that salinity is a crucial environmental factor in regulating AnAOB communities [7]. Most experiments exploring the effects of salinity on anaerobic systems are conducted at low salinity levels (5 to 20 g NaCl/L), with 15 g NaCl/L generally regarded as the threshold at which the treatment efficiency of anaerobic systems begins to be suppressed [9]. For instance, Su and Yang (2022) carried out a comparative experiment in a sequencing batch reactor (SBR) and found that salinity above 14 g NaCl/L inhibited the contaminant-handling capacity of AnAOB [10]. Previous research has shown that when the salinity increased to 21 g NaCl/L, the ratio of the change in nitrous nitrogen to the change in ammonia nitrogen ($\triangle NO2^{-}$-N: $\triangle NH4^{+}$-N) in the system decreased to 0.68, significantly deviating from the theoretical value of 1.32, indicating a substantial impact on AnAOB activity [11]. At salinities above 30 g NaCl/L, sludge activity is lost [12]. Even after an extended period of adaptation, a salinity of 35 g NaCl/L led to the breakdown of Anammox performance, and salinity also caused microorganisms to develop more compact and complex structures [13].

Heterotrophic nitrification sludge has certain advantages compared to other types of sludge. It has a short inoculation time [14] and contains abundant nitrifying bacteria such as Proteobacteria and Chloroflexi, which are associated with nitrogen removal [15]. Some studies suggest that autotrophic nitrifying bacteria are often eliminated over long periods in high-salt environments, while certain heterotrophic nitrification strains display good salt tolerance [16]. Additionally, when used as inoculation sludge, nitrobacteria have a better effect on enriching nitrogen-metabolism-related functional genes [17]. The up-flow anaerobic sludge blanket (UASB), a typical anaerobic reactor, has a lower risk of sludge granule washout and can maintain a high biomass concentration, resulting in a high-quality effluent [18]. Given that the inoculated sludge varies across different studies, the Anammox system's response to salinity also differs. While a few studies have enabled Anammox sludge to tolerate 15 or 20 g NaCl/L salinity through extended salinity acclimation [19, 20], most experimental results indicate that Anammox sludge activity is severely impaired or the system gradually collapses under high salinity [21, 22]. Based on these findings, it is urgent to study the response mechanism of AnAOB and microbial strategies in high-salt environments.

In this experiment, a UASB reactor was utilized to inoculate heterotrophic nitrified sludge, and the salinity was gradually increased from 5 g NaCl/L to 40 g NaCl/L. The study examined changes in the extracellular polymer substance (EPS) composition, the PN/PS ratio, microbial adaptability, and the ammonium removal load per unit sludge within the Anammox - UASB system under high-salinity conditions (30 and 40 g NaCl/L). Furthermore, the changes in the content and composition of EPS were analyzed, and an effort was made to explain the changes in microbial community dynamics. These results offer a fundamental reference for the application of the Anammox process in practical engineering.

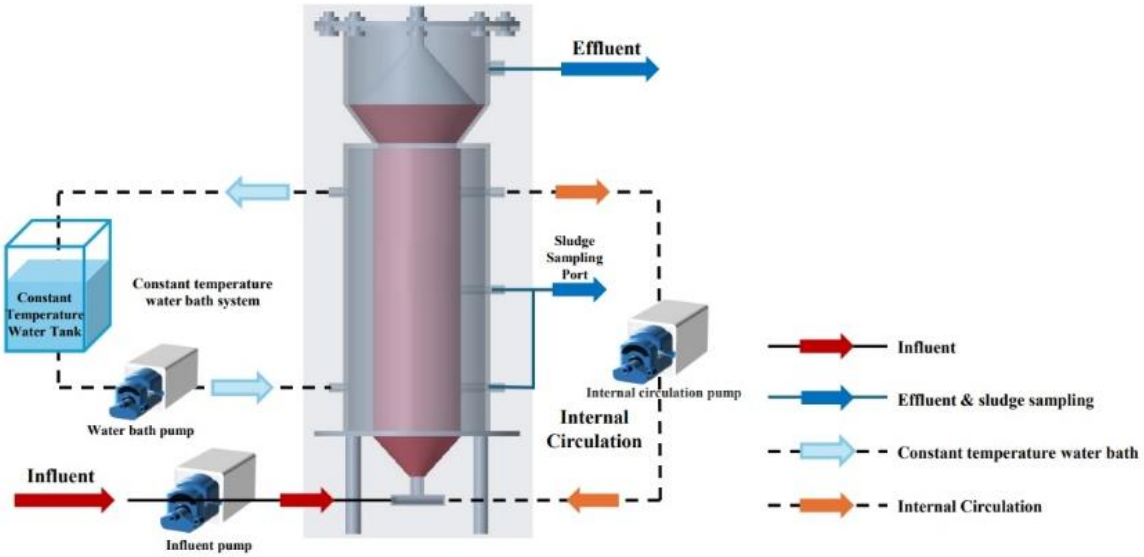

Figure 1. Anammox-UASB reaction system

2. Materials and methods

2.1 Design and operation of the reactor

Under gradient salinity conditions, the setup for initiating the Anammox process is depicted in Fig. 1 The UASB reactor is made of organic glass, with a total volume of 4 L and an effective volume of 2.5 L. This reactor is equipped with a solid - liquid - gas three - stage separator. Water is pumped in at the bottom using a peristaltic pump and exits from the top. Any gas generated during the reaction is captured by the separator and then vented out. Some of the activated sludge is separated and accumulates in the separator; subsequently, it sinks back down under the influence of gravity. A thermostatic water bath is installed outside the reactor (30 ± 1°C). The outer layer of the reactor is covered with a black light - shield to minimize the potential impact of light on the reaction. The hydraulic residence time (HRT) is set at 0.085 days. Throughout the operation, the dissolved oxygen (DO) level is carefully kept below 0.2 mg/L.

2.2 The synthetic wastewater and protocol

Artificial simulated wastewater served as the test inlet water. Its components, measured in mg/L, were as follows: KH_2PO_4 at 8.77, $NaHCO_3$ at 500, $CaCl_2 \cdot 2H_2O$ at 50, $MgSO_4 \cdot 7H_2O$ at 50, along with trace elements. For the settings of the salinity gradient, and the concentrations of NH_4Cl and $NaNO_2$, refer to Table 1. Notably, the salinity gradient in this experiment was solely adjusted by additional NaCl. Other nitrogen-containing salts (NH_4Cl, $NaNO_2$) maintained stable and low concentrations, and their contribution to total salinity was negligible. The trace element solution, added at a rate of 1 ml/L, had the following components and content (mg/L): EDTA at 1000, $FeSO_4 \cdot 7\ H_2O$ at 5000, $ZnSO_4 \cdot 7\ H_2O$ at 430, $MnCl_2 \cdot 4H_2O$ at 990, $CuSO_4 \cdot 5H_2O$ at 250, H_3BO_3 at 14, $NiCl_2 \cdot 6\ H_2O$ at 190, $CoCl_2 \cdot 6\ H_2O$ at 240.

Table 4. Quantitative analysis of EDS spectra (weight % and atomic %)

Stages	Time (d)	NaCl (g/L)	NH_4^+-N (mg/L)	NO_2^--N (mg/L)
1	1 - 10	5	200	220
	11 - 20	10		
	21 - 30	15		
	31 - 49	30		
2	50 - 70	30	100	110
3	71 - 100	40		

2.3 The characteristics of inoculating sludge

The inoculated sludge was obtained from heterotrophic nitrification sludge that had been steadily cultured in the laboratory. Prior to the current experiment, the UASB reactor operated with synthetic wastewater stably for over a year. The sludge morphology was brick-red flocculent at the beginning of the test. The concentration of mixed suspended solids (MLSS) in the reactor at the beginning of the test was about 9420 mg/L, the mass concentration of volatile suspended solids (MLVSS) in the mixed solution was about 7728 mg/L. These initial characteristics of the inoculated sludge were fundamental parameters that would influence the performance of the Anammox-UASB system under the varying salinity conditions investigated in the study.

2.4 Analytical methods

Daily water samples from both the inlet and outlet of the system were collected. The concentrations of NH_4^+-N, NO_2^--N, and nitrate nitrogen (NO_3^--N) in these samples were measured. Based on these measurements, the volumetric nitrogen load, volumetric nitrogen removal load, and volumetric nitrogen removal efficiency within the system were calculated. The sludge concentration was also monitored at regular intervals to analyze the changes in denitrification.

For NH_4^+-N, Nessler's reagent spectrophotometry was used. The determination of NO_2^--N was determined by N-(1-naphthyl)-ethylenediamine spectrophotometry while NO_3^--N was measured by ultraviolet spectrophotometry. MLSS and MLVSS were determined by weight method.

Regarding EPS, the extraction and determination methods followed those described by Li et al. Li [23]. The method was used to extract tight bound EPS (TB-EPS) and loose bound EPS (LB-EPS), respectively. The content of PS was determined using sulfuric acid-anthrone colorimetric method, and the content of PN was determined by Coomassie brilliant blue (G-250) method [24].

The collected sludge samples were entrusted to Sangon Biotech (Shanghai) Co., Ltd. for sample DNA extraction, PCR amplification and high-throughput sequencing. High-throughput sequencing was performed through the Illumina platform. DNA was extracted from the samples using E.Z.N.A MAG-BINDSOIL DNA KIT, using bacterial 16S rRNA gene V4 amplified region with primers of 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT).

The calculation methods for the volumetric nitrogen loading rates (NLR, kg•N/(m^3•d)), volumetric nitrogen removal rates (NRR, kg•N/(m^3•d)) and volumetric nitrogen removal efficiency (NRE, %) in the in-reaction system are shown in Eqs. (1-3), respectively [25].

$$NLR = TN_{\text{influent}}/(1000 \times HRT) \quad (1)$$

$$NRR = (TN_{\text{influent}} - TN_{\text{effluent}})/(1000 \times HRT) \quad (2)$$

$$NRR = (TN_{\text{influent}} - TN_{\text{effluent}})/TN_{\text{influent}} \times 100 \quad (3)$$

The calculation method of TN (mg/L) in the inlet and outlet water is shown in Eqs. (4-5).

$$TN_{\text{influent}} = (NH_4^+ - N)_{\text{influent}} + (NO_2^- - N)_{\text{influent}} \quad (4)$$

$$TN_{\text{effluent}} = (NH_4^+ - N)_{\text{effluent}} + (NO_2^- - N)_{\text{effluent}} + (NO_3^- - N)_{\text{effluent}} \quad (5)$$

3. Results and discussions

3.1 Effects of salinity on NH4+-N and TN conversion

The concentration changes of NH_4^+-N, NO_2^--N, and NO_3^--N in the inlet and outlet water, the conversion rates of NH_4^+-N and NO_2^--N, and the TN changes during the treatment in the Anammox-UASB reactor are shown in Fig. 2 As shown in Fig. 2a, during the first stage (0-49 d), the sludge was in an adaptation stage. Overall, the NH_4^+ - N conversion efficiency decreased gradually as the salinity gradient changed. At a salinity of 10 g NaCl/L, the concentrations of NH_4^+-N and NO_2^--N in the effluent started to rise, and the corresponding conversion rate dropped significantly. Specifically, it decreased by approximately 11.90% compared to the initial reduction rate. Nevertheless, around 20 d, the conversion rates of NH_4^+-N and NO_2^--N increased. In this experiment, this increase occurred towards the end of the 10 g NaCl/L salinity phase. This phenomenon could be attributed to the fact that the inoculated sludge in the system was heterotrophic nitrified sludge. This type of sludge has a relatively high tolerance to salinity, causing a delay in the microorganisms' response to salinity changes [26].

When the salinity was 15 g NaCl/L, the effluent NH_4^+-N concentration peaked at around 150.00 mg/L. This corresponded to the most substantial decline in NH_4^+-N conversion rate, with an overall reduction of approximately 20.00%. Moreover, the decline in the TN conversion rate was the most pronounced at this point, decreasing by 13.14%. The activity of Anammox was obviously inhibited in the system.

When the salinity reached 30 g NaCl/L, the conversion rates of NH_4^+-N and NO_2^--N hit their lowest points, approximately 30% and 40%, respectively. This clearly demonstrated that 30 g NaCl/L exerted a strong inhibitory effect on the system. At this salinity level, the effluent TN concentration fluctuated between 22.74% and 42.76%. The likely cause was that the 30 g NaCl/L salinity inhibited microorganisms. However, the system's resistance changed abruptly, struggling to maintain stability under high salinity and high inlet nitrogen pressure. Generally, a salinity of 30 g NaCl/L is regarded as an acceptable threshold for the Anammox system [27].

The second stage (50-70 d) was the sludge stabilization stage. After reducing the TN in the influent, the TN conversion gradually increased. It rose from 20.82% on day 49 to 48.59% on 70 d, and the effluent TN concentration reached a minimum of 109.40 mg/L during this stage. The increase in the conversion rate indicated that at 30 g NaCl/L, the system's capacity to handle pollutants had decreased. During this stage, the system gradually stabilized, and the conversion of NH_4^+-N and NO_2^--N increased to around 51.90% and 71.80%, respectively.

The third stage (71-100 d) was the sludge limitation stage, with the salinity rising to 40 g

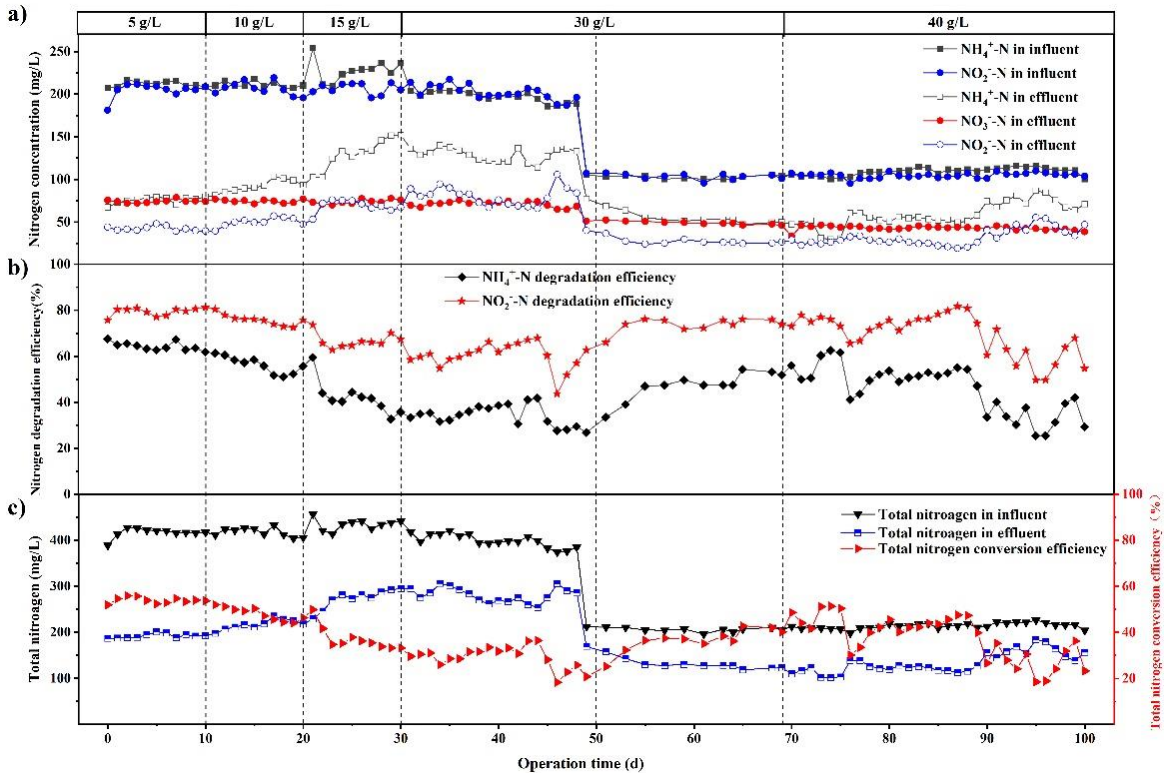

Figure 2. Effect of salinity on the NH_4^+-N removal capacity of the system (a. NH_4^+-N, NO_2^--N, and NO_3^--N in influent and effluent; b. Conversion rates of NH_4^+-N and NO_2^--N; c. TN and conversion rate of influent and effluent)

NaCl/L. Between 71 and 90 d, the ammonium removal efficiency dropped sharply and then gradually increased, eventually maintaining a similar level to that in the second stage. From 90 to 100 d, the overall concentration of NH_4^+-N in the effluent showed an upward trend, ultimately reaching nearly 80.00 mg/L. The conversion efficiency of NH_4^+-N and NO_2^--N also continued to decline, with the final conversion rate of NH_4^+-N being 29.30%. This indicated that a salinity of 40 g NaCl/L had a strong impact on the system, strongly inhibiting the sludge's activity.

The change trend of TN in effluent was similar to that of NH_4^+-N and NO_2^--N in effluent. This was likely because the NO_3^--N concentration in the effluent was relatively stable, and NH_4^+-N and NO_2^--N changed synchronously. The trend of TN thus depended on the combined changes of NH_4^+-N and NO_2^--N.

Additionally, the performance (TN conversion efficiency) from 20 - 30 days (at 15 g NaCl/L) was like that from 30-50 days (at 30 g NaCl/L). Even though the salinity doubled, the conversion efficiency did not deteriorate as anticipated. This could be closely related to the characteristics of the inoculated sludge. As described in section 2.3, the heterotrophic nitrified sludge has demonstrated good salt tolerance in other experiments. Hence, the inoculated sludge was able to adopt a more gradual approach in coping with the high - salt environment.

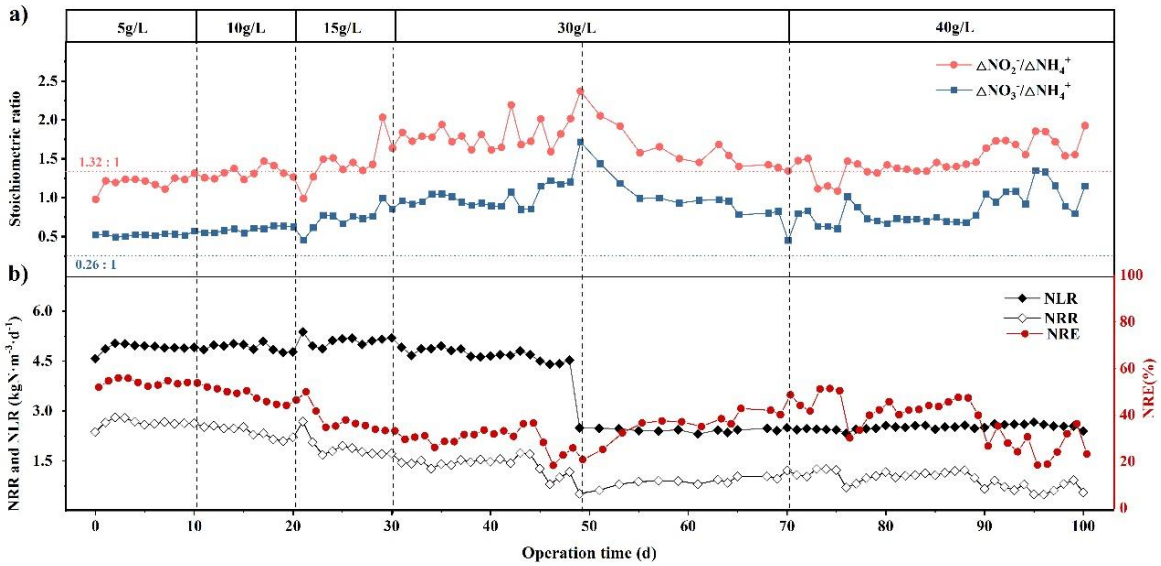

Figure 3. Effect of salinity on the stoichiometric ratio of Anammox reaction and the change of system volumetric nitrogen load (a. change in NH_4^+-N, NO_2^--N, and NO_3^--N metering ratio; b. NLR, NRR, NRE change)

3.2 Impacts of salinity on changes in Anammox reaction stoichiometry and system volumetric nitrogen load

The empirical reaction equation for anaerobic ammonia oxidation is presented in Eq. (6) [28]. According to this, the theoretical ratio of the Anammox reaction should be $\triangle NH_4^+$-N $_{consumption}$: $\triangle NO_2^-$-N $_{consumption}$: $\triangle NO_3^-$-N $_{generation}$ =1:1.32:0.26.

As shown in Fig. 3a, two reference lines are marked. From the trend of the fold line, it is evident that as the salinity gradient rises to 15 g NaCl/L, the ratio of $\triangle NO_2^-$-N: $\triangle NH_4^+$-N is approximately near the theoretical value. However, the ratio of $\triangle NO_3^-$-N: $\triangle NH_4^+$-N gradually deviates from the theoretical value. This could be attributed to the stimulation of salt - tolerant bacteria within the nitrifying bacteria by the salinity.

At a salinity of 30 g NaCl/L, in the first half (30 - 49 d), the Anammox reaction was further inhibited. The ratio of $\triangle NO_2^-$-N: $\triangle NH_4^+$-N was 1.82 ± 0.20, which is 34.90% higher than the theoretical value of 1.32. Simultaneously, the ratio of$\triangle NO_3^-$-N: $\triangle NH_4^+$-N was 1.03 ± 0.20, far exceeding the theoretical value. This indicates that the Anammox reaction was more competitive, and the deamination effect of AnAOB was notably reduced.

During the second half of the 30 g NaCl/L salinity period (50 - 70 d), the ammonium removal efficiency improved, and the ratio of $\triangle NH_4^+$-N: $\triangle NO_2^-$-N: $\triangle NO_3^-$-N gradually approached the theoretical value. This suggests that reducing the concentration of NH_4^+-N and NO_2^--N in the influent may assist the Anammox reaction in dominating microbial metabolisms. The ratio of $\triangle NO_3^-$-N: $\triangle NH_4^+$-N ratio was consistently higher than the theoretical value of 0.26, signifying continuous competition between anaerobic nitrifying bacteria and AOB. The competition was most intense at a salinity of 30 g NaCl/L and weakened after the influent nitrogen decreased.

$$H_4^+ + 1.32NO_2^- + 0.066HNO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{2.5}N_{0.25} + 2.03H_2O \quad (6)$$

As shown in Fig. 3b, during stage 1 (0 to 49d), the NLR remained at 1.22 ± 0.05 kg•N/(m^3•d). At a salinity of 5 g NaCl/L, the NRR remained relatively stable at 0.66 kg•N/(m^3•d), and the NRE fluctuated around 53.00%. At salinities of 10 and 15g NaCl/L, both NRR and NRE showed a downward trend. The NRR decreased from 0.55 kg•N/(m^3•d) to 0.43 kg•N/(m^3•d), and the NRE decreased from 46.37% to 33.23%, with a relatively mild decline.

However, at 30 g NaCl/L, both NRR and NRE reached their lowest values in the first stage. The NRR decreased from 1.73 kg•N/(m^3•d) to 0.52 kg•N/(m^3•d), a decrease of 69.83%. The NRE decreased from 33.23% to 20.82%, a decrease of 37.33%. This phenomenon was consistent with the previous analysis. Microorganisms in the Anammox-UASB system were less inhibited at low salinity. When the salinity exceeded 10 g NaCl/L, the system's denitrification was more inhibited by salinity, and the amount of pollutants that could be removed by sludge per unit volume and unit time continued to decline.

At a salinity of 30 g NaCl/L, the inhibitory effect peaked. During the second stage (50 - 70 d), the NLR was halved to (0.62 ± 0.02 kg•N/(m^3•d)). The NLR then maintained at about 0.35 kg•N/(m^3•d), which is more effective than the 0.28 kg•N/(m^3•d) reported in previous studies [15,29]. During this stage, the NRE increased from 20.82% to 48.59%, demonstrating good system stability.

During stage 3 (71-100 d), the NLR maintained the load from stage 2. A further increase in salinity enabled the system's NRR and NRE to continue the upward trend from stage 2. The decrease of NRR and NRE on 75 d might be due to the microorganisms' delayed response to the higher salinity environment. From 75 to 90 d, the NRE gradually increased to about 50.00%, indicating that microorganisms could adapt to and tolerate high salinity wastewater. After the 90 d, NRE and NRR began to decline erratically. This could be because AOB and AnAOB in microorganisms could only resist the inhibition of high salinity for a limited period, although this requires further verification. There could also be other reasons, such as the accumulation of high salinity in continuous reactors or that the inhibition is a process that may take time.

3.3 Effects of salinity on changes of sludge concentration and ammonium removal load per unit sludge

In the salinity range of 5-10 g NaCl/L, the sludge concentration increased. Salinity stimulated the production of extracellular polymeric substances (EPS) outside the sludge cells. This led to the cells forming a more compact structure, thereby increasing sludge concentration. Additionally, the rise in MLSS could be attributed to the adsorption of salt by microorganisms, further compressing the sludge volume.

However, when the salinity increased from 10 to 15 g NaCl/L, a significant decline in sludge concentration was observed. Once the salinity exceeded 10 g NaCl/L, sludge growth was inhibited. and the ammonium removal load per unit of sludge dropped sharply, from 20.21 to 5.27 mg NH_4^+-N/g VSS.

The ratio of MLVSS to MLSS is an indicator of sludge activity. Generally, this ratio ranges from 0.70 - 0.80 [30]. and a lower value indicates insufficient sludge activity [31]. As shown in Fig. 4, at 5 g NaCl/L, salt stimulated sludge activity. But beyond 10 g NaCl/L, the MLVSS/MLSS ratio decreased with increasing salinity, mirroring the trend of the sludge concentration. At 30 g

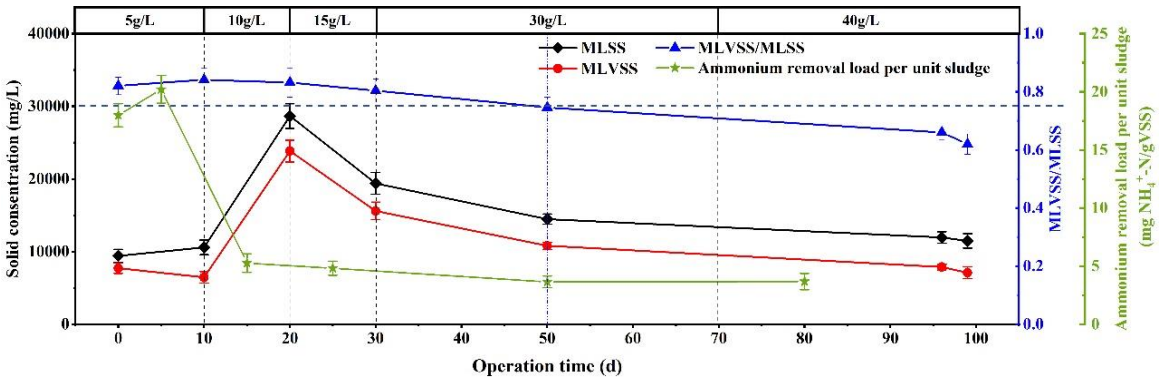

Figure. 4 Changes of MLSS and MLVSS and the denitrification load per unit of sludge

NaCl/L salinity, the MLVSS/MLSS ratios fell below 0.75 and eventually approached 0.60. This implies that at salinity of 30 and 40 g NaCl/L, sludge activity was strongly inhibited, while at lower salinities, the growth and metabolism of microorganisms could be sustained.

Conversely, when the salinity was above 10 g NaCl/L, the ammonium removal load of unit sludge shows a relatively trend ranging from 4.83 to 3.65 and then to 3.69 mg NH_4^+-N /g VSS). This suggests that, after the salinity exceeds a certain level, the ammonium removal load per unit of sludge in this system can maintain relative stability.

The likely reason for this is that as the salinity gradient increased, the system gradually eliminated microorganisms unable to tolerate the saline environment, retaining those species capable of participating in the ammonium removal reaction in the saline conditions. As shown in Fig. 4, this screening process seemed to be completed at 30 g NaCl/L. Prior to this salinity level, the ammonium removal load per unit of sludge decreased. After the salinity continued to rise, the ammonium removal load of the selected bacteria increased. The ammonium removal load per unit of sludge remained stable at high salinities (30 and 40 g NaCl/L).

In the future, if researchers can develop methods for rapidly amplifying and culturing the relevant salt - tolerant anaerobic dominant species and increase the total amount of sludge in the reactor, the system's volumetric nitrogen removal load may achieve stable and potentially doubled results. Furthermore, the adaptive EPS secretion strategy and microbial community succession law obtained in this lab-scale experiment can provide practical guidance for the operation of full-scale saline wastewater treatment plants. Engineering operators can adopt gradual salinity acclimation mode, retain salt-tolerant functional bacteria such as *Candidatus Kuenenia* and *Halomonas*, and optimize sludge retention time to improve the stability of the Anammox-UASB system in high-salinity actual wastewater.

3.4 Change characteristics of EPS at high salinity

EPS are macromolecular organic substances secreted by microorganisms. They adhere to the cell membrane surface and primarily contain components such as proteins PN and polysaccharides (PS). EPS are vital for microbial aggregation, maintaining cell structure, and ensuring cell membrane stability [32]. As salinity rises, the EPS content increased greatly, growing from 11.40 to 14.04 mg/g VSS, and ultimately reaching 37.26 mg/g VSS. This trend indicates that during the

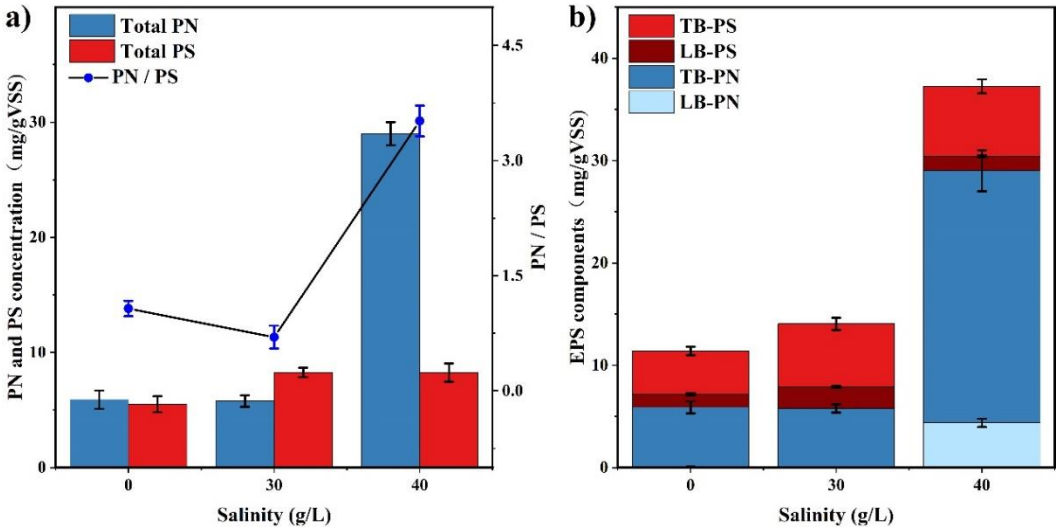

Figure 5. Changes in EPS content at : (a) PN, PS and PN/PS ratio, (b) TB-EPS and LB-EPS

increasing salinity process, microorganisms secrete substantial amounts of EPS to safeguard their cell structure and metabolic activities against the adverse environment.

According to the extraction method, EPS can be classified into tight bound EPS (TB-EPS) and loose bound EPS(LB-EPS). In this experiment, the PN and PS contents of these two EPS were measured.

Fig. 5a shows the changes in PN and PS. At salinities of 30 g NaCl/L and below, the PN content remained relatively stable, while the PS content increased from 5.50 to 8.26 mg/g VSS. This indicated that as salinity reaches 30 g NaCl/L, microorganisms mainly rely on the increase in PS to cope with the high-salt environment. Research indicates that in a high osmotic pressure environment, an increase in PS content helps cells reduce water loss, serving as a mechanism for cells to counteract the rise in external osmotic pressure [33]. As shown in Fig. 4b, among the increased PS, the growth rate of TB-PS was higher than that of LB-PS. This is because salinity stimulation prompts microorganisms to form compact structures to maintain cell - structure stability. Compact EPS, in turn, enhance the sludge's shear resistance, facilitating microbial accumulation [34].

At a salinity of 40 g NaCl/L salinity, the PN content increased markedly (5.78 to 29.01 mg/g VSS), while the PS content did not change proportionally. This could be due to the restricted regulatory capacity of PS in the face of continuously increasing osmotic pressure. After the salinity increased from 30 g NaCl/L to 40 g NaCl/L, microorganisms further produced a large quantity of PN to maintain their structure. The growth of PN was relatively more substantial than that of PS because a higher content of PN can reduce the hydrophilicity of cell surface. This, in turn, alleviates the pressure of salinity environment on cells and improves the sludge's flocculation ability [35]. In Fig. 5b, although both LB-PN and TB-PN increased, the increase in TB-PN (18.59 mg/g VSS) was 4.3 times that of LB-PN (4.37 mg/g VSS), suggesting that TB-PN plays a crucial role as a protective structure for cells in adverse environments. Other factors, such as the inherent properties of the inoculated sludge, intracellular enzyme activity, or the protective effect of secreted compatible substances, may also contribute to this phenomenon.

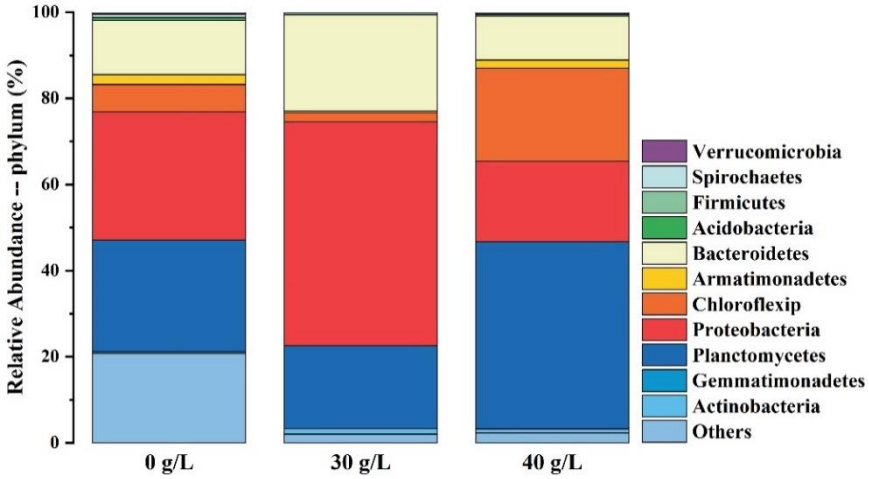

Figure 6. Relative abundance of the microbial community at the phylum levels

The PN/PS ratio varies with the values of PN and PS. As Fan et al. suggested, the PN/PS ratio in Anammox can range from 1.25 to 10.00 [36]. The PN/PS ratio decreased as the salinity gradually increased to 30 g NaCl/L, indicating that PS growth was stimulated at this stage. Fang et al. obtained similar results when studying the impact of salinity on Anammox granules [37]. However, in this experiment, at the 30 g NaCl/L, the PS content was higher than that of the PN content, which may be attributed to the different morphology of the inoculated sludge. When the salinity increased to 40 g NaCl/L, the PN/PS ratio increased sharply, indicating that micro-organisms secreted a large amount of PN to stabilize their structures.

3.5 Effects of high salinity on microbial phylum level

Fig. 6 reveals that *Bacteroidetes*, *Chloroflexi*, *Proteobacteria* and *Planctomycetes* are the dominant phyla during the start-up of the UASB-Anammox system. At salinities of 0 g NaCl/L, 30 g NaCl/L, and 40 g NaCl/L, the percentages of *Bacteroidetes* and *Proteobacteria* increased. Initially, at 0 g NaCl/L, they were 12.62% and 29.85% respectively. These percentages increased to 22.36% and 51.89% at 30 g NaCl/L, but then decreased to 10.20% and 18.76% at 40 g NaCl/L. Among *Bacteroidetes*, *Halomonas*, a heterotrophic anaerobe, first increased to 12.18% and then decreased at 40 g NaCl/L.

Proteobacteria includes numerous bacteria with denitrification capabilities [38]. Some researchers suggest a close association between salinity and *Proteobacteria*. Moreover, *Proteobacteria* occupies a dominant position in marine microbial communities [39]. This also explains why *Proteobacteria* had a high relative abundance when the system entered the high salt condition of 30 g NaCl/L.

Chloroflexi, being facultative anaerobes, the abundance of decreased from 6.32% to 2.22%. However, it then rapidly increased to 21.60%. Studies indicate that *Chloroflexi* can utilize the inactive biomass of AnAOB as a food source. As heterotrophic bacteria, they can also metabolize soluble microbial products (SMP) and EPS released by autotrophic bacteria, thereby enhancing the microbial particle structure [40]. In this experiment, *Chloroflexi* may have contributed to maintaining the sludge structure. Its sharp increase at 40 g NaCl/L might be one of the key factors in preserving

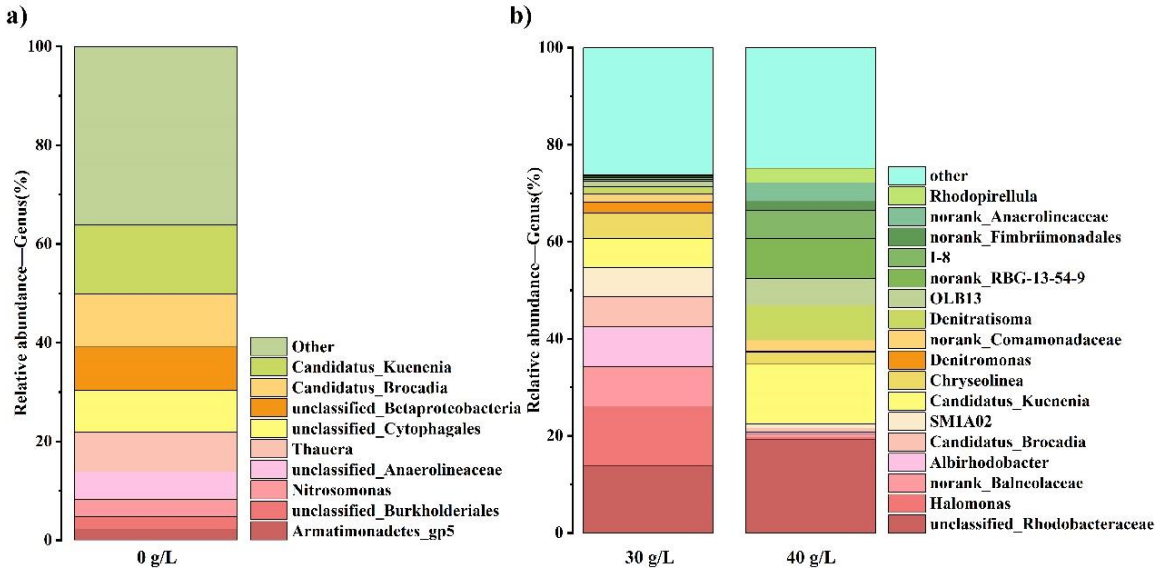

Figure 7. Relative abundance of the microbial community at the genus levels at : (a) 0 g NaCl/L, (b) 30 g NaCl/L and 40 g NaCl/L

the system's denitrification capacity.

Planctomycetes, which contain Anammox bacteria, experienced a slow decrease in abundance from 25.91% to 19.28% as the salinity increased to 30 g NaCl/L. At 40 g NaCl/L, it abruptly rose to 43.35%. This suggests a decline in the system's denitrification performance and severe sludge calcification. The likely reason is that species from other phyla were more sensitive to the high salinity of 40 g NaCl/L compared to *Planctomycetes*. As a result, the number of microorganisms from other phyla decreased more significantly, leading to a relatively higher abundance of *Planctomycetes*.

3.6 Effects of high salinity on microbial genus level

At the experiment's initial stage, the system was dominated by anaerobic ammonia oxidation bacteria, denitrifying bacteria, unclassified beta-deformation phylum, unclassified *fibrophagia*, unclassified anaerobic rope bacteria order and halophilic bacteria as shown in Fig. 7. In the assay system, two types of AnAOB, *Candidatus_Brocadia* and *Candidatus_Kuenenia*, were detected. When the salinity increased to 30 g NaCl/L, the relative abundances of *Candidatus_Brocadia* and *Candidatus_ Kuenenia* were 6.25% and 5.97%, respectively. Compared to the start of the experiment, they decreased by 4.55% and 8.02%, respectively. However, upon reaching a higher salinity of 40 g NaCl/L, the relative abundance of *Candidatus_Kuenenia* increased to 12.29%. *Candidatus_Kuenenia* shows greater tolerance to high salinity environments, while *Candidatus_ Brocadia* is more sensitive to salinity changes.

SM1A02, a member of the *Planctomycetes*, is often found co-existing with the Anammox genus. It has good nitrification ability and is regarded as a new type of anaerobic ammonia-oxidizing bacterium [41]. As the salinity increased from 30 g NaCl/L to 40 g NaCl/L, the relative

abundance of *SM1A02* decreased, which might be related to the decline in *Candidatus_Brocadia*.

OLB13, belonging to the *Chloroflexi*, is a core genus in anaerobic digestion and has denitrification capabilities. It is reported to be a key factor in nitrite accumulation [42], which could explain the high nitrite content in the effluent during this test.

Throughout all stages, the effluent NO_3^--N concentration was relatively stable. This stability might be attributed to the increase in the relative abundance of *Halomonas* within nitrifying bacteria (NOB) as the salinity gradient increased. This maintained the effluent NO_3^--N at a stable level [43, 44]. At salinity of 30 g NaCl/L, the relative abundance of *Halomonas* reached 12.17%, making it the dominant species. This can be attributed to its excellent salt tolerance. It also validates the finding in section 3.2.1 that the anaerobic ammonia oxidation reaction faced competition and was enhanced as the salinity increased to 30 g NaCl/L. After entering a higher salinity, its relative abundance dropped sharply, likely due to substrate competition caused by the halving of influent nitrogen.

With a further increase in salinity, the relative abundance of *I-8*, a member of *Planctomycetes* identified as *Sphingomonas*, increased from 0.35% to 5.79%. *Sphingomonas* is widely distributed in nature and possesses genes conferring good saline-alkali tolerance [45]. It can utilize multiple carbon and nitrogen substrate to ferment into that extracellular PS velan gum [46]. Studies indicate that this bacterium can not only as a denitrifying microorganism to convert NH_4^+-N into NO_2^-- N but also convert NH_4^+-N into N_2, achieving shortcut nitrification-denitrification. Thus, the increase in the relative abundance of the *I-8* strain may support the system's denitrification and structural stability. *Unclassified_Rhodobacteraceae*, *Denitratisoma*, and *norank_RBG-13-54-9* all have denitrification capacity [47-49]. Their relative abundances increased from 13.75%, 1.52% and .42% to 19.18%, 7.12% and 8.19%, respectively. After reaching the salinity of 40 g NaCl/L, denitrifying bacteria became the main deamination system, ensuring the system still maintains a certain treatment effect under high salt conditions.

4. Conclusions

After an increase in salinity, the ammonium removal load per unit sludge remained relatively stable within the 30-40 g NaCl/L range, specifically varying from 3.65 to 3.69 mg NH_4^+-N/g VSS. Moreover, 20 days into the high-salinity exposure, the microorganisms' capacity to remove total nitrogen (TN) also stabilized and was unaffected by further salinity increments. This stability indicates that the system can adapt to high-salinity conditions for these nitrogen - removal processes.

At high salinity, the growth of PN and PS in microorganisms followed a sequential pattern. At 30 g NaCl/L, the system's microorganisms in the system preferentially grown more PS (from 5.50 to 8.26 mg/g VSS) to resist the external high osmotic pressure environment. At 40 g NaCl/L, the microorganisms secreted a substantial amount of extracellular PN, rising from 5.78 to 29.01 mg/g VSS. This significant increase in PN was crucial for stabilizing the cellular structure and maintaining metabolic activities.

The sludge acclimation process provides an explanation for the system's behavior under high salinity. Initially, salinity inhibited or eliminated some of the salt-tolerant flora, leading to a decrease in the MLVSS, which is an indicator of sludge volume. Subsequently, the salt-tolerant flora gradually became the dominant group. In the system, bacteria and genera with higher salinity tolerance, such as *Candidatus_Kuenenia*, *Halomonas*, *unclassified_Rhodobacteracea*e, and

Denitratisoma, increased in abundance to 12.29%, 12.17%, 19.18%, and 7.12%, respectively. This shift in the microbial community enabled the system to sustain its ammonium - removal function.

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