

# Responses of microbial interactions to elevated salinity in activated sludge microbial community

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## HIGHLIGHTS

- Salinity led to the elevation of NAR over 99.72%.
- Elevated salinity resulted in a small, complex, and more competitive network.
- Various AOB or denitrifiers responded differently to elevated salinity.
- Putative keystone taxa were dynamic and less abundant among various networks.

## ARTICLE INFO

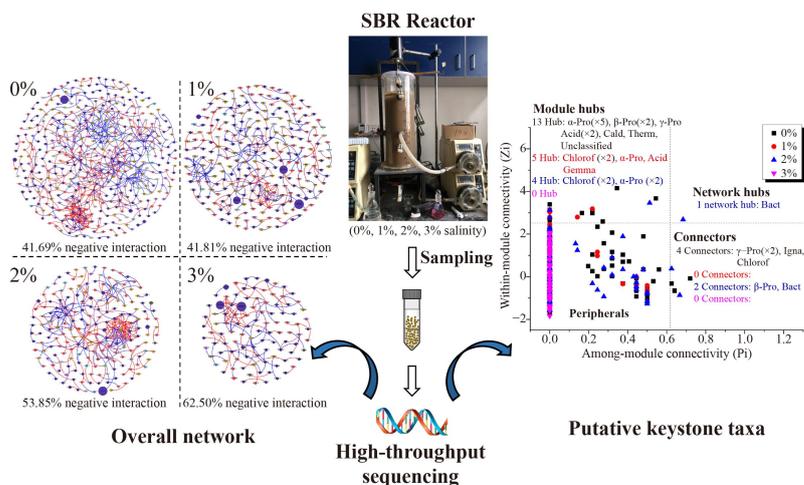
### Article history:

Received 7 June 2022  
Revised 24 October 2022  
Accepted 26 October 2022  
Available online 18 December 2022

### Keywords:

Elevated salinity  
Activated sludge system  
Pollution removal  
Microbial interactions  
Competitive relationship

## GRAPHIC ABSTRACT



## ABSTRACT

Biological treatment processes are critical for sewage purification, wherein microbial interactions are tightly associated with treatment performance. Previous studies have focused on assessing how environmental factors (such as salinity) affect the diversity and composition of the microbial community but ignore the connections among microorganisms. Here, we described the microbial interactions in response to elevated salinity in an activated sludge system by performing an association network analysis. It was found that higher salinity resulted in low microbial diversity, and small, complex, more competitive overall networks, leading to poor performance of the treatment process. Subnetworks of major phyla (Proteobacteria, Bacteroidetes, and Chloroflexi) and functional bacteria (such as AOB, NOB and denitrifiers) differed substantially under elevated salinity process. Compared with subnetworks of *Nitrosomonadaceae*, *Nitrosomonas* (AOB) made a greater contribution to nitrification under higher salinity (especially 3%) in the activated sludge system. Denitrifiers established more proportion of cooperative relationships with other bacteria to resist 3% salinity stress. Furthermore, identified keystone species playing crucial roles in maintaining process stability were dynamics and less abundant under salinity disturbance. Knowledge gleaned from this study deepened our understanding of microbial interaction in response to elevated salinity in activated sludge systems.

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## 1 Introduction

Wastewater salinity is a noteworthy problem in biological

treatment processes all over the world. Very large amounts of high saline wastewater (mainly NaCl) are generated in tanning, petroleum, chemical production, food processing as well as textile industry (Lefebvre and Moletta, 2006; Kishor et al., 2021). Also, in many coastal areas, seawater is used to flush toilets to alleviate the shortage of freshwater, which could lead to increased salinity in wastewater treatment plants (WWTPs) (Liu et al., 2019; Li et al., 2022).

Biological treatment processes, especially activated sludge systems, are widely used in the removal of pollutants on account of their low cost and high efficiency. But high salinity could cause poor treatment performance of biological WWTPs, since high osmotic pressure is lethal to microbes and can inhibit the enzyme activity (Duan et al., 2015; Maqbool et al., 2018; Han et al., 2021; Luo et al., 2021). Previous researches showed that salinity reduced the diversity of bacterial community, altered the compositions of nitrifiers and denitrifiers, and further inhibited the activity of nitrifying bacteria (Luo et al., 2015; Zhao et al., 2016; Wang et al., 2017a; García-Ruiz et al., 2018; Wang et al., 2020a). To date, the activated sludge microbial community has been investigated extensively, but seldom focuses on the microbial interactions among microbial taxa during system operation.

Activated sludge system is a complex micro-ecosystem, in which various bacterial taxa interact with each other through energy transfer and exchange of matter and information to form a large, complex ecological network (Yuan et al., 2021). Through such network connections, the biological treatment process can achieve desirable performance in removal of organic matters and nutrients (Wang et al., 2019; Ya et al., 2021). For example, there is a symbiotic relationship between ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), as AOB provides nitrite for NOB as the metabolic substrate, while NOB removes nitrite to reduce its inhibitory effect on AOB. For another example, heterotrophic *Saprosiraceae* can hydrolyze protein to small molecular amino acids, which can be utilized by other heterotrophic bacteria as metabolic substrates (Xia et al., 2008). Other typical microbial cooperative interactions in activated sludge systems include: 1) AOB and NOB can provide substrates such as nitrite and nitrate for denitrifying bacteria (Teske et al., 1994; Baumann et al., 1997; Mota et al., 2005); 2) CO<sub>2</sub> released by heterotrophic bacteria can be assimilated by autotrophic AOB and NOB (Peng et al., 2015); and 3) soluble microbial products released after cell death and the lysis of AOB and NOB can be used as organic carbon source for denitrifying bacteria (Miao et al., 2016). It is expected that some physicochemical changes (e.g. influent salinity) might disturb the microbial interactions among various functional populations (e.g., heterotrophic bacteria, nitrifiers, denitrifiers, polyphosphate accumulating organisms (PAO), etc.), and accordingly impact the performance efficiency in activated sludge systems (Wang et al., 2017b; Wang et al., 2019). Hence, understanding the response of microbial interactions to salinity

perturbation is significant to develop operational strategies to resist unfavorable disturbance and further improve the nitrification-denitrification processes of activated sludge systems. However, information regarding microbial interactions and how they respond to elevated salinity has been rarely reported. Recently, by performing a novel RMT-based network analysis, the ecological interactions among members of the community can be assessed and keystone populations of the complex community can be identified, providing a novel insight into the dynamic changes of microbial interaction under physicochemical changes (Sun et al., 2020; Yuan et al., 2021).

In this study, the potential relationship between system performance and microbial interaction dynamics was evaluated and the keystone species were identified by using network analysis in activated sludge microbial communities during a salinity gradient of 0%–3%. A dynamic variation of molecular ecological networks (MENs) was detected to explore the following questions: 1) How does the overall network structure respond to elevated salinity? 2) How does the subnetwork structure of different phylogenetic taxa respond to elevated salinity? 3) How do the functional bacteria and keystone species respond to elevated salinity?

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## 2 Materials and methods

### 2.1 Reactor construction and operational strategy

A sequencing biological reactor (SBR) (Fig. S1) with 7.7 L effective volume was operated at 25 ± 1 °C for 80 d. Seed sludge was activated sludge that had been domesticated by a low C/N ratio environment. The synthetic wastewater contained 400 mg/L COD, 80 mg/L NH<sub>4</sub><sup>+</sup>-N, 4 mg/L TP, 36 mg/L KCl, 14 mg/L CaCl<sub>2</sub>·2H<sub>2</sub>O, 90 mg/L MgSO<sub>4</sub>·7H<sub>2</sub>O and 0.3 mL/L trace elements solution. The composition of trace elements solution was according to Smolders et al. (1994). The system was continuously exposed to 0%, 1%, 2%, and 3% salinities (using NaCl), named phases A, B, C and D, respectively, and each phase was conducted for 20 d. The above-mentioned reagents were analytical reagents and purchased from Tianjin Guangfu Technology Development Co., Ltd., China.

The reactor ran for 6 h in one cycle, pumping wastewater for 10 min, stirring for 1.5 h, aeration for 1.5 h, stirring for 2 h, settling for 0.5 h, drainage for 10 min, and idle stage for 10 min. The rotation speed in the stirring stage was 200 (± 20) r/min, and the aeration volume in the aeration stage was 1.6 (± 0.3) L/min. And the wastewater intake and drainage were 4 L per cycle. MLSS was maintained at 3000 (± 500) mg/L.

### 2.2 Analytical methods

COD, NH<sub>4</sub><sup>+</sup>-N, NO<sub>2</sub><sup>-</sup>-N, and NO<sub>3</sub><sup>-</sup>-N levels were measured every three days. NH<sub>4</sub><sup>+</sup>-N from aerobic-stage

start,  $\text{NO}_2^-$ -N and  $\text{NO}_3^-$ -N from aerobic-stage end were measured every three days.  $\text{NH}_4^+$ -N,  $\text{NO}_2^-$ -N, and  $\text{NO}_3^-$ -N in a cycle at the end of each phase were measured every 30 min. Total nitrogen (TN) was the sum of the concentrations of  $\text{NH}_4^+$ -N,  $\text{NO}_2^-$ -N, and  $\text{NO}_3^-$ -N. pH and dissolved oxygen (DO) were measured every day. MLSS and MLVSS were measured every 10 d. COD,  $\text{NH}_4^+$ -N,  $\text{NO}_2^-$ -N, and  $\text{NO}_3^-$ -N were detected in accordance with Standard Method (Wang et al., 2017b).

### 2.3 Microbial DNA extraction and high throughput sequencing

A total of 32 activated sludge samples were generated from four phases during the final eight consecutive days of each phase. DNA extraction was performed using the FastDNA SPIN Kit for Soil (MP Biotechnology, France) following the manufacturer's recommendations (Hu et al., 2021). 16S rRNA gene amplicon sequencing was performed on Illumina MiSeq PE300 platform in Majorbio Co., Ltd. (Shanghai, China) by using the primers 338F and 806R for bacteria in the V3–V4 hypervariable region. Then raw data were quality-filtered according to our previous literature (Wang et al., 2017b). Operational taxonomic units (OTUs) were obtained by using UPARSE (v11) with 97% sequence similarity. And then the OTUs were taxonomically annotated using RDP Classifier (v2.13) against the 16S rRNA database (Silva v138) with a confidence threshold of 0.7. Raw sequences generated in this paper have been deposited in NCBI SRA under the accession number PRJNA732228.

### 2.4 Data analysis and network construction

Sobs, Shannon, Shannoneven indices represented the diversity of the bacterial community. The statistical analysis was proposed in R (v.3.5.1). In addition, four MENs (named 0%, 1%, 2%, and 3% networks) were constructed to reveal the possible co-occurrence patterns among species by Molecular Ecological Network Analysis Pipeline. The detailed methods of the construction of experimental and random networks were shown in Text S1, and iDIRECT (Inference of Direct and Indirect Relationships with Effective Copula-based Transitivity) was applied to disentangle direct from indirect relationships in the constructed networks according to Xiao et al. (2022) in Text S2. Gephi 0.9.2 and Cytoscape 3.6.1 were used for visualization of the networks.

## 3 Results and discussion

### 3.1 System performance

As shown in Fig. 1(a), the salinity gradient (1%–3%) had no obvious impact on organic matter removal, as the

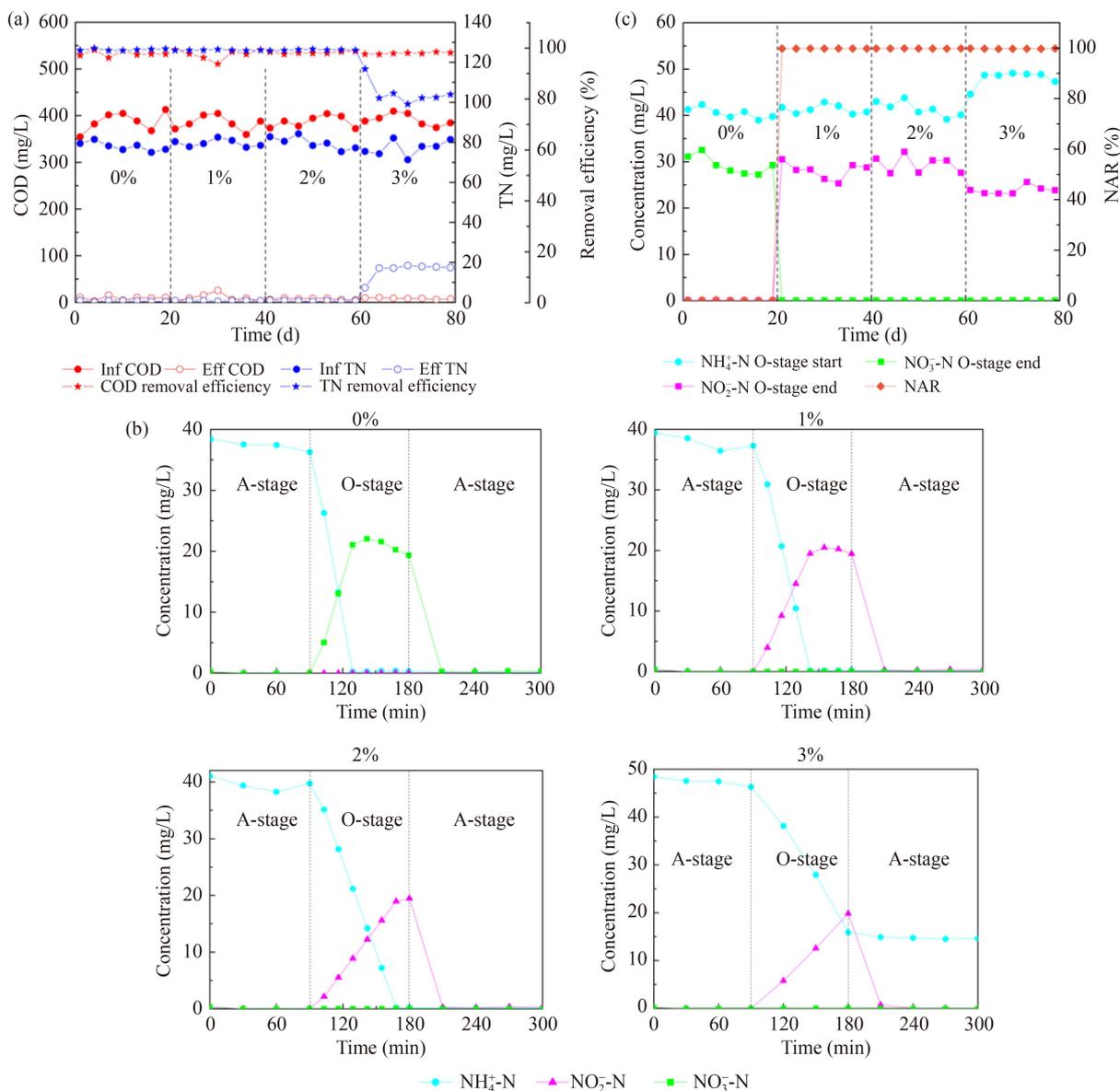
COD removal efficiency was always higher than 94% in all phases. In comparison, although the effluent TN was less than 0.93 mg/L at lower salinity (1% and 2%), the TN concentration suddenly increased to 14.93 mg/L under 3% salinity. Fig. 1(b) showed the concentrations of  $\text{NH}_4^+$ -N,  $\text{NO}_2^-$ -N, and  $\text{NO}_3^-$ -N in a cycle at 0%–3% salinities.  $\text{NH}_4^+$ -N remained at a relatively high level (14.64 mg/L) after O-stage when exposed to 3% salinity, while the concentrations of  $\text{NO}_2^-$ -N and  $\text{NO}_3^-$ -N were very low and even couldn't be detected at the end of the anoxic stage, which revealed that nitrification was more severely inhibited than denitrification under high salinity. Fig. 1(c) further illustrated that  $\text{NO}_3^-$ -N was the main nitrification product after O-stage under 0% salinity, and the NAR (the accumulation rate of nitrous nitrogen:  $\text{NO}_2^-$ -N/( $\text{NO}_2^-$ -N +  $\text{NO}_3^-$ -N)) was less than 0.23%. While, the NAR increased to 99.72% along with the salinity from 1% to 3%, and  $\text{NO}_3^-$ -N was undetectable after O-stage, suggesting  $\text{NO}_2^-$ -N was the main product of nitrification. These results demonstrated shortcut nitrification-denitrification was achieved under salt conditions, which was consistent with previous research (Ye et al., 2009). In addition, the production of  $\text{NO}_2^-$ -N after O-stage declined in the presence of 3% salinity, indicating higher salinity also inhibited the shortcut nitrification.

### 3.2 Community structure

All samples were rarefied to 33264 sequences for subsequent comparative analyses. A total of 891 bacterial OTUs were determined based on 97% sequence similarity level. The rarefaction curves were exhibited in Fig. S2(a). Sobs and Shannon indices were both significantly negatively correlated ( $P < 0.01$ ) with elevated salinity (Fig. S3), implying that salinity could reduce the abundance and diversity of bacterial communities. Besides, high salinity (3%) greatly altered the bacterial community compositions (Fig. S2). For example,  $\alpha$ -Proteobacteria (59.49%) was the dominant population under 0%–2% salinity, while  $\beta$ -Proteobacteria (70.17%) became the most abundant class in the present of 3% salinity. The above results showed that salinity had a significant influence on microbial diversity and composition.

### 3.3 Characteristics of association networks

To explore the effect of elevated salinity on microbe-microbe interactions in an activated sludge system, we constructed four bacterial networks for each salinity and removed spurious/indirect links by using iDIRECT method (Fig. 2). The network topological features of original and iDIRECT-processed networks were exhibited in Table S1. A considerable portion of the links (23.18%, 22.64%, 30.95%, and 38.80%, corresponding to 0%–3% salinity) was removed in elevated salinity networks. As a



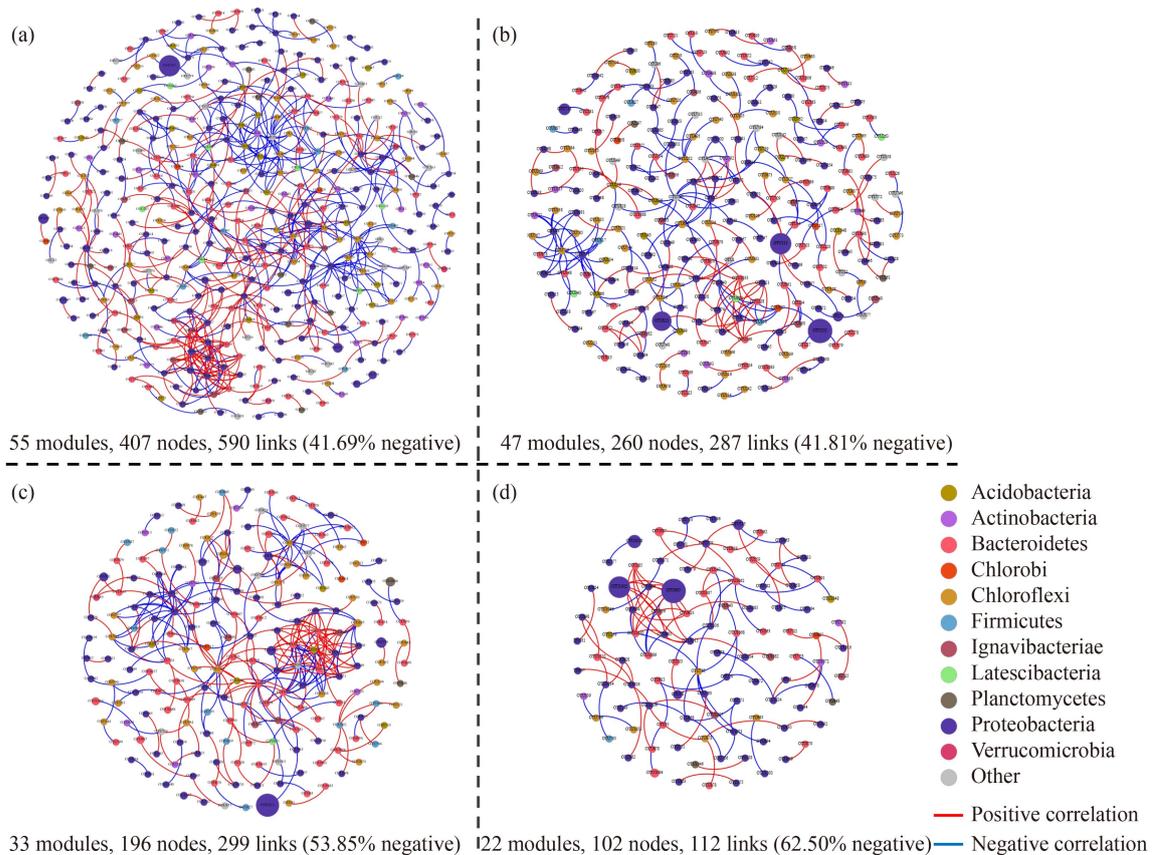
**Fig. 1** Dynamic changes of nitrogenous substances in SBR along a salinity gradient. Removal efficiencies of COD and TN (a); the concentrations of  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^-\text{-N}$ , and  $\text{NO}_3^-\text{-N}$  in one cycle (b); dynamic changes of nitrogenous substances before and after O-stage (c); under different salinity. A-stage, O-stage and A-stage in a cycle represented anaerobic stage, aerobic stage and anoxic stage, respectively.

consequence, the average clustering coefficient (avgCC) significantly decreased under 0%–3% salinity compared to the original networks, while the modularity of the iDIRECT-derived networks increased significantly compared to the original networks. Subsequent analyses were based on the networks generated by iDIRECT. Besides, the iDIRECT-derived bacterial networks significantly ( $P < 0.05$ ) differed from corresponding randomized networks, suggesting the empirical network structures were nonrandom. The topology of all networks fit the power-law distribution very well ( $R^2$  from 0.802 to 0.961), and the topological features were higher than those in corresponding random networks. These metrics indicated

that these four networks clearly exhibited small world, scale-free, and modularity properties (Girvan and Newman, 2002; Blonder et al., 2012; Deng et al., 2012).

### 3.4 Effects of elevated salinity on overall networks

Although the identical threshold ( $St$ ) 0.92 was adopted to construct networks, these four microbial networks differed profoundly (Fig. 2). Higher salinity formed smaller networks with fewer nodes, as there were 407, 260, 196, and 102 nodes with 590, 287, 299, and 112 links across four salinity elevated networks. The average degree (avgK) and avgCC decreased at 1% salinity, and



**Fig. 2** Variation of activated sludge microbial networks generated by iDIRECT under 0% (a), 1% (b), 2% (c) and 3% (d) salinities. The node size was proportional to the corresponding microorganism (OTU) relative abundance in microbial community.

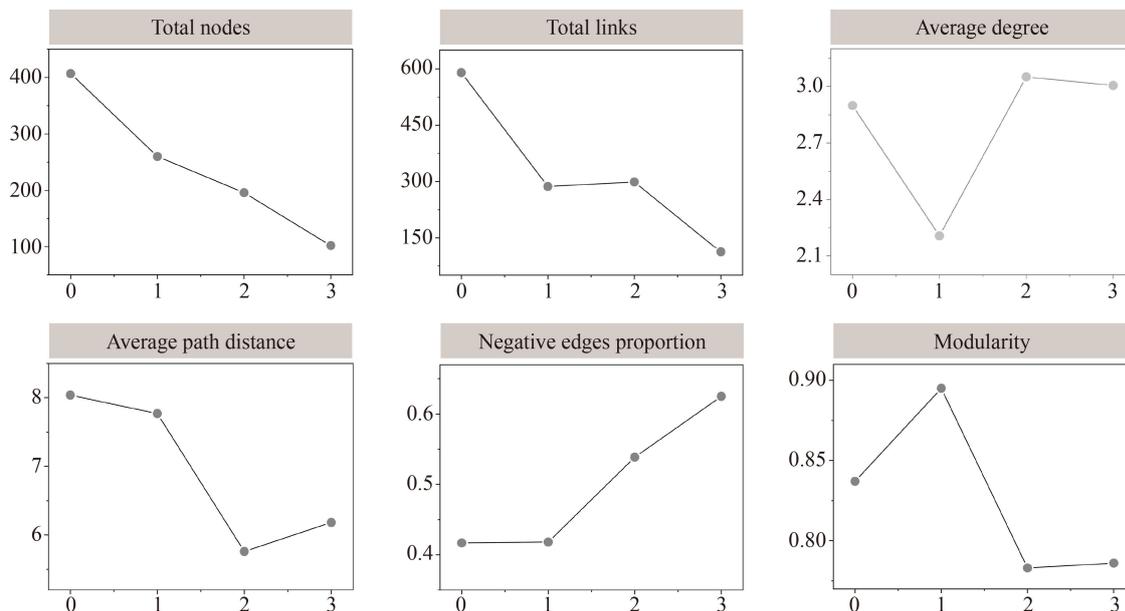
then significantly increased under higher salinities, while the modularity presented an opposite trend (Fig. 3 and Table S1). Among these indexes, avgK represents the microbial interaction complexity, and avgCC indicates the connectivity between a node and its neighbors. The average path distance (GD) not only describes the shortest path between two nodes, but also represents the exchange speed of substances and information between two nodes (Luo et al., 2006). The variations of network topological indexes indicated 1% salinity weakened the connection between nodes. However, when exposed to higher salinity (2% or 3% salinity), the increase in avgK and avgCC, as well as the decrease in GD led to more complex and tighter network connection, suggesting the intense communications among microorganisms under higher salinities.

In microbial networks, organic matter and nutrient removal depend on various types of interactions, which generally fall into two categories, positive connection and negative connection. Positive relationships mainly include mutualism, while negative relationships are generally due to competition, as predation and amensalism are unlikely to emerge in activated sludge systems (Deng et al., 2016; Wu et al., 2016; Ya et al., 2021). In this study, the proportion of negative links increased from 41.69% to

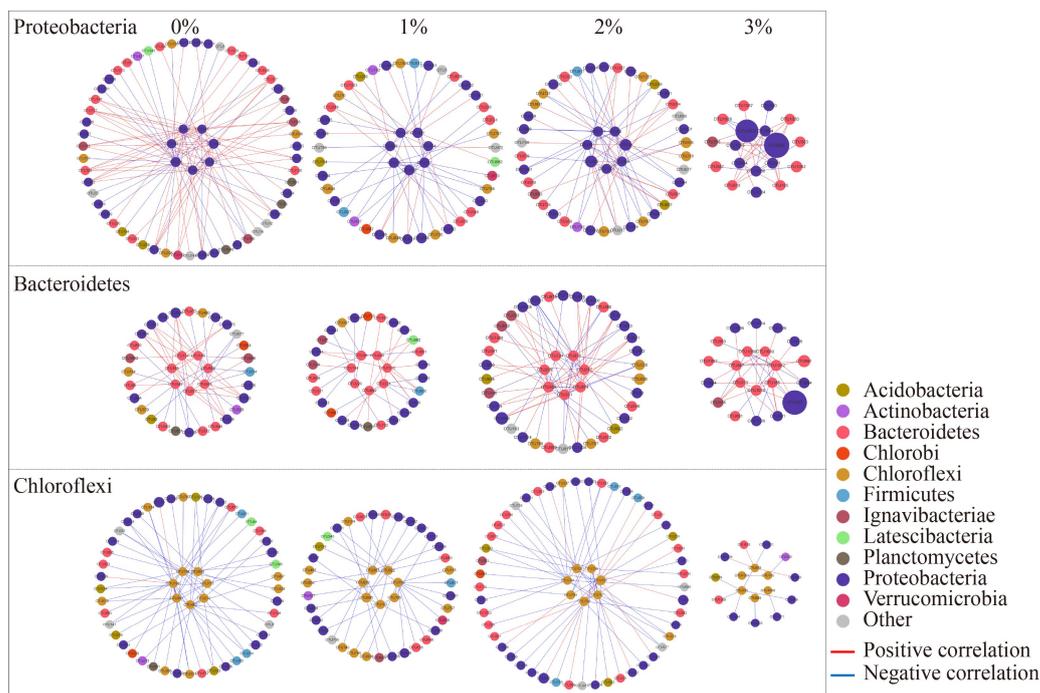
41.81%, 53.85%, and 62.50% when exposed to elevated salinity from 0% to 3% (Fig. 3). The highest proportion of competitive interactions under 3% salinity might lead to the deterioration of TN removal. In addition, the higher salinities (2% and 3%) also reduced the modularity, as the module numbers of four networks (0%, 1%, 2%, and 3% networks) were 55, 47, 33, and 22 with modularity values of 0.837, 0.894, 0.782 and 0.786. Modularity, describing how well a community could be divided into modules, is regarded as a topological indicator and is crucial for system stability and resilience (Olesen et al., 2007; Wu et al., 2016). The module could be considered as a functional unit since the OTUs within one module might tend to own similar ecological niches (Luo et al., 2006; Eiler et al., 2012). It appeared that 2%–3% salinity decreased the modules (i.e., functional units) and this could be another potential reason causing the poor performance of nitrogen removal.

### 3.5 Effects of elevated salinity on subnetworks

The subnetworks of major phyla containing the top seven nodes with the highest connectivity were generated in Fig. 4. Proteobacteria was the dominant phylum in the SBR system, and in the subnetworks of Proteobacteria,



**Fig. 3** Topological properties of different networks (*X*-axis: values of salinity (%)).



**Fig. 4** Variation of subnetworks of Proteobacteria, Bacteroidetes, and Chloroflexi under 0%, 1%, 2% and 3% salinities.

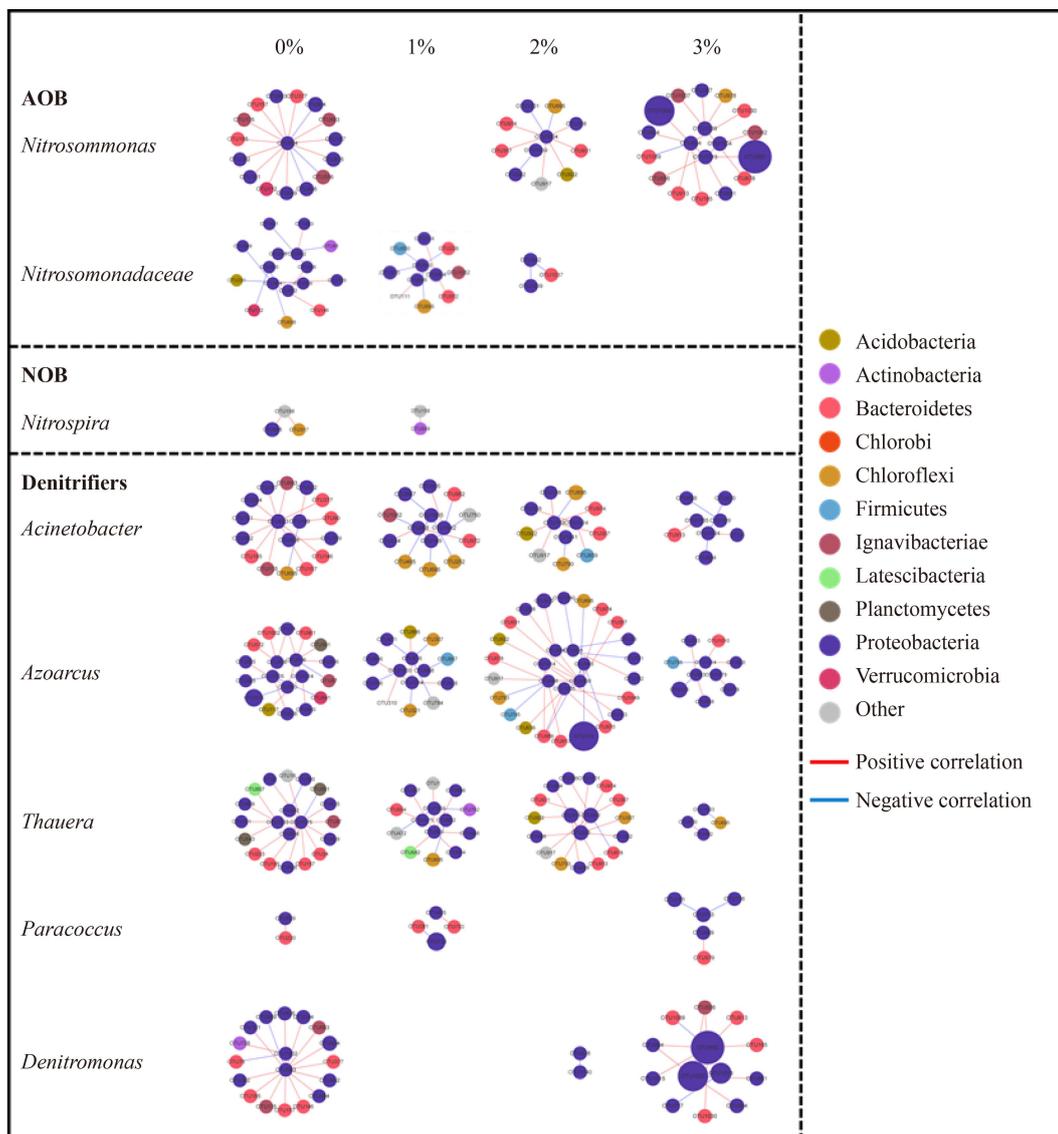
the number of nodes declined from 62 to 46, 45, and 18, respectively, and the proportions of negative relationship were 70.71%, 38.78%, 45.31%, and 78.79%, respectively, under elevated salinity process. It appeared that higher salinity formed a smaller size network and the most intensive competition was found in 3% salinity. Proteobacteria mainly contributed to the removal of organic matter and nutrients in the activated sludge system, therefore, the minimum network size and the most intensive competi-

tion probably led to the deterioration performance at 3% salinity condition. Within Bacteroidetes, the subnetwork size became bigger at 2% salinity and then turned smaller at 3% salinity, as there were 35, 33, 41, and 21 nodes according to 0%–3% salinities. For Chloroflexi, 3% salinity also dramatically reduced the size and interactions, while promoted the competitive relationships in the corresponding subnetworks. The smallest network size and complexity, as well as the most intensive competition

among these major phyla under 3% salinity probably led to the deterioration of system performance.

Although higher salinity levels reduced the community diversity and simplified the network connection, functional organisms could still form interactive networks in high salinity environments. Therefore, we further discussed the potential effects of the salinity gradient on some functional bacteria subnetworks. For example, *Nitrosomonas* and *Nitrosomonadaceae* were two typical AOB, while their response to elevated salinity was different. The relative abundance of *Nitrosomonas* was 0.02%, 0.03%, 0.05% and 1.55%, while *Nitrosomonadaceae* was 1.29%, 0.47%, 0.10% and undetected with salinity increasing from 0% to 3%. Based on the changes in abundance, *Nitrosomonadaceae* was gradually replaced by *Nitrosomonas*, which might make a greater contribution to nitrification under

higher salinity (especially 3%) in the activated sludge system. Consistently, 1, 0, 2, and 4 OTUs belonged to *Nitrosomonas*, and 7, 3, 1, and 0 OTUs belonged to *Nitrosomonadaceae* were detected in the salinity elevated networks. These OTUs and their first neighbors were used to construct the subnetworks of *Nitrosomonas* and *Nitrosomonadaceae* (Fig. 5). At the salinity gradient of 0%–2%, the percentage of negative links decreased from 80.00% to 66.67% in the *Nitrosomonas* subnetworks and also reduced from 40.00% to 0.00% in *Nitrosomonadaceae* subnetworks. When salinity increased to 3%, the negative link proportions rapidly rose to 93.73% in *Nitrosomonas* subnetwork, and no nodes related to *Nitrosomonadaceae* were detected. It suggested that higher salinity completely inhibited the relationship between *Nitrosomonadaceae* and other populations, and it also started to suppress



**Fig. 5** Succession of subnetworks of AOB, NOB and denitrifiers under 0%, 1%, 2% and 3% salinities. The nodes derived from AOB, NOB and denitrifiers were in the center.

*Nitrosomonas* simultaneously, leading to the decline of  $\text{NO}_2^-$ -N production after O-stage under 3% salinity shown in Fig. 1(b). In addition, *Nitrospira*, as the representative NOB, was only found in 0% network, which manifested that salinity seriously inhibited the nitrification of  $\text{NO}_2^-$ -N resulting in the elevation of NAR over 99.72% under 1%–3% salinity in activated sludge system.

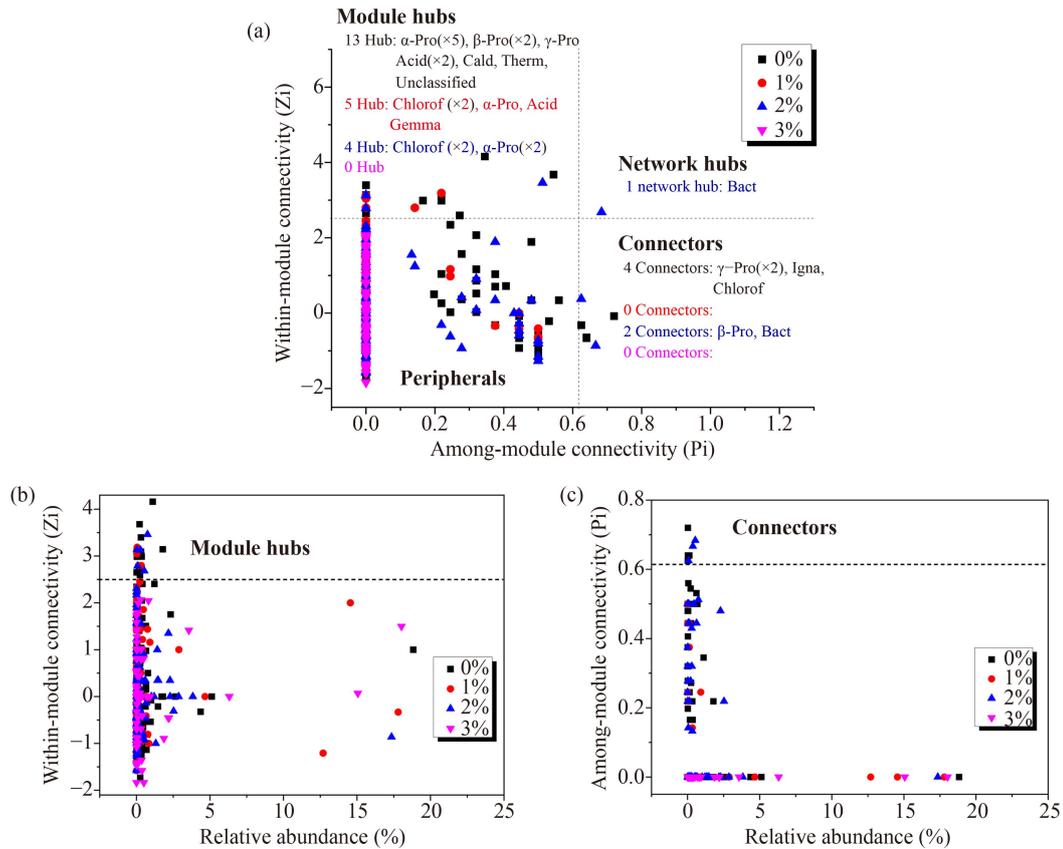
Some denitrifiers, such as *Acinetobacter*, *Thauera*, *Azoarcus*, *Paracoccus*, *Denitromonas*, and *Defluviicoccus*, were also used to construct subnetworks (Fig. 5). The size and complexity of subnetworks of *Acinetobacter* and *Thauera* were both declined by elevating salinity. For example, the four *Acinetobacter* subnetworks contained 17, 14, 12, and 8 nodes with 14, 11, 9, and 5 links, respectively, along with a salinity gradient. Besides, the elevated salinity stress decreased the abundance of *Acinetobacter* (from 2.10% to 0.06%–0.61%), whereas enhanced *Thauera* abundance (from 1.70% to 2.66%–11.18%). Although a similar trend of these two subnetworks was observed, the abundance trends of *Acinetobacter* and *Thauera* were different. This inconsistency highlighted the necessity of studying the bacterial interaction, as it provided a pivotal dimension to understand the microbial assembly process rather than the simple community richness and diversity. In addition, the most complex subnetwork of *Azoarcus* appeared at 2% salinity, and the most complex subnetwork of *Paracoccus* appeared at 3% salinity, while the most complex subnetwork of *Denitromonas* appeared at 0% salinity. *Defluviicoccus*, an endogenous denitrifier, was found dominant at 0%–2% salinity (relative abundance: 37.15%, 52.07%, 35.01%, and 8.80% according to 0%–3% salinity) under this intermittent aeration sewage treatment system (Wang et al., 2020b). However, it appeared that 1%–3% salinity seriously reduced the microbial interactions in *Defluviicoccus* subnetworks, as the subnetworks contained 74, 32, 44, and 14 nodes with 63, 25, 41, and 10 links, respectively (Fig. S4). It is worth noting that the percentage of negative relationships in subnetworks of *Acinetobacter*, *Thauera*, *Azoarcus*, *Paracoccus*, and *Denitromonas* reduced from 92.86%, 88.24%, 75.00%, 100%, and 87.50% to 40%, 50%, 71.43%, 33.33%, and 84.62%, respectively, according to 0% and 3% salinities, indicating denitrifiers established more proportion of cooperative relationships with other bacteria to resist 3% salinity stress. The above results indicated that elevated salinity could have different influences on the microbial interactions of various bacteria with the same function to ensure the stability of the activated sludge system, even at 3% salinity.

### 3.6 Putative keystone taxa of association networks

In addition to the above-mentioned functional microorganisms, topological keystone species also play irreplaceable roles in ecological networks. The topological role of these

species could be defined by Zi-Pi parameter (Fig. 6(a)). In this study, over 97.12% of the nodes were classified as peripherals, while only 22 module hubs and six connectors as well as only one network hub were identified in the elevated salinity networks (Tables S2). Among these 22 module hubs, 11 derived from Proteobacteria, 6 belonged to Chloroflexi, 3 related to Actinobacteria, 1 related to Gemmatimonadetes, and 1 belonged to unclassified bacteria, all of which merely amounted to very low abundance ranging from 0.01% to 1.79% (Fig. 6(b)). Six connectors from four networks were derived from four phyla, 50.00% of connectors were Proteobacteria, and the others were derived from Bacteroidetes, Chloroflexi, and Ignavibacteriae. Similarly, all connectors also presented relatively low abundance (ranging from 0.01% to 0.37%) (Fig. 6(c)). Only one OTU (OTU 959) was identified as the network hub at 2% salinity in this study, and this is the only few times that network hubs were identified by network analysis (Wu et al., 2016; Ya et al., 2021). Previous studies have suggested that connectors and hubs could be regarded as key species, as they play over-proportional ecological role in maintaining community structure and function stability than some abundant species (Olesen et al., 2007; Shi et al., 2016). Among these key species, eight module hubs (OTUs 28, 1075, 599, 198, 581, 663, 750, and 771) as well as one connector (OTU 1005) were closely related to *Defluviicoccus*, *Thauera*, norank\_f\_Blastocatellaceae\_Subgroup\_4, norank\_o\_JG30-KF-CM45, norank\_f\_Gemmatimonadaceae, *Gemmobacter*, and *Azoarcus*, all of which were denitrifiers (Kämpfer et al., 2015; Wang et al., 2020b; Wang et al., 2020c; Huang et al., 2022). Hub OTU 955 as well as connector OTU 635, derived from the Candidatus *Competibacter*, were considered as denitrifying glycogen accumulating organisms (DGAO) (Xu et al., 2020). Besides, two module hubs (OTUs 252 and 736) and one connector (OTU 704) attributed to the family Caldilineaceae, as well as one connector OTU 898 attributed to *Pseudoxanthomonas* were capable of degrading lignocellulose (Zhang et al., 2019; Tang et al., 2020). Two hubs (OTUs 793 and 744) belonged to the family Anaerolineaceae. Members of Anaerolineaceae were found abundant in anaerobic system and were responsible for the removal of carbohydrates and cell tissues (Miao et al., 2016). Connector of OTU 555 was related to order Ignavibacteriales, and they were key players in the observed shift from denitrification to dissimilatory reduction of nitrate to ammonium (Grießmeier et al., 2017). Furthermore, two module hubs (OTUs 117 and 259) attributed to Acidobacteria and one connector OTU 380 attributed to Bacteroidetes were found to degrade some complicated organic substances. OTU 629 was related to order SC-I-84 and OTUs 601, 43 and 577 belonged to unclassified bacteria, and their specific functions remain unknown.

Interestingly, three nodes (OTUs 28, 771, and 736) served as module hubs in different networks. OTU 28



**Fig. 6** Z-P plot showing distribution of nodes topological roles (a), the relative abundance of (b) module hubs and (c) connectors under 0%, 1%, 2% and 3% salinity networks.

functioned in 0% and 2% networks, and OTU 771 functioned in 1% and 2% networks, and OTU 736 functioned in 0% and 1% networks. The other 89.66% of hubs and connectors were unique. No putative keystone species were found in 3% network. Notably, OTU 28 was derived from *Defluviococcus* and considered as an endogenous denitrifier as well as a glycogen accumulating organism (GAO), and OTU 771 was also responsible for denitrification. OTUs 28 and 771 and their first neighbors were presented in Fig. S5. There were 21, 18, 14, and 3 nodes with 20, 20, 24, and 2 links corresponding to 100% positive correlations with salinity increasing, which suggested 1%–2% salinity reduced the subnetwork size but promoted the complexity and collaboration between populations to resist the high salinity, however, OTUs no longer served as key taxa and more species could not be detected under 3% salinity. Although 0%–2% salinities greatly altered the mostly putative key microbial populations, various species could successively perform the same function under elevated salinity. For example, OTUs 1075, 599, 955, 198, 581, 663, 635 from 0% network, OTUs 750, 771 from 1% network and OTUs 28, 1005 from 2% network all functioned as denitrifiers. Besides, OTUs 117, 736, 704, 898 from 0% network, OTUs 252, 259 from 1% network, and OTUs 793, 794, 380 from 2% network were all

responsible for degrading organic substances. Hence, functional redundancy might be a potential factor for the keystone taxa dynamics with the salinity increasing. Specially, the relatively low abundances of these putative keystone species in this study indicated that some low-abundant microorganisms could play a disproportionately more significant role than abundant species in maintaining microbial community stability in activated sludge systems.

The above network analysis could identify that the putative keystone species were critical in maintaining the community structure and functional stability of the activated sludge system in response to a salinity gradient, although they were dynamic and less abundant. Therefore, network analysis has much potential for exploring these keystone populations interactions to gain a deeper understanding of the critical roles of these species.

## 4 Conclusions

The mechanism underlying the succession of ecological network is often overlooked in microbial ecology. Here, we evaluated the effects of elevated salinity on ecological networks of activated sludge system by performing a novel RMT-based network analysis. Our results found

3% salinity inhibited TN removal and reduced the diversity of microbial communities. Network analysis revealed that overall networks under higher salinity conditions (2% and 3%) exhibited more complex, tighter networks, and more competitive bacterial interactions. Subnetworks of bacteria with same function (such as AOB, NOB, and denitrifiers) differed substantially when exposed to elevated salinity. The connection between *Nitrospira* (NOB) and other species was seriously inhibited under 1%–3% salinity resulting in the elevation of NAR over 99.72%. In addition, keystone species (hubs and connectors) were dynamics when exposed to different salinity and played crucial roles in maintaining system stability, despite the low abundances in microbial community. Collectively, this work improved our understanding of the relationship between system performance and microbial interaction dynamics of activated sludge microbial communities in response to elevated salinity. Further studies are necessary to verify the salinity effects in more parallel reactors with control.

**Acknowledgements** This study was supported by the Open Research Fund Program of State Environmental Protection Key Laboratory of Food Chain Pollution Control (China) (FC2022YB08) and the Fundamental Research Funds for the Central Universities (China) (JD2227).

**Electronic Supplementary Material** Supplementary material is available in the online version of this article at <https://doi.org/10.1007/s11783-023-1660-x> and is accessible for authorized users.

## References

- Baumann B, Snozzi M, Van Der Meer J R, Zehnder A J B (1997). Development of stable denitrifying cultures during repeated aerobic-anaerobic transient periods. *Water Research*, 31(8): 1947–1954
- Blonder B, Wey T W, Dornhaus A, James R, Sih A (2012). Temporal dynamics and network analysis. *Methods in Ecology and Evolution*, 3(6): 958–972
- Deng Y, Jiang Y H, Yang Y, He Z, Luo F, Zhou J (2012). Molecular ecological network analyses. *BMC Bioinformatics*, 13: 113
- Deng Y, Zhang P, Qin Y, Tu Q, Yang Y, He Z, Schadt C W, Zhou J (2016). Network succession reveals the importance of competition in response to emulsified vegetable oil amendment for uranium bioremediation. *Environmental Microbiology*, 18(1): 205–218
- Duan J, Fang H, Su B, Chen J, Lin J (2015). Characterization of a halophilic heterotrophic nitrification-aerobic denitrification bacterium and its application on treatment of saline wastewater. *Bioresource Technology*, 179: 421–428
- Eiler A, Heinrich F, Bertilsson S (2012). Coherent dynamics and association networks among lake bacterioplankton taxa. *ISME Journal*, 6(2): 330–342
- García-Ruiz, Castellano-Hinojosa, Gonzalez-Lopez, Osorio (2018). Effects of salinity on the nitrogen removal efficiency and bacterial community structure in fixed-bed biofilm CANON bioreactors. *Chemical Engineering Journal*, 347: 156–164
- Girvan M, Newman M E J (2002). Community structure in social and biological networks. *Proceedings of the National Academy of Sciences of the United States of America*, 99(12): 7821–7826
- Grießmeier V, Bremges A, McHardy A C, Gescher J (2017). Investigation of different nitrogen reduction routes and their key microbial players in wood chip-driven denitrification beds. *Scientific Reports*, 7: 17028
- Han F, Zhang M, Liu Z, Shang H, Li Q, Zhou W (2021). Dynamic characteristics of microbial community and soluble microbial products in partial nitrification biofilm system developed from marine sediments treating high salinity wastewater. *Journal of Environmental Management*, 290: 112586
- Hu Y R, Jiang L, Sun X Y, Wu J Q, Ma L, Zhou Y B, Lin K F, Luo Y, Cui C Z (2021). Risk assessment of antibiotic resistance genes in the drinking water system. *Science of the Total Environment*, 800: 149650
- Huang X, Xing Y X, Wang H J, Dai Z Y, Chen T T (2022). Nitrogen advanced treatment of urban sewage by denitrification deep-bed filter: removal performance and metabolic pathway. *Frontiers in Microbiology*, 12: 811697
- Kämpfer P, Jerzak L, Wilharm G, Golke J, Busse H J, Glaeser S P (2015). *Gemmobacter intermedius* sp. nov., isolated from a white stork (*Ciconia ciconia*). *International Journal of Systematic and Evolutionary Microbiology*, 65(Pt\_3): 778–783
- Kishor R, Purchase D, Saratale G D, Saratale R G, Ferreira L F R, Bilal M, Chandra R, Bharagava R N (2021). Ecotoxicological and health concerns of persistent coloring pollutants of textile industry wastewater and treatment approaches for environmental safety. *Journal of Environmental Chemical Engineering*, 9(2): 105012
- Lefebvre O, Moletta R (2006). Treatment of organic pollution in industrial saline wastewater: a literature review. *Water Research*, 40(20): 3671–3682
- Li J B, Ma J X, Sun L, Liu X, Liao H Y, He D (2022). Mechanistic insight into the biofilm formation and process performance of a passive aeration ditch (PAD) for decentralized wastewater treatment. *Frontiers of Environmental Science & Engineering*, 16(7): 86
- Liu X, Dai J, Ng T L, Chen G (2019). Evaluation of potential environmental benefits from seawater toilet flushing. *Water Research*, 162: 505–515
- Luo F, Zhong J, Yang Y, Scheuermann R H, Zhou J (2006). Application of random matrix theory to biological networks. *Physics Letters. [Part A]*, 357(6): 420–423
- Luo L, Zhou W, Yuan Y, Zhong H, Zhong C (2021). Effects of salinity shock on simultaneous nitrification and denitrification by a membrane bioreactor: performance, sludge activity, and functional microflora. *Science of the Total Environment*, 801: 149748
- Luo W, Hai F I, Kang J, Price W E, Guo W, Ngo H H, Yamamoto K, Nghiem L D (2015). Effects of salinity build-up on biomass characteristics and trace organic chemical removal: implications on the development of high retention membrane bioreactors. *Bioresource Technology*, 177: 274–281
- Maqbool T, Cho J, Hur J (2018). Changes in spectroscopic signatures in soluble microbial products of activated sludge under different osmotic stress conditions. *Bioresource Technology*, 255: 29–38
- Miao L, Wang S, Li B, Cao T, Zhang F, Wang Z, Peng Y (2016).

- Effect of carbon source type on intracellular stored polymers during endogenous denitrification (ED) treating landfill leachate. *Water Research*, 100: 405–412
- Mota C, Head M A, Ridenoure J A, Cheng J J, de Los Reyes F L 3rd (2005). Effects of aeration cycles on nitrifying bacterial populations and nitrogen removal in intermittently aerated reactors. *Applied and Environmental Microbiology*, 71(12): 8565–8572
- Olesen J M, Bascompte J, Dupont Y L, Jordano P (2007). The modularity of pollination networks. *Proceedings of the National Academy of Sciences of the United States of America*, 104(50): 19891–19896
- Peng L, Ni B J, Ye L, Yuan Z (2015). N<sub>2</sub>O production by ammonia oxidizing bacteria in an enriched nitrifying sludge linearly depends on inorganic carbon concentration. *Water Research*, 74: 58–66
- Shi S, Nuccio E E, Shi Z J, He Z, Zhou J, Firestone M K (2016). The interconnected rhizosphere: high network complexity dominates rhizosphere assemblages. *Ecology Letters*, 19(8): 926–936
- Smolders G J F, van der Meij J, van Loosdrecht M C, Heijnen J J (1994). Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. *Biotechnology and Bioengineering*, 44(7): 837–848
- Sun X, Xu R, Dong Y, Li F, Tao W, Kong T, Zhang M, Qiu L, Wang X, Sun W (2020). Investigation of the ecological roles of putative keystone taxa during tailing revegetation. *Environmental Science & Technology*, 54(18): 11258–11270
- Tang Z, Lin Z, Wang Y, Zhao P, Kuang F, Zhou J (2020). Coupling of thermophilic biofilm-based systems and ozonation for enhanced organics removal from high-temperature pulping wastewater: performance, microbial communities, and pollutant transformations. *Science of the Total Environment*, 714: 136802
- Teske A, Alm E, Regan J M, Toze S, Rittmann B E, Stahl D A (1994). Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. *Journal of Bacteriology*, 176(21): 6623–6630
- Wang J, Gong B, Huang W, Wang Y, Zhou J (2017a). Bacterial community structure in simultaneous nitrification, denitrification and organic matter removal process treating saline mustard tuber wastewater as revealed by 16S rRNA sequencing. *Bioresource Technology*, 228: 31–38
- Wang J, Liu Q, Wu B, Hu H, Dong D, Yin J, Ren H (2020a). Effect of salinity on mature wastewater treatment biofilm microbial community assembly and metabolite characteristics. *Science of the Total Environment*, 711: 134437
- Wang J, Zhou J, Wang Y, Wen Y, He L, He Q (2020b). Efficient nitrogen removal in a modified sequencing batch biofilm reactor treating hypersaline mustard tuber wastewater: The potential multiple pathways and key microorganisms. *Water Research*, 177: 115734
- Wang X, Li J, Liu R, Hai R, Zou D, Zhu X, Luo N (2017b). Responses of bacterial communities to CuO nanoparticles in activated sludge system. *Environmental Science & Technology*, 51(10): 5368–5376
- Wang X, Ya T, Zhang M, Liu L, Hou P, Lu S (2019). Cadmium (II) alters the microbial community structure and molecular ecological network in activated sludge system. *Environmental Pollution*, 255(Pt 2): 113225
- Wang Y, Shen L, Wu J, Zhong F, Cheng S (2020c). Step-feeding ratios affect nitrogen removal and related microbial communities in multi-stage vertical flow constructed wetlands. *Science of the Total Environment*, 721: 137689
- Wu L, Yang Y, Chen S, Zhao M, Zhu Z, Yang S, Qu Y, Ma Q, He Z, Zhou J, He Q (2016). Long-term successional dynamics of microbial association networks in anaerobic digestion processes. *Water Research*, 104: 1–10
- Xia Y, Kong Y, Thomsen T R, Halkjaer Nielsen P (2008). Identification and ecophysiological characterization of epiphytic protein-hydrolyzing saprospiraceae (*Candidatus Epiflobacter* spp.) in activated sludge. *Applied and Environmental Microbiology*, 74(7): 2229–2238
- Xiao N, Zhou A, Kempfer M L, Zhou B Y, Shi Z J, Yuan M, Guo X, Wu L, Ning D, Van Nostrand J, Firestone M K, Zhou J (2022). Disentangling direct from indirect relationships in association networks. *Proceedings of the National Academy of Sciences of the United States of America*, 119(2): e2109995119
- Xu J, Pang H L, He J G, Nan J (2020). The effect of supporting matrix on sludge granulation under low hydraulic shear force: performance, microbial community dynamics and microorganisms migration. *Science of the Total Environment*, 712: 136562
- Ya T, Du S, Li Z, Liu S, Zhu M, Liu X, Jing Z, Hai R, Wang X (2021). Successional dynamics of molecular ecological network of anammox microbial communities under elevated salinity. *Water Research*, 188: 116540
- Ye L, Peng C, Tang B, Wang S, Zhao K, Peng Y (2009). Determination effect of influent salinity and inhibition time on partial nitrification in a sequencing batch reactor treating saline sewage. *Desalination*, 246(1–3): 556–566
- Yuan M M, Guo X, Wu L W, Zhang Y, Xiao N J, Ning D L, Shi Z, Zhou X S, Wu L Y, Yang Y F, et al. (2021). Climate warming enhances microbial network complexity and stability. *Nature Climate Change*, 11(4): 343–348
- Zhang L L, Ding L L, He X M, Ma H J, Fu H M, Wang J F, Ren H Q (2019). Effect of continuous and intermittent electric current on lignin wastewater treatment and microbial community structure in electro-microbial system. *Scientific Reports*, 9: 805
- Zhao Y, Park H D, Park J H, Zhang F, Chen C, Li X, Zhao D, Zhao F (2016). Effect of different salinity adaptation on the performance and microbial community in a sequencing batch reactor. *Bioresource Technology*, 216: 808–816