Response of Nitrifier and Denitrifier Abundance to Salinity Gradients in Agricultural Soils at the Yellow River Estuary

Daqing Huang 1,2,†, Xiang Li 1,2,† and Xuesong Luo 1,2,*

1 Key Laboratory of Subtropical Agricultural Resource and Environment, Ministry of Agriculture and Rural Affairs, College of Resources and Environment, Huazhong Agricultural University, Wuhan 430070, China; huangdaqing@webmail.hzau.edu.cn (D.H.); li.xiang@webmail.hzau.edu.cn (X.L.)
2 State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China
* Correspondence: xuesongluo@mail.hzau.edu.cn
† These authors contributed equally to this work.

Abstract: Salinization is considered a threat to agricultural soil and decreases crop yield worldwide. Nitrification and denitrification are the core processes of soil N-cycle. However, the response of nitrifiers and denitrifiers to salinity in agricultural soils remains ambiguous. The study aimed to explore the effect of salinity on nitrifiers and denitrifiers communities in agricultural soils along a naturally occurring salinity gradient. The effects of salinity on the abundance, composition, and interactions of nitrifiers and denitrifiers in surface soils were investigated. The abundance of nitrifiers significantly decreased in response to the increase in salinity. Ammonia-oxidizing archaea (AOA) were more susceptible to salinity elevation than ammonia-oxidizing bacteria (AOB). Nitrospira and Nitrobacter showed a similar trend to the salinity gradient, but the relative abundance of Nitrobacter was increased in the saline soils. High salinity decreased the abundance of napA and nirK, but had no significant effect on other marker genes for denitrification. Besides electrical conductivity, total sulfur (TS)+available potassium (AK) and TN+TS+C/N+total phosphorus (TP)+AK significantly explained the variation in denitrifier and nitrifier communities. We also found that high salinity decreased the connections between different N functional genes. These results implied the alteration of the nitrogen cycling community by high salinity mainly through decreasing AOA, NOB, and some denitrifiers with nitrate or nitrite reduction potentials and weakening the connectivity between nitrogen cycling drivers.

Keywords: salinity; nitrifiers; denitrifiers; community structure; microbial interaction

1. Introduction

Soil salinization is a global problem, affecting 833 million hectares of cropland in more than 100 countries [1]. Saline soils, commonly referred to as soils with electrical conductivity (EC) > 4 dS m−1 in saturated soil extracts, are widespread in agricultural ecosystems [2]. The salinized soil area is expected to increase due to climate change and human activities, such as irrigation with inferior water or overuse of chemical fertilizers [3,4]. Salinity is considered one of the vital environmental factors affecting global microbial community composition [5]. Previous research has indicated that increased salinity can impact microbial physiology and activity by directly altering microbial communities or indirectly via environmental factors [6,7]. Unfortunately, our understanding of the effect of salinity on nitrogen cycling bacterial communities remains limited.

The soil nitrogen cycle is a critical component of the biogeochemical cycle and affects soil productivity, sustainable development, and climate changes. The core steps in nitrogen cycling include nitrogen fixation, nitrification, and denitrification. Nitrification is a rate limit step and driven by the microorganisms oxidizing ammonia (NH4+) to nitrite (NO2−) and then to nitrate (NO3−), including ammonia-oxidizing bacteria (AOB), ammonia-oxidizing
archaea (AOA), nitrite oxidizing bacteria (NOB) and comammox *Nitrobacter* [8,9]. Denitrification occurs primarily in anaerobic environments, causing nitrogen to be lost in gaseous forms, including nitric oxide, nitrous oxide, which traps heat, and nitrogen [10]. The nitrate is reduced to N\(_2\) by denitrifiers with NO\(_2\)-N, NO, and N\(_2\)O intermediaries. The critical functional enzymes involve nitrate reductase (*napA* and *narG*), nitrite reductase (*nirS* and *nirK*), nitric oxide reductase, and nitrous oxide reductase (*nosZ*) [11].

Several studies explored the response of nitrifiers and denitrifiers communities to salinity in mangrove sediments [12], coastal wetlands [13–15], and desert soils [16]. In some cases, it was found that salinity harmed nitrification [17]. Chandra et al. [18] observed that nitrogen nitrification and mineralization were enhanced before a salinity threshold. This finding was reasonable because nitrifying bacteria grew well at 5–10 ppt and were inhibited when the salinity was higher than 10 ppt [19]. However, the effect of salinity on soil nitrification could be inconsistent behind different environmental backgrounds [20]. The differences in salt tolerance or range and the populations of nitrifiers diverged in different environments. Based on molecular studies, the abundance of AOA and AOB is reported to decrease with salinity [16]. In contrast, other studies found that AOA abundance and potential nitrification rates increased under moderate salinity (10–20 ppt), while AOB abundance was negatively or not correlated with salinity [15,21]. Nitrite oxidizing bacteria (NOB) have been reported to be more susceptible to salinity than ammonia-oxidizing bacteria (AOB) [22]. Yue et al. (2020) found that *Nitrobacter* played a critical role in saline soil nitrification, and its abundance positively correlates with soil salinity [23]. The interactions between NOB members were likely blocked by high salinity [24]. Environmental factors explain nitrifying bacterial community structure in environments with different salinity backgrounds [12,16,25].

Understanding the relationship between denitrifiers and salinity is essential for predicting nitrogen cycling in response to environmental changes. Denitrification rates decreased with salinity from estuarine sediments [26]. Two reasons could explain the negative correlation between denitrification and salinity. One is that inhibition of nitrification by salinization reduces the substrate for denitrification [27]. The other is that some denitrifiers could be sensitive to high salinity. In contrast, differences in denitrification rates are not significantly explained by salinity in the Eutrophic Neuse Estuary in the United States [28]. This suggested that salinity may not drive the variation in the activity of salt-tolerant denitrifying microbial communities [29]. Previous studies showed that *nirK* genotype denitrifying bacteria seem less tolerant than *nirS* [30]. Indeed, besides *nirK* and *nirS* nitrite-reducers, *nosZ* possessing bacterial communities in coastal tidal wetlands were also influenced by salinity [31]. The diversity of the denitrifying community was inversely correlated with salinity [32,33]. We consider that salinity is a crucial factor in shaping soil nitrogen cycling. The responses of nitrifiers and denitrifiers along naturally occurring salinity gradients in agricultural ecosystems remain unclear. It is necessary to improve our understanding of nitrifiers and denitrifiers’ ecophysiology and salinization’s effects on the nitrogen cycling community in soil. This study aims to reveal the role of salinity gradients in shaping the abundance, community structure, and network of nitrifiers and denitrifiers in agricultural soils near the Yellow River estuary, China. We hypothesized that: (i) most nitrifiers and denitrifiers can decrease with an increase in salinity, but different functional groups could diverge in their sensitivity to salt stress. (ii) Salinity and other soil properties could shape nitrifier and denitrifier communities, and the interaction among nitrifiers and denitrifiers would be weakened under high salinity.

2. Materials and Methods
2.1. Study Area and Soil Background

The research site is at the yellow river estuary, Dongying city, Shandong Province, China (37°30′ N, 118°15′ E). The area is a warm temperate semi-humid monsoon climate with an average annual temperature of 14.2 °C and an average yearly rainfall of 634 mm.
Low altitude with seawater flooding results in salinization. The wheat–maize rotation system is widely applied in this area.

Soils were sampled from 40 plots (10 m × 10 m) near the seashore. We collected five cores from 0–20 cm soils in each plot and mixed them to form a composite sample. First, 40 soil samples were on ice and delivered to the laboratory within 24 h. Each sample was divided into several aliquots. One aliquot was air-dried and used to analyze the physochemical factors. The physochemical factors, including total carbon (TC), total nitrogen (TN), total sulfur (TS), Electrical conductivity (EC), soil organic matter (SOM), total phosphorus (TP), total potassium (TK), available phosphorus (AP), available potassium (AK), soil pH, NH\textsubscript{4}\textsuperscript{+}-N and NO\textsubscript{3}\textsuperscript{-}-N have been determined in our previous study [24]. The other was stored under −80 °C for DNA extraction. Saline soils (EC < 4 dS/m, n = 20) and non-saline soils (EC > 4 dS/m, n = 20) were defined as previously described by Rath et al. [2].

2.2. DNA Extraction and PCR Amplification

Soil DNA was extracted from 0.5 g samples, and the purification and detection were performed as described by Li et al. [34]. The quality and purity of extracted DNA products were determined by a Nanodrop-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) for purity and concentration. The obtained DNA was subsequently stored at −20 °C for quantitative PCR assays.

Quantitative PCR assays were carried out by an ABI7500 FAST Real-time PCR system. The primers used to amplify nitrifiers and denitrifiers are shown in Table 1. A standard curve was generated using 10-fold serial dilutions of the plasmid encoding a target gene. Each sample to be tested was added to three wells as technical replicates. Gel electrophoresis analysis was performed to validate the specificity of the amplification.

Table 1. Primer information for functional gene amplification.

| Genes   | Primer Name | Amplicon Size (bp) | Reference |
|---------|-------------|--------------------|-----------|
| for nitrifiers | amoA (AOA) | amoA23F/amoA616R   | 593 [35]  |
|         | amoA (AOB) | amoA-1F/amoA-2R    | 491 [36]  |
|         | nxrA        | F1norA/R2norA      | 282 [37]  |
|         | nxrB        | nxrB169F/nxrB638R  | 469 [38]  |
| for denitrifiers | narG        | narGf/narGr        | 173 [39]  |
|         | napA        | V17m/napA4r        | 152 [39]  |
|         | nirK        | F1aCu/R3Cu         | 473 [40]  |
|         | nirS        | cd3aF/R3cd         | 425 [41]  |
|         | qnorB       | qnorB2F/qnorB5R    | 262 [42]  |
|         | nosZ        | nosZ1F/nosZ1R      | 259 [43]  |

2.3. Statistical Analysis

This study demonstrates the community structure of nitrogen-cycling bacteria using the relative abundance of specific genes. Gene abundance data were log\textsubscript{10} transformed if necessary. Spearman’s correlation between gene abundance and physochemical factors was performed using the SPSS 20 statistical software (IBM Co., Armonk, NY, USA). Correlations between community composition and physochemical properties were tested using Mantel tests between Bray–Curtis dissimilarity matrices and Euclidean distance matrices of physochemical properties distribution. Canonical analysis of principal coordinates (CAP) based on Bray–Curtis heterogeneity was applied to calculate the extent to which physochemical factors explain the composition of the nitrifying bacteria community using the “capscale” function in the “vegan” package in R. Co-occurrence networks were applied to examine the complexity of the nitrogen cycling bacteria community. Spearman’s correlation between two genes was considered statistically if Spearman’s coefficient (\(\rho\)) > 0.6 and
the p-value was < 0.05. Furthermore, the networks were visualized using the interactive platform Gephi 0.9.2.

3. Results

3.1. Characteristics of the Soil Properties

The naturally occurring soil salinity ranges from 0.22 to 19.98 ds m$^{-1}$. Soil C/N ranged from 1.2 to 41.0. Along the gradients, soil EC was significantly positively correlated with soil C/N ($R^2 = 0.212$, $p < 0.01$). Soil organic matter content ranged from 4.0 to 51.6 g kg$^{-1}$ and was negatively correlated with soil EC ($R^2 = 0.18$, $p < 0.01$). Moreover, the soil EC showed a significant negative correlation with soil NH$_4^+$-N ($R^2 = 0.200$, $p < 0.01$) and TN ($R^2 = 0.247$, $p < 0.01$) content [24].

3.2. The Correlation between EC and N-Cycling Gene Abundance

The abundance of some nitrifiers and denitrifiers was significantly decreased along the salinity gradients (Figure 1). The abundances of amoA genes of AOA and AOB decreased from $3.6 \times 10^7$ to $1.0 \times 10^6$ and $1.2 \times 10^7$ to $6.7 \times 10^5$ copies g$^{-1}$ dry soil, respectively (Figure 1A,B). A similar trend was also observed for amoA from Nitrobacter and nxrB from Nitrobacter (Figure 1C,D). The abundance of AOA ($Y = 1.6 \times 10^7 X^{−0.3694}$, Spearman’s $\varrho = −0.797$, $p < 0.001$) was more sensitive to salinity than AOB ($Y = 4.6 \times 10^7 X^{−0.2283}$, $\varrho = −0.599$, $p < 0.001$), while the abundance of Nitrobacter ($Y = −2.8 \times 10^6 X + 5.9 \times 10^7$, $\varrho = −0.606$, $p < 0.001$) was slightly more sensitive to salinity than Nitrospira ($Y = −2.6 \times 10^6 X + 4.7 \times 10^7$, $\varrho = −0.780$, $p < 0.001$). For the denitrifying genes, napA and narG ranged from $7.9 \times 10^6$ to $2.3 \times 10^4$ and $3.1 \times 10^8$ to $1.9 \times 10^6$ copies g$^{-1}$ dry soil, respectively (Figure 1E,F). NirK and nirS ranged from $2.6 \times 10^8$ to $1.2 \times 10^6$ and $2.9 \times 10^8$ to $6.2 \times 10^3$ copies g$^{-1}$ dry soil, respectively (Figure 1G,H). NosZ and qnorB ranged from $1.8 \times 10^7$ to $1.4 \times 10^3$ and $4.7 \times 10^7$ to $6.8 \times 10^4$ copies g$^{-1}$ dry soil, respectively (Figure 1I,J). We also observed negative correlations between EC and napA ($\varrho = −0.600$, $p < 0.001$), between EC and nirK ($\varrho = −0.558$, $p < 0.001$). NarG, nirS, qnorB, and nosZ were not correlated with EC.

3.3. The Effect of Salinity on Community Structure

It was considered that EC = 4 ds/m is a threshold for salinity. We compared the community composition of nitrifiers and denitrifiers in the non-saline soils (EC < 4 ds/m) with those in the saline (EC > 4 ds/m) ones (Figure 2). For the nitrifying microbial community, the relative abundances of AOA and Nitrobacter decreased while the relative abundance of Nitrobacter increased in high salinity soils (Figure 2A). For the denitrifying bacteria community, the relative abundance of nirK-denitrifying bacteria in saline soil decreased significantly while the changes of nirS-denitrifying bacteria and narG-denitrifying bacteria were opposite (Figure 2B). In conclusion, soil salinity affected nitrifier and denitrifier’s community composition.
Figure 1. The correlation of EC and N-cycle related gene abundance, AOA amoA (A), AOB amoA (B), nxrA (C), nxrB (D), narG (E), napA (F), nirK (G), nirS (H), qnorB (I), nosZ (J). X stands for salinity, and Y stands for gene copy number.
Mantel test data (Figure 3) showed that soil C/N \( (r = 0.28, p < 0.01) \) and soil EC \( (r = 0.53, p < 0.001) \) significantly correlated with nitrifiers community. Denitrifiers community was significantly correlated with soil TS \( (r = 0.41, p < 0.01) \), AK \( (r = 0.31, p < 0.01) \) and EC \( (r = 0.27, p < 0.01) \). Among these soil physicochemical properties, soil EC emerged as a dominant factor linked to N-cycle-related microbial community composition. The first and second axis explain 40.71% and 17.49% of the total changes in nitrifying bacteria community composition, respectively, in CAP (Figure 4A). For the denitrifier community, the first and second axes explained 20.39% and 6.82% of the total change in community composition, respectively (Figure 4B). The first and second axes explained 17.76% and 14.02% of the total change for the whole nitrogen cycling microbial community (Figure 4C).

Figure 2. Relative gene abundance of nitrifiers (A), denitrifiers (B), and N-cycle-related microorganisms (C) under low (EC < 4 ds/m) and high (EC > 4 ds/m) salinity conditions.

Figure 3. Pearson correlations among soil physicochemical factors. Mantel tests linked the Nitrifiers community, denitrifiers community, and N-cycle-related microbial community to each factor. Links width is proportional to the Mantel’s \( r \), and link color signifies significance. Sub 1 indicates nitrifier community; Sub 2 indicates denitrifier community; Sub 3 indicates N-cycle-related microbial community displayed in this study. TC, total carbon; TN, total nitrogen; TS, total sulfur; EC, electron conductivity, SOM, soil organic matter; TP, total phosphorus, AP, available phosphorus (Olsen P); TK, total potassium; AK, available potassium.
3.4. Co-Occurrence Network Analysis

We assessed the effect of salinity on the potential interactions or linkages among different N-cycle-related microorganisms by co-occurrence network analysis (Figure 5). For the non-saline soils, the connectivity of the nitrogen cycler network was denser than that for the saline soil (Figure 5A,B). A less close correlation in saline soils indicated that high salinity might weaken the interaction or the functional coupling among nitrogen cycle drivers.

Figure 4. Canonical analyses of the principal coordinates (CAP) show the relationships between environmental factors and nitrifiers community (A), denitrifiers community (B), and N-cycle-related microbial community (C) in saline and non-saline soils. The number near each axis represents an explanation for variation in community composition. The significance of environmental variables was measured using Adonis and is indicated by asterisks next to the variable names (* \( p < 0.05; ** p < 0.01; *** p < 0.001 \)). NS: non-saline soil; S: saline soil.

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Figure 5. Co-occurrence networks of the N-function genes for saline soils (A) and non-saline soils (B) based on correlation analysis. A connection stands for a strong (Spearman’s \( \rho > 0.6 \)) and significant \( (p < 0.01) \) correlation. The size of each node is proportional to the degree of the abundance.
4. Discussion

4.1. Responses of Gene Abundance to Salinity

The abundance of nitrifiers was considerably decreased with salinity in this study, suggesting that salinity inhibits nitrifiers growth, consistent with Guo et al. [16], who reported that the abundance of AOA and AOB decreased with elevated salinity. He et al. [44] observed that the potential nitrification rate decreased significantly with salinity. Our data showed that the abundance of AOA and *Nitrobacter* was higher than that of AOB and *Nitrospira*. In less fertile soils, the AOA *amoA* gene copy number is usually higher than AOB [45–47]. Bernhard et al. [21] observed that the abundance of AOA was always higher than that of AOB along Plum Island Sound estuary salinity gradient. The higher AOA abundance suggests that AOA dominates the ammonia oxidation community and may be a major contributor to ammonia oxidation in sediments under a wide range of salinity [15]. In contrast to these studies, Wang et al. [12] reported that AOB played a dominant role in nitrification because the abundance of AOB exceeded that of AOA. Those inconsistent results may be explained by their differences in environmental backgrounds. We found that the AOA exhibited a more robust response to salinity than AOB (Figure 1), suggesting that AOA could be more sensitive to salinity in this soil. This data agrees with a previous finding in alluvial grey desert soils [16]. It was wildly accepted that *Nitrobacter* is more resistant to salt stress than *Nitrospira* [48,49]. Here, we found that the response level of *Nitrobacter* showed a similar trend to *Nitrospira*, but displayed a higher relative abundance in the saline soils, supporting this hypothesis.

Depending on their salinity tolerance, different denitrifier species can grow at various salinities [11]. In this study, the abundance of denitrifiers (*napA* and *nirK*) was significantly influenced by salinity. However, no significant relationship between other denitrifiers with salinity was found, suggesting salinization selectively inhibited both *napA* and *nirK* denitrifiers, possibly due to their weaker tolerance to salinity. Wang et al. [12] also found that increased salinity inhibits the abundance of *nirK* genes. In contrast, Zhai et al. [11] found that *napA* genes have a higher abundance in biofilm electrode reactors under different salinities. The *nirS* and *nirK* genes encode nitrite reductase, which converts nitrite to NO. In our study, the relative abundance of the *nirK* genes was greater than that of *nirS* under low salinity (EC < 4 ds/m), whereas the opposite trend was observed under high salinity (EC > 4 ds/m). Although some results supported a hypothesis that *nirS* type denitrifiers could be more tolerant of salt stress [11,50,51], others disagree. Our study showed no significant correlation between salinity and *nosZ* denitrifiers, inconsistent with two previous reports [12,52]. The varying salinity concentration and ecosystem may partly explain these different results. It is suggested that the level of salinity considered in a particular study, along with the nature of the habitat soil vs. water ecosystem, may be critical factors when integrating results of the influence of salinity in microbial community structure from different investigations [53]. As a result, salinity could reduce the abundance of nitrifiers and *napA/nirK* genotype denitrifiers.

4.2. Impact of Salinity on the Community Structure of Nitrifiers and Denitrifiers

Previous studies in saline ecosystems have shown that salinity alters the community composition and reduces the phylogenetic diversity [54–56]. This phenomenon is primarily because some microorganisms adapt to high osmotic stress caused by high salinity and toxicities of sodium and other accompanying ions such as HCO$_3^-$ under sodic conditions, while others do not [37,38]. In this study, we observed that the community composition of nitrifiers and denitrifiers has a significant shift in saline soils compared with their non-saline precursors. Compared with non-saline soil, the relative abundance of *Nitrospira* and *nirS*-type denitrifying bacteria in saline soil increased significantly, while *Nitrobacter* and *nirK*-type denitrifying bacteria decreased. Rath et al. [59] reported that soil microbial communities quickly adapt to fluctuating salt concentrations. The shifting community salt tolerance is partly driven by changes in community composition, with more salt-tolerant species replacing the sensitive ones. We speculated that *Nitrospira* and *nirS*-type
denitrifying bacteria are moderately halotolerant. They may partly replace *Nitrobacter* and *nirK*-type denitrifying bacteria along the salinity gradient for the ecosystem function. Previous studies have confirmed that salinity directly affects the structure and diversity of bacterial communities in coastal wetlands [60,61]. Besides EC, we also found other factors influencing the composition of the nitrifiers and denitrifiers community. For instance, TN, TS, and C/N significantly explained variations in the nitrifier community, while TS and AK did that in the denitrifiers. Considering that soil EC was also significantly correlated with TN, C/N, TS, and SOM, we infer that soil salinization directly affects the activity of nitrogen cycling microorganisms and indirectly by altering some key physicochemical factors. We found that saline soils had fewer network edges than the non-saline soils, indicating that salinity reduced network connectivity for nitrifiers to denitrifiers. Shifts in the composition in nitrifiers and denitrifiers and the salinity increase indicated that N-cycling-related microbes have strong niche differentiation due to their salt tolerance variation. Although this was discussed, we would like to mention the niche hypothesis to discuss our network analysis further. Indeed, the salinization process could allow more salt-tolerant microbes to survive and less salt-tolerant ones to decrease. Considering that weak niche differentiation possibly results in stronger interactions between soil microorganisms [62], it is reasonable to see that salinity decreased correlations between N-cycle-related microbes since niche differentiation became strong under a higher salinity. Our previous study showed that the network within *Nitrospira* collapsed while *Nitrobacter* was resistant to high salinity [24].

5. Conclusions

The present study found that salinity significantly influences nitrifiers and denitrifiers in agricultural soil from the Yellow River estuary. Elevated salinity decreased the abundance of nitrifiers and partial denitrifiers represented by the napA and nirK genotypes. AOA abundance exhibited a higher sensitivity to elevated salinity than AOB, while *Nitrobacter* showed a similar resistance level to *Nitrospira*. nirS type denitrifiers were probably more salt-tolerance than nirK. EC+TS+AK and EC+TN+TS+C/N+TP+AK significantly explained the variation in denitrifier and nitrifier communities. High salinity reduced the network complexity of nitrifier to denitrifier microbial community. These results prove that soil salinization can reduce AOA, NOB, some denitrifiers, and their network connectivity, probably weakening the nitrogen cycling.

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