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Original Research Article

Intensified river salinization alters nitrogen-cycling microbial communities in arid and semi-arid regions of China

Qingqing Pang [a], Dan Wang [a,b], Zewei Jiang [c], Mohamed Abdalla [d], Lei Xie [a,e], Xiang Zhu [a,b], Fuquan Peng [a], Pete Smith [d], Longmian Wang [a,*], Lingzhan Miao [b], Jun Hou [b,*], Peng Yu [f], Fei He [a], Bin Xu [a]

[a] Nanjing Institute of Environmental Sciences, Ministry of Ecology and Environment, Nanjing 210042, China
[b] College of Environment, Hohai University, Nanjing 210098, China
[c] College of Agricultural Science and Engineering, Hohai University, Nanjing 210098, China
[d] Institute of Biological & Environmental Sciences, University of Aberdeen, 23 St Machar Dr., Aberdeen AB24 3UU, UK
[e] School of Environmental Science and Engineering, Nanjing University of Information Science and Technology, Nanjing 210044, China
[f] Shandong Academy of Environmental Sciences Co., Ltd, Jinan 250100, China

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ABSTRACT

Freshwater salinization is receiving increasing global attention due to its profound influence on nitrogen cycling in aquatic ecosystems and the accessibility of water resources. However, a comprehensive understanding of the changes in river salinization and the impacts of salinity on nitrogen cycling in arid and semi-arid regions of China is currently lacking. A meta-analysis was first conducted based on previous investigations and found an intensification in river salinization that altered hydrochemical characteristics. To further analyze the impact of salinity on nitrogen metabolism processes, we evaluated rivers with long-term salinity gradients based on in situ observations. The genes and enzymes that were inhibited generally by salinity, especially those involved in nitrogen fixation and nitrification, showed low abundances in three salinity levels. The abundance of genes and enzymes with denitrification and dissimilatory nitrate reduction to ammonium functions still maintained a high proportion, especially for denitrification genes/enzymes that were enriched under medium salinity. Denitrifying bacteria exhibited various relationships with salinity, while dissimilatory nitrate reduction to ammonium bacterium (such as *Hydrogenophaga* and *Curvibacter* carrying *nirB*) were more inhibited by salinity, indicating that diverse denitrifying bacteria could be used to regulate nitrogen concentration. Most genera exhibited symbiotic and mutual relationships, and the highest proportion of significant positive correlations of abundant genera was found under medium salinity. This study emphasizes the role of river salinity on environment characteristics and nitrogen transformation rules, and our results are useful for improving the availability of river water resources in arid and semi-arid regions.

1. Introduction

Surface freshwater resources for agriculture production are particularly scarce in arid and semi-arid areas [1]. Although about 70% of the Earth is covered by water, only 2.5% of it is fresh [2]. Due to the underlying geology and high evaporation, the phenomenon of freshwater salinization is more prominent in arid and semi-arid regions [3,4]. Surface water can be divided according to electrical conductivity (EC) as follows: freshwater <0.7 mS/cm, slightly saline 0.7–2.0 mS/cm, moderately saline water 2.0–10.0 mS/cm, highly saline water 10.0–20.5 mS/cm, very highly saline water 20.5–45.0 mS/cm, and brine water >45.0 mS/cm [5]. The salinity of river is gaining increasing attention and has been measured in situ, including Ningxia Hui Autonomous Region [6] and Xinjiang Uygur Autonomous Region [7,8], with salinity levels ranging from 0.05 to 9.9 mS/cm. The difference emphasizes the need for a comprehensive evaluation of river's salinity at local or regional scales. However, increasing attention is being paid to the phenomenon of freshwater salinization, and a notable research gap persists in our understanding of river salinity changes in China's arid and semi-arid regions.

* Corresponding authors.
E-mail addresses: wlm@nies.org (L. Wang), hjy_hj@hhu.edu.cn (J. Hou).

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Nitrogen is crucial in maintaining ecosystem functionality [9]. Increasing levels of inorganic nitrogen, especially nitrate nitrogen (NO_3^--N, from 0.15 to 108.76 mg/L in surface water) [10–12], result in water eutrophication and a lack of freshwater resources [13,14]. Moreover, excessive nitrogen inputs from irrigation water cause overfertilization [15]. Salinization alters nitrogen metabolic processes [3,16]. A meta-analysis found that salinization decreased denitrification and dissimilatory nitrate reduction to ammonium (DNRA) by about 10% and 38%, respectively, and increased nitrification rates by about 15% under slightly saline conditions [17]. This elevated NO_3^--N further exacerbates the risk of eutrophication. However, studies on the impacts of salinity on nitrogen transformation mainly focused on coastal estuaries or wetlands [18,19]. Unlike in coastal areas, river salinization in arid and semi-arid rivers is mainly caused by the long-term accumulation of climate change. Moreover, the salinity of rivers (0.05–9.9 mS/cm) is generally lower than that of coastal areas (4.3–33.3 mS/cm) [20–22]. However, the transformation of NO_3^--N in rivers at relatively low salinity levels and specific climatic conditions is still unclear.

Microbes play an irreplaceable role in river nitrogen cycling. Among abiotic environmental factors, salinity is considered the key factor determining the composition of the nitrogen transformation microbial community [23]. However, examinations of the adaptability and composition of microbial communities along river salinity gradients in arid and semi-arid regions are still lacking. Functional enzymes and genes are the main factors driving biological nitrogen transformation [24]. The high salinity significantly inhibited the relative abundances of key enzymes, such as nitrogenase (NG, EC 1.18.6.1), ammonia monooxygenase (AMO, EC 1.14.99.39), hydroxylamine reductase (HAO, EC 1.7.99.1), and nitrite reductase [NiR (NO-forming), EC 1.7.2.1] [25]. However, Chi et al. [26] found that the enzymes involved in DNRA were enriched under high salinity levels. Thus, the effects of salinity on nitrogen-transforming functional genes and enzymes are still subject to debate. In addition, because of changes in environmental conditions (such as nitrogen concentration and pH) [27], the interactions of nitrogen-transforming bacteria differ. Therefore, it is important to evaluate the effects of different salinity levels caused by climate or geology on the genes and microorganisms related to nitrogen metabolism, and to disclose the relationship between salinity and nitrogen conversion for rivers in arid and semi-arid regions.

Qingshui River is the primary tributary of the Yellow River. It has a surface area of 13,511 km^2 and a stream length of 320 km. The area is mainly influenced by arid and semi-arid climates, with average annual rainfall and evaporation of 300 mm and 2,300 mm [28], respectively. Due to the varying geological conditions and climates, spatial salinity gradients were established. It has been reported that the river salinities ranged from 0.86 to 1 mS/cm upstream, 4.29 to 7.14 mS/cm midstream, and 7.17 to 7.86 mS/cm downstream in 1993, respectively [6]. The main land use types are cultivated land (39.5%), which imposes considerable irrigation water demands [29]. Therefore, Qingshui River, characterized by long-term salinity gradients, was chosen for field research. Detailed background information on Qingshui River is available in Text S1.

Here, the change trends of salinity at the regional level in arid and semi-arid regions of China were compiled. Subsequently, the impact of changes in salinity levels on nitrogen conversion processes in the Qingshui River was examined. The objectives of this study were to (1) characterize the changing trends of river salinity in arid and semi-arid regions of China since the 1990s, (2) elucidate the effect of salinity on the environmental characteristics of rivers in arid and semi-arid regions of China, (3) explore the responses of functional genes and communities to salinity, especially the network relationship, (4) establish the relationship between salinity, inorganic nitrogen, and microbial community in terms of nitrogen biotransformation. This study provides a theoretical basis for using molecular biology methods to accurately reduce nitrogen concentration under changes in freshwater salinization.

2. Materials and methods

2.1. Data collection and compilation in arid and semi-arid regions of China

Articles published in peer-reviewed journals in the arid and semi-arid regions of China were gathered using the Web of Science and the China National Knowledge Infrastructure with a period of 1990–2022. The following keywords were used: “river” (or “stream”) and “salinity” (or “conductivity” or “total dissolved solids”) and “hydrochemical characteristics” (or “environmental characteristics” or “nitrogen” or “environmental characteristics” or “dissolved organic nitrogen” or “chemical oxygen demand”) and “affect” (or “effect” or “impact” or “correlation”) and “arid region” (or “arid area”) and “China”. This allowed for the analyses of long-term changing trends in river salinity in arid and semi-arid regions of China. The average data was used if the original value in papers contained monthly or quarterly data. The following criteria were used to determine appropriate reports: (1) only on-site observations were collected, excluding laboratory simulations; (2) at least one of the selected indicators was detected; (3) studies from coastal estuaries or wetlands were excluded, because only aquatic ecosystems with salinity caused by climate or geology in arid and semi-arid regions were investigated. Reported salinity values were converted to mS/cm; if reported by weight, a factor of 0.7 is used to convert to EC [4]. The collected literature that is suitable for this study is shown in Table S1.

In addition, to assess the impact of salinity on environmental characteristics, the following criteria were used to select suitable studies: (1) river samples along the salinity gradient were directly collected, and (2) the lowest salinity gradient was <0.7 mS/cm. The environmental characteristics in non-saline samples (salinity < 0.7 mS/cm) were selected as control, while those in high salinity samples (> 0.7 mS/cm) were treated as experimental groups for salinity comparison with the same study [16].

2.2. Sample collection

Water sampling was carried out along the river (Fig. S1) in May 2020, which is the typical month of perennial runoff in semi-arid regions. The average water temperature at the time of sampling was 14.89 °C. Based on the current salinity of water samples and the future trend of river salinization, the points were categorized into three salinity levels, classified as low salinity (LS, EC < 2 mS/cm) (L1–L5), medium salinity (MS, 2 < EC < 8 mS/cm) (M1–M4), and high salinity (HS, EC > 8 mS/cm) (H1–H5) ($p < 0.05$). Samples were collected from the surface (top 30 cm) using 2 L sampling bottles in three replicates, obtaining a total of 14 samples. Water samples were stored at 4 °C, filtered through a 0.22 μm filter membrane, and transported to the laboratory within 24 h for water analysis. The filter membrane, which was used for metagenome analysis, was delivered to the laboratory using a cooling box fitted with an ice pack.

2.3. Data analysis

2.3.1. Meta-analysis

The effects of increased salinity on the environmental characteristics of rivers were evaluated by calculating the natural logarithm of the response ratio (ln RR). The calculation formula is as follows:

$$\ln RR = \ln X_t - \ln X_c \quad (1)$$

where X_t and X_c denote the mean values in the treatment and control groups, respectively [16]. The ln RR was further calculated by the variance (v):

$$v = \frac{S_t^2}{n_t X_t^2} + \frac{S_c^2}{n_c X_c^2} \quad (2)$$

where S_t and S_c denote the standard deviations of the treatment and control groups, respectively; n_t and n_c are the sample number of treatment and control groups, respectively.

The response ratio and 95% confidence interval were calculated by MetaWin 2.1 and R software package Metafor 2.0 [16,30]. A weighted resampling method with 9999 iterations was used to estimate the random effects model in MetaWin. A multivariate model of mixed effects was constructed by rma.uni function and restricted maximum likelihood estimation.

2.3.2. Water quality analysis

Based on the actual situation of the study area, water quality indices, including EC, pH, dissolved oxygen (DO), total nitrogen (TN), NO_3^--N, nitrite nitrogen (NO_2^--N), ammonia nitrogen (NH_4^+-N), and chemical oxygen demand (COD), were determined and analyzed (Table S2). The parameters of EC, pH, and DO were determined in situ using a YSI Multiparameter Water Quality Monitor (YSI ProPlus, YSI Inc., USA); TN was measured via potassium persulfate ultraviolet spectrophotometry; NO_3^--N was determined via phenol sulfonic acid spectrophotometry; NO_2^--N was measured with the spectrophotometric method, and COD was determined using potassium dichromate.

2.3.3. Metagenomic analysis

Genomic DNA was extracted from 0.5 g of the aqueous filter membranes using the FastDNA SPIN Kit (MP Biomedicals, USA) according to the manufacturer's instructions. The concentration of extracted DNA was quantified with a NanoDrop 2000. The DNA integrity was assessed using gel electrophoresis (1% agarose). The raw sequencing data were quality-controlled using the FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc). The extracted DNA was processed to construct fragments approximately 400 bp by using Illumina TruSeq Nano DNA LT Library Preparation Kit (Illumina, San Diego, CA, USA) for metagenome shotgun sequencing libraries. Each library was performed using the Illumina NovaSeq platform (Illumina, USA) with PE150 strategy at Personal Biotechnology Co., Ltd. (Shanghai, China).

To ensure the reliability of the gene data, low-quality reads (length < 50 bp, a quality value of <20, or having N bases) were removed using Sickle. Once quality-filtered reads were obtained, the de Bruijn graph Assembler was assembled to construct the metagenome. For each sample, at least 200-bp scaftigs sequences were selected using MetaGeneMark (http://exon.gatech.edu/GeneMark/metagenome) [31] to predict metagenomic gene sequences and identify the open reading frame to obtain corresponding gene prediction results. Subsequently, CD-HIT software was employed to merge the above protein sequences according to 90% sequence similarity to remove redundancy [32]. The taxonomic annotation of the non-redundant genes was obtained by aligning them against the NCBI-NT database using BLAST software (BLAST2lca) (e-value: 0.0001). Similarly, the functional profiles were obtained by annotating against the Kyoto Encyclopedia of Genes and Genomes (KEGG ver. 94.2, http://www.genome.jp/kegg/) by using Diamond. The targeted genes involved in the nitrogen cycle were filtered out based on the main pathways of nitrogen cycling across all the samples. Finally, the taxonomic annotation for the subset of selected genes was processed with Diamond and BLASTP against the NCBI-NR database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/). For statistical analysis, the abundances of single genes are presented as transcripts per kilobase per million (TPM), calculated by the number of the single gene reads divided by the total reads of the sample and multiplied by 1 million. The metagenomic sequencing data were archived in the NCBI database under registration number SRP329549, the detailed numbers of the Biosample were provided in Table S3.

2.4. Statistical analysis

A one-way analysis of variance (ANOVA) was used to test for significant differences among LS, MS, and HS. Based on Spearman correlation analysis, the relationship between salinity and nitrogen-transforming bacteria was determined. Network analysis of dominant nitrogen-transforming functional bacteria was performed using "psych" of R 3.5.1 and Gephi 0.9.3, according to significant Spearman's correlation coefficients ($|r| > 0.6, p < 0.05$). The relationships among environmental factors and bacterial community structure were evaluated via structural equation modeling (SEM) using SPSS 22.0 and Amos 24.0.

3. Results

3.1. River salinity variation and their impact on water environment characteristics

As depicted in Fig. 1, the salinity of rivers in arid and semi-arid regions of China was displayed throughout the past three decades. The results showed that the average salinity of rivers increased by 0.64 mS/cm (with a maximum increase of 2.44 mS/cm), indicating an overall increasing tendency in salinity. It should be noted that differences in the spatial distribution of sampling locations and densities led to significant variations in salinity changes across various provinces (Fig. S2). The number of studies in Xinjiang had significantly increased from 2010 to 2022, but with no significant changes in salinity. This meant that salinity had gradually become a key environmental characteristic indicator in arid regions. Although the sampling points remained unchanged in Gansu, the average salinity significantly increased by 2.44 mS/cm (Fig. S3).

The relationship between river salinity and water environment characteristics in arid and semi-arid regions of China was evaluated (Fig. 1b). The concentrations of NH_4^+-N and NO_3^--N were reduced by 82% and 11%, respectively, with increasing salinity. In contrast, the contents of COD, dissolved organic carbon (TOC), NO_2^--N, and TN were increased by 75% (95%CI: 11%–139%), 35% (95%CI: 20%–50%), 63% (95%CI: 27%–98%), and 23% (95%CI: 0%–47%), respectively.

3.2. Responses of dominant nitrogen-cycling functional enzymes, genes, and bacteria to salinity

Since the impact of salinity on water environmental characteristics in rivers was identified in arid and semi-arid regions of China, we further evaluated whether salinity regulates nitrogen transformation processes. As shown in Fig. 2, the relative abundances of AMO and HAO were lower than those of other enzymes, especially under HS (0.08 and 0.01, respectively). In agreement with the changing trend of enzyme catalyzation in nitrification, the relative abundance of genes related to ammonia oxidation (*amoABC* and *hao*) and nitrobacteria [*Nitrosopumilus* (*amoB*), *Nitrospira* (*amoC*), and *Sulfurifustis* (*hao*)] were also low under HS (Figs. 3 and 4).

The NO_3^--N reduction mainly included traditional denitrification, DNRA, and assimilatory nitrate reduction to ammonium (ANRA) (Fig. 2). Compared to denitrification and DNRA, the abundances of ANRA-related enzymes [NR (EC 1.7.1.1, EC 1.7.1.2, EC 1.7.1.3) and NAR (EC 1.7.7.2)] and genes (*NR* and *narB*) were low (Figs. 2 and 3), indicating that ANRA was weak in the Qingshui River. However, considering salinity, there was an increasing trend of enzymes [NR, aNiR (EC 1.7.7.1)] related to ANRA ($p < 0.05$) (Table S4). As a key genus for ANRA, the relative abundance of *Thalassosira* was highest under HS (6.52) (Fig. 4).

Notably, *narG*, the most dominant functional gene in the Qingshui River, gradually increased with increasing salinity ($p < 0.05$), theoretically leading to a reduced NO_3^--N concentration. Conversely, the abundances of *Hydrogenophaga* (1.38–4.65) and *Variovorax* (0.38–0.76), which contained the *narG* gene, gradually decreased with increasing salinity ($p > 0.05$) (Table S5), and *Pseudomonas* and *Desulfuromonas* were enriched under MS, reaching 0.81 and 1.05, respectively (Fig. 4, Table S6). The abundances of the other three functional enzymes associated with denitrification [NiR, NOR (EC 1.7.2.5), and N_2OR (EC 1.7.2.4)] were highest under MS (24.84, 16.49, and 13.11) and lowest under LS. However, due to significant changes within the group, there was no significant difference between the three salinities ($p > 0.05$). The corresponding functional gene (*nirKS*, *norBD*, and *nosZ*) involved in the reduction of NO_2^--N to N_2 showed a consistent trend. In addition, the dominant functional genera *Silicimonas* (*nirK*), *Luteimonas* (*nirS*),

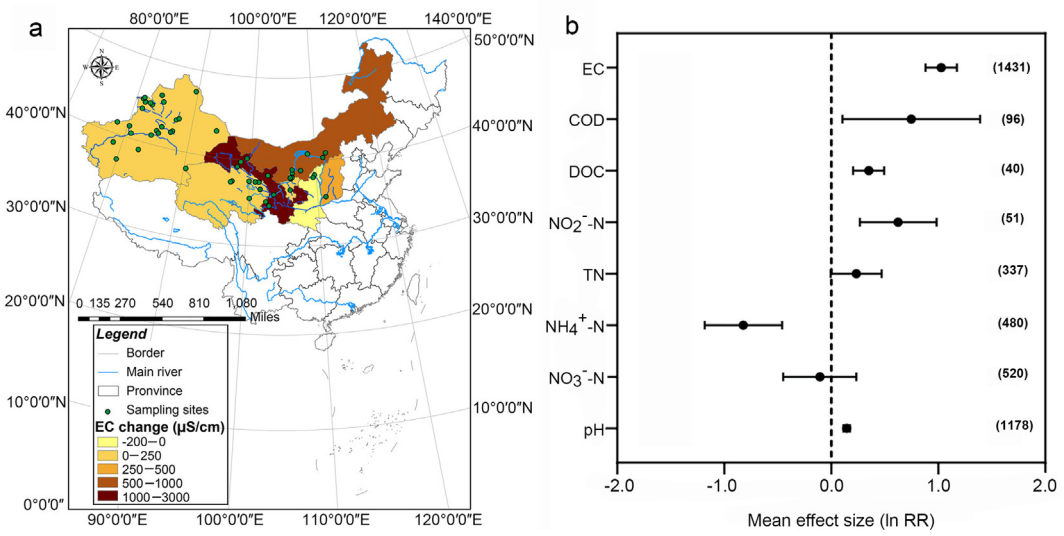

Fig. 1. Sampling points included in the dataset and salinity changes before and after 2010 in arid and semi-arid regions of China (a). Effects of salinity on environmental characteristics for river in arid and semi-arid regions of China (b). The data before 2010 is represented as the 20-year average from 1990 to 2009, and the data after 2010 is represented as the 13-year average from 2010 to 2022. The error bar represents 95% confidence interval. The observation numbers for each environmental variable are given in parentheses.

Sulfuritalea (*nirS*), *Rhodoferax* (*norB*), and *Gemmatimonas* (*nosZ*) had the highest abundances under MS.

Regarding DNRA, the relative abundance of NiR (NADH) (EC 1.7.1.15) was more sensitive to salinity, especially in HS ($p < 0.05$). The relative abundance of the dominant functional gene *nirBD* (corresponding to the process of NO_2^--N conversion to NH_4^+-N) and two most abundant genera (*Hydrogenophaga* and *Curvibacter*) involved in the DNRA process was similar to that of NiR (NADH), with a consistent decreased tendency with increasing salinity (Fig. 2). In contrast to *nirBD*, the genera containing *nrfAH* were mainly positively correlated with salinity ($p > 0.05$).

3.3. Co-occurrence networks of dominant nitrogen-cycling bacteria and their relationships with environmental factors

The river microbial network was constructed based on the dominant genera (top 5) among various nitrogen transformation functional genes (Fig. S4). The results indicated that river micro food web complexity was influenced by salinity. The networks identified in this study consisted of 313, 799, and 374 significant edges under LS, MS, and HS, respectively. In addition, most of the associations were dominated by positive correlations (96.40%, 84.78%, and 86.43% for LS, MS, and HS,

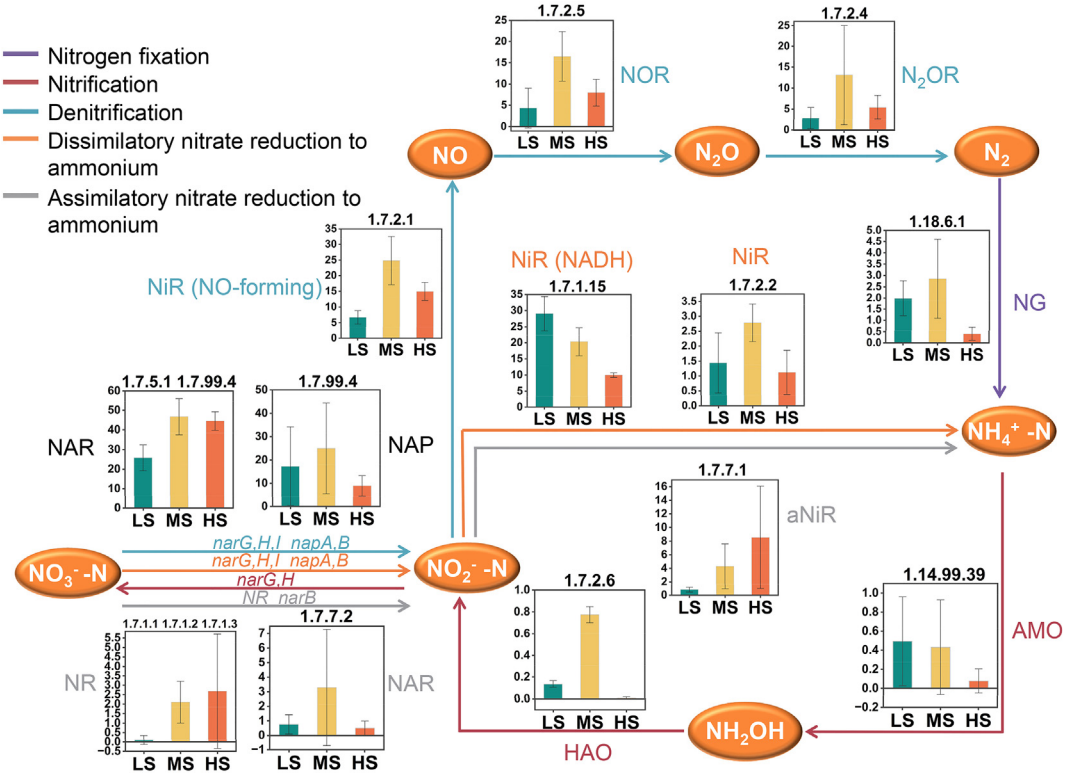

Fig. 2. Abundance of functional enzymes involved in nitrogen transformation based on metagenomic datasets. Abundance is presented as transcripts per kilobase per million.

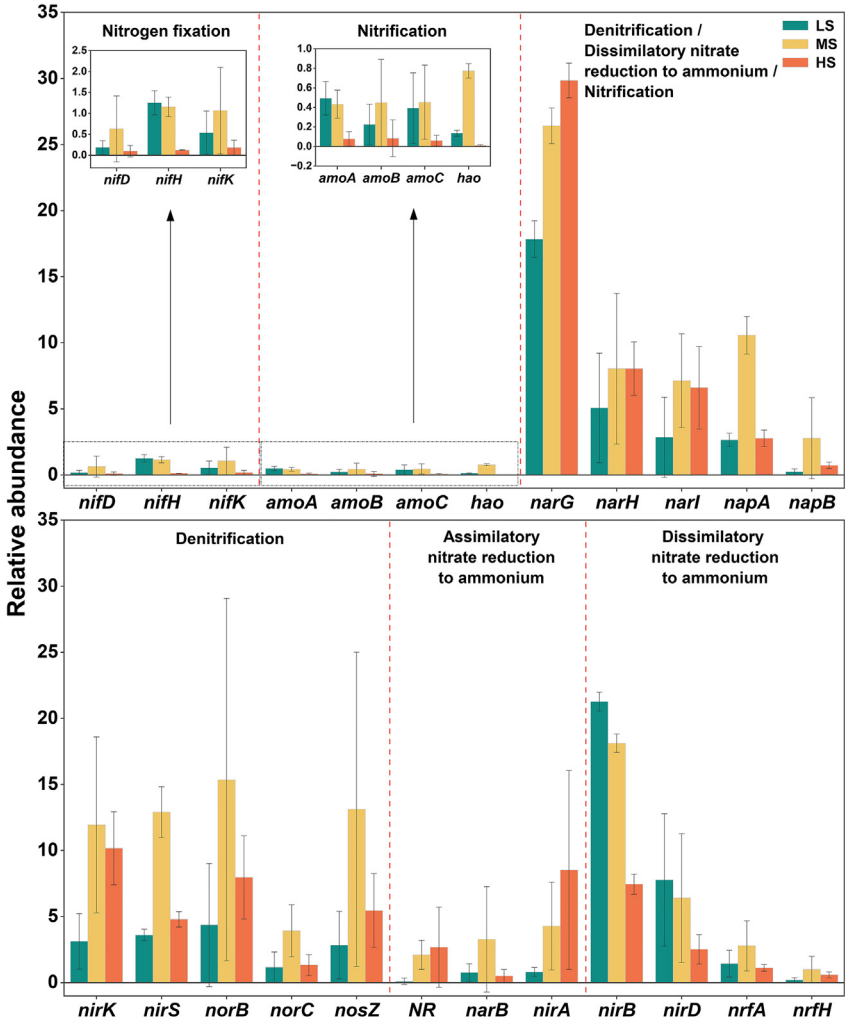

Fig. 3. Abundance of nitrogen transformation functional genes involved in nitrogen transformation based on metagenomic datasets. Abundance is presented as transcripts per kilobase per million.

respectively), illustrating that they coexisted in a mutually reinforcing pattern. Notably, salinity increased the percentage of negative correlations of network in the river in MS and HS, which was approximately 10% higher than under LS. As for the links between functional communities, the specific genera with more associations exhibited differences in salinity levels. In LS, nitrogen-fixing bacteria (*Azoarcus* and *Azospira*), nitrobacteria (*Nitrosopumilus*), denitrifying bacteria (*Lacunisphaera*, *Luteimonas*, *Sulfuritalea*, *Pseudoxanthomonas*, *Pseudomonas*, and *Hydrogenophaga*), DNRA bacteria (*Sulfuritalea*, *Methylotenera*, and *Hydrogenophaga*), and denitrifying/DNRA/nitrifying bacteria (*Hydrogenophaga* and *Variovorax*) exhibited more connections with other bacterial genera. Under MS and HS, denitrifying bacteria (*Lacunisphaera* under MS and *Pseudomonas* under HS) and denitrifying/DNRA/nitrifying bacteria (*Acidovorax* under MS and HS) exhibited a relatively close relationship with other genera.

The response changes of different nitrogen-transforming bacteria to various environmental factors are shown in Fig. 5. Salinity had a negative effect on nitrification, ANRA, and DNRA bacteria. Nitrogen-fixing and denitrifying bacteria were positively correlated with salinity. The correlation between the denitrifying community and salinity was weak (standardized coefficient = 0.22). Although salinity drives the microbial community structure, the impact of other environmental factors (such as inorganic nitrogen forms) on functional genera not be ignored. In this study, the pH was significantly negatively correlated with bacteria related to denitrification and positively correlated with ANRA. Although the relative abundances of microorganisms related to nitrification and ANRA were low in the Qingshui River, they were significantly negatively correlated with NO_3^--N and positively correlated with TN. Denitrification and DNRA microbial communities showed no significant correlation with NO_3^--N and TN. ANRA bacteria were significantly positively correlated to NH_4^+-N (Fig. 5e).

4. Discussion

4.1. The effect of river salinity on water environment characteristics

The chemical characteristics of river water are generally controlled by atmospheric precipitation, evaporation crystallization, and rocks [33]. For rivers in arid and semi-arid regions of China, salinity in river water has increased due to strong evaporation in recent years (Fig. 1a). This trend aligned with the global pattern of increasing surface water salinity [34], indicating that freshwater salinization was an increasingly serious water quality problem. Certainly, some areas did not show significant changes in salinity (with an average increase of only 0.27 mS/cm in Xinjiang, as shown in Fig. S3), due to relatively high runoff rivers around the Tianshan Mountains (such as the Ili River). Despite uncertainty in data due to differences in research points, the salinity of rivers in arid and semi-arid regions had significantly increased regionally. Furthermore, freshwater salinization is expected to rise in the future due to both climate change and human activities, with the potential to exert a substantial influence on the quality of river water [35,36].

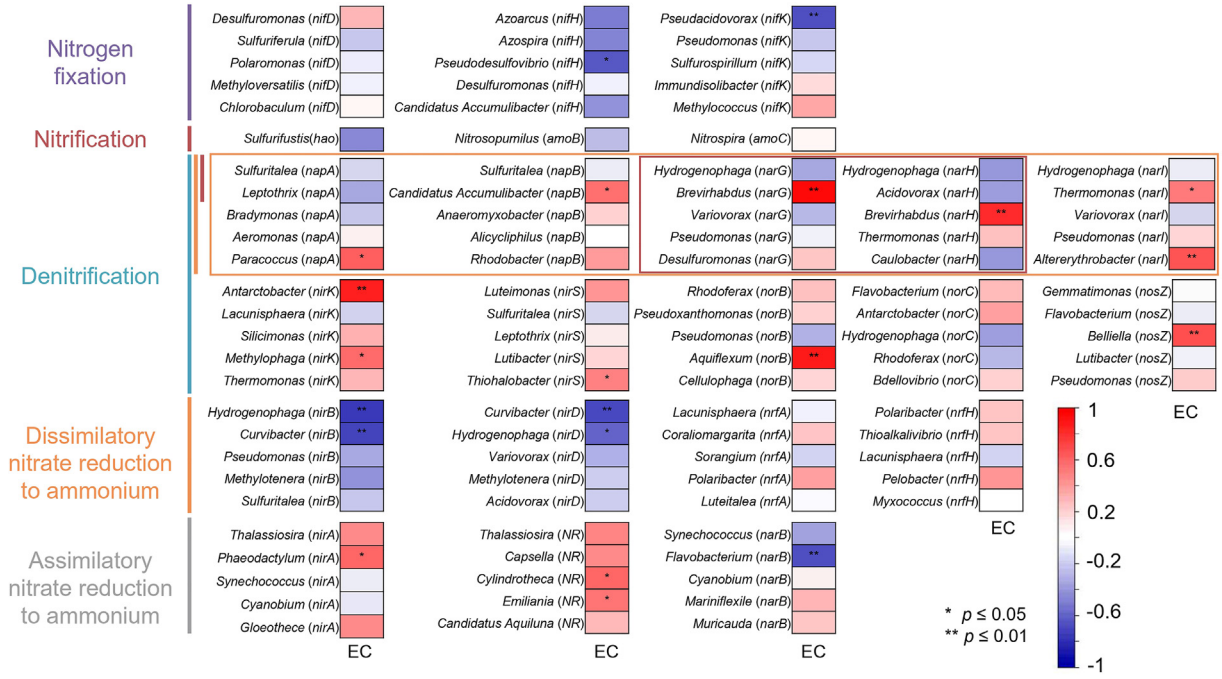

Fig. 4. Responses of top 5 dominant functional bacteria annotated with different functional genes with salinity.

The meta-analysis revealed a correlation between environmental characteristics and elevated salinity in river ecosystems (Fig. 1b). The reduction of NO_3^--N concentration was mainly attributed to the enrichment of diverse denitrifying bacteria. Additionally, increased DOC content (35%) under the salinization river could promote denitrification through organic carbon availability. The salinization of rivers reduced NH_4^+-N concentration, whereas increased NO_2^--N content. This meant that the nitritation process was stimulated by salinity, which was consistent with the results that the nitrification rate notably increased in a soil micro-salt environment [17]. However, due to the stronger inhibition of nitrite oxidizers by salinity [37], the related NO_2^--N accumulated. In river ecosystems experiencing salinization, the strong selective pressure exerted by salinity leads to a decrease in microbial structure and function [25]. The concentration of TN increased with increasing

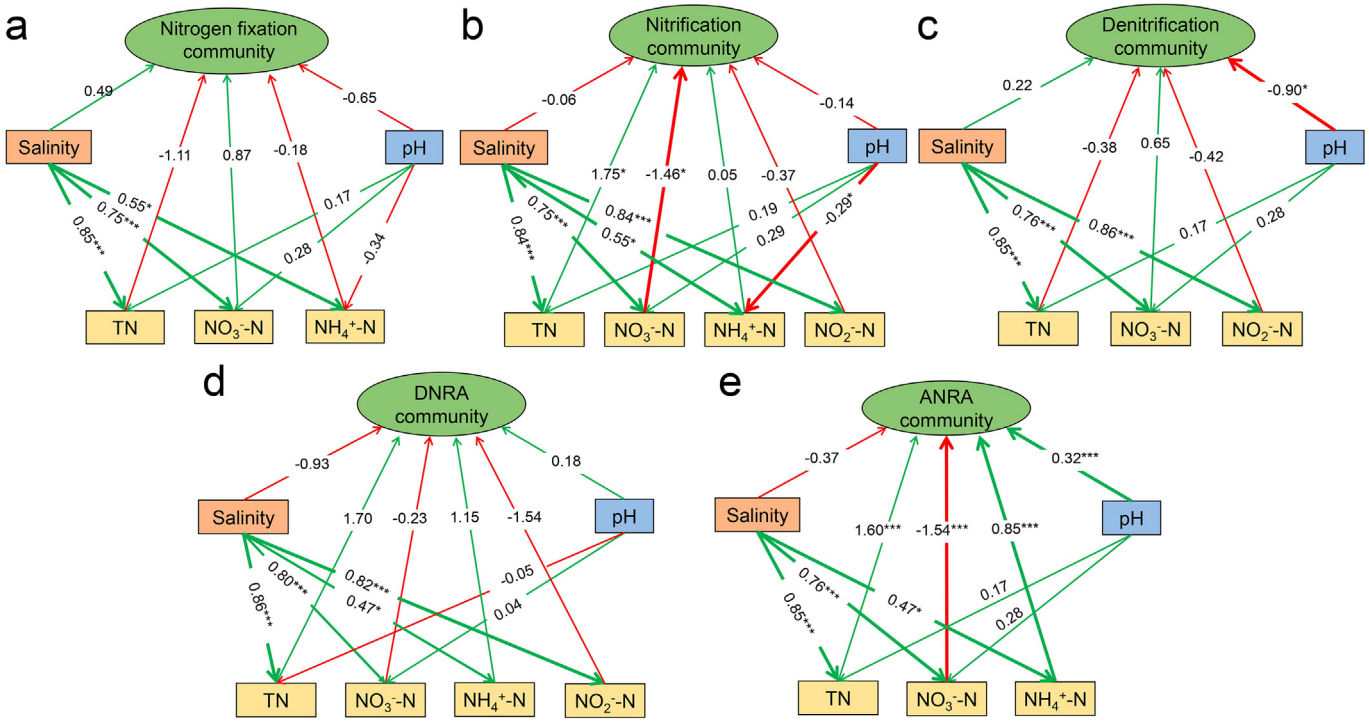

Fig. 5. The relationship between environmental factors and the nitrogen-transforming functional community involved in nitrogen fixation (a), nitrification (b), denitrification (c), dissimilatory nitrate reduction to ammonium (DNRA) (d), and assimilatory nitrate reduction to ammonium (ANRA) (e) based on structural equation modeling. Green and red arrows indicate positive and negative relationships, respectively. The numbers next to the arrows indicate the normalized path coefficient. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

salinity, which attributed to the selection pressure from salinity that caused some bacterial communities to dormant or die [38], thereby limiting nitrogen removal.

In general, salinity changed environmental characteristics, which affected the water quality of rivers in arid and semi-arid regions of China. However, this finding does come with some limitations. First, the current studies focused on rivers with low salinity levels (<4 mS/cm), and there was a lack of results on nitrogen form caused by high salinity levels. Second, due to the limitations of current research, the analysis only focused on physical and chemical characteristics. Thus, it is necessary to further explore the potential mechanisms involved in nitrogen cycling (such as genes and microorganisms) for rivers with a wider range of salinity in arid and semi-arid regions.

4.2. Response of nitrogen metabolism process to salinity

Increasing salinity greatly changed the nitrogen cycling process in the Qingshui River (Figs. 2–4), thus affecting the availability of irrigation water [36]. As the main form of nitrogen, the conversion of NO_3^--N was the focus of this study. Nitrification is a crucial process contributing to NO_3^--N concentration. The oxidation of NH_4^+-N to NO_2^--N is considered to be the first and rate-limiting step of nitrification [39]. The extremely low abundance of key enzymes (AMO), genes (*amoABC*, and *hao*), and genera (*Nitrosopumilus*, *Nitrospira*, and *Sulfurifustis*) involved in the step indicates that the nitritation process was limited, which explained the high NH_4^+-N concentration in HS (up to 1.41 mg/L). Similar results have been reported for soil, e.g., HS significantly altered *amoAB* abundance and weakened ammonia oxidation activity [25]. In addition, because of the limited conversion of NH_4^+-N to NO_2^--N, NO_2^--N-oxidizing bacteria lacked substrate for nitratation. Nitrification was not facilitated even under high abundances of amphiphilic *narGH*. However, to meet nitrogen input on crop production in high-salinity regions, residual NH_4^+-N would be utilized by crops along irrigation water, thus promoting the development of agriculture in surrounding areas.

The relatively low abundances of NR and NAR indicated that ANRA had a weak contribution to the reduction of NO_3^--N in the Qingshui River. However, with the increase in salinity, the abundance of the typical salt-tolerant bacterium *Thalassiosira* was the highest in HS, reaching 6.52. Although the genus contributed to the transformation of NO_3^--N, the higher concentration under HS indicated that the ANRA process cannot reduce the nitrogen content. Effective regulation genera for ANRA in HS areas play an important role in reducing NO_3^--N concentration and improving water quality.

Regarding denitrification, the genes *narGHI* and *napAB* are most commonly used as functional marker genes for cytoplasmic NO_3^--N reductase [NAR (EC 1.7.5.1, EC 1.7.99.4)] and periplasmic NO_3^--N reductase [NAP (EC 1.7.99.4)], completing the conversion of NO_3^--N to NO_2^--N, which also occurred in the DNRA process [40]. However, as the most advantageous functional genes, *narG* and NO_3^--N did not show a corresponding negative relationship with salinity changes. This indicated that, despite salinity promoting the directional selection of salt-tolerant bacteria (such as *Brevirhabdus*, Fig. 4) in *narG*, further evaluation of their function is necessary. *Hydrogenophaga* has been proven to be metabolically diverse [9]. The genus contained multiple genes (*narG*, *narH*, *narI*, *norC*, *nirB*, and *nirD*) and participated in multiple nitrogen transformation processes in this study. *Hydrogenophaga* remained dominant even though its relative abundance decreased with increasing salinity. The reason was that the increasing salinity resulted in a slight increase in the alkalinity of the Qingshui River (8.60 for pH in HS, Table S2). While this increase in alkalinity interfered with the occurrence of hydrogen as an electron donor, it did not significantly inhibit it. Thus, we can speculate that utilizing multifunctional communities (such as *Hydrogenophaga*) can regulate the changes in water quality, thereby reducing the risk of irrigation water. In addition, salinity selectively enriched specific denitrifying bacteria in MS, such as *Silicimonas* (*nirK*), *Luteimonas* (*nirS*), *Rhodoferax* (*norB*), and *Gemmatimomonas* (*nosZ*), though there was no significant correlation between the above bacteria and salinity. This also suggested that the aforementioned denitrification genes were in a state of optimal abundance under MS, which facilitated denitrification. However, this pattern was not observed in tidal freshwater wetlands, in which the activities of the enzymes (NiR, NOR, and N_2OR) decreased with increasing salinity [41]. In contrast, Wang et al. [42] reported that increasing the salinity from 0 to 30 ppt reduced the abundances of *nirK* and *nosZ* in mangrove sediment. This discrepancy might be due to differences in environmental media, salinity range, and nutrient availability, which are important factors affecting denitrification. Although denitrification has been promoted and may provide adaptive strategies for reducing nitrogen load under MS, the emission of a large volume of greenhouse gases, especially N_2O, is still a problem that needs to be solved [17].

NiR (NADH) was a more advantageous enzyme in the ANRA process but was more sensitive to salinity in HS ($p < 0.05$). It explained the significant increase in NO_3^--N to a certain extent under HS. This was mainly because the blocked reaction impeded the conversion of NO_3^--N in the previous enzyme reaction process (NO_3^--N → NO_2^--N) promptly, resulting in its accumulation. The two most abundant genera (*Hydrogenophaga* and *Curvibacter*) containing *nirB* were significantly inhibited by salinity ($p < 0.01$). This result contrasted with the findings that DNRA microorganisms are more salt-tolerant [43]. These discrepancies could be explained by the differences in specific DNRA genera caused by changes in environmental conditions, such as DO and organic carbon availability. Chi et al. [26] also found that different DNRA genera were significantly influenced by salinity; e.g., *Aeromonas* and *Geobacterium* occurred in LS areas (salinity of 1.5%), whereas *Caldithrix* was enriched in HS areas (salinity of 13.9%). Conversely, the genera containing *nrfAH* were mainly positively influenced by salinity even though not significant. A possible explanation is that *nirB*(*D*) is located in the cytoplasm of DNRA bacteria, whereas *nrfA*(*H*) is periplasmic (outside of the cell membrane) [40]. Compared to these genera containing *nrfAB*, although genera carrying *nirBD* cannot tolerate the pressure of salinity, their high abundance confirms its metabolic function (Table S6). Nitrogen required for agricultural irrigation (with an average TN concentration of 3.69 mg/L) was retained because, under LS, DNRA outcompetes denitrification for NO_3^--N.

The results of this study provide new data reference for the nitrogen conversion process of rivers primarily used for agricultural irrigation in semi-arid regions. The nitrogen conversion process varied under different salinity levels, thus affecting the concentration and form of inorganic nitrogen, which significantly impacted irrigation water use. However, to further regulate the nitrogen concentration required for crops and safeguard the irrigation water quality for farmlands, it is necessary to consider the symbiotic relationship between nitrogen-cycling microorganisms under different nitrogen transformation processes.

4.3. Influence of salinity on microbial community structure

In response to elevated salinity levels, nitrogen-cycling bacteria showed co-occurrence, which ultimately affected the microbial nitrogen-transforming process. The salinity had different effects on the network complexity of nitrogen-transforming bacteria, and the highest stability was found under MS (Fig. S4). This disagrees with the findings that high salinity (EC > 4 mS/cm) in salinization soil reduced the stability of bacterial communities [25]. This discrepancy might be attributed to differences in environmental media and salinity. The low stability under LS and HS meant that it was easier to reduce the nitrogen concentration through microbial regulation, thereby reducing the risk of deteriorating irrigation water quality. The positive connections indicate niche overlap and reciprocal/promoting interactions [44]. In this study, the higher cooperative behavior may help promote the resistance of all members, thereby exhibiting more complexity.

However, the percentage of negative correlation increased with elevated salinity, indicating that genera tend to mutually repel in HS. In addition, elevated salinity levels limit the availability of biological resources; the lower positive interactions may also indicate intensified competition and niche differentiation in this stage. Thus, if the nitrogen conversion process is not changed, the nitrogen applied to farmland inevitably enters the Qingshui River again by soil leaching or surface runoff. When salinity increases, even if it disrupts the stability of the network, specific bacterial communities still emerge to maintain system stability. The specific selection was mainly due to conditions differences under different salinity (such as pH, DO, nitrogen, and COD), which potentially impact microbial community connections. For example, as the most dominant functional bacterium (*nar*G) in the Qingshui River, *Hydrogenophaga* was significantly correlated with 17 genera under LS and with 10 and 6 genera under MS and HS, respectively, most likely because it is a typical autotrophic bacterial genus [45]. *Hydrogenophaga* can use H^+ as an electron donor and sole energy source under LS and slightly alkaline conditions, providing available carbon for other heterotrophic microorganisms. HS reduces the availability of nutrient resources (such as COD) and filters bacterial communities [46], further weakening the network connections. Overall, specific environmental filters with higher abundance and effective functional microorganisms formed under different salinity conditions. This is important for effectively removing nutrients from surface water.

The nitrification, ANRA, and DNRA bacteria were inhibited by salinity, which can be explained by variations in dominant functional genes and bacteria at different salinity levels. This was consistent with the results of previous studies determining the characteristics of functional bacterial genera by dominant bacteria [47]. Even though denitrifying bacteria were positively correlated with salinity, it was not significant ($p > 0.05$). This was attributed to the higher diversity of functional genes and the community involved in denitrification. Despite a significant positive correlation between salinity and both the dominant gene (*narG*) and genus (*Brevirhabdus*) associated with denitrification, the denitrifying bacterial community encompassed multiple phyla and engaged in diverse nitrogen conversion processes. This diversity offset the salinity-driven positive responses observed in the primary bacterial genera, including *Brevirhabdus*. The results verified that certain genera were halophilic but not significantly positively correlated with salinity. This applies even if the abundance of dominant denitrifying bacteria decreased under LS and HS, and the addition of a variety of rare denitrifying bacteria could promote the denitrification process and then permanently remove nitrogen.

The higher pH value occurred in HS, and the increase in alkalinity promoted the enrichment of halophilic bacteria of ANRA. Denitrifying bacteria show better growth and survival under neutral or weak alkaline conditions with a pH range of 7.0–8.0 [48]. The higher pH was not conducive to the reproduction of denitrifying bacteria. Perhaps for this reason, the denitrification process was inhibited under HS, which undoubtedly increased the difficulty of reducing the NO_3^--N concentration and required further research. As a product of nitrification, at high concentrations, NO_3^--N inhibited the abundance of dominant nitrobacteria (such as *Nitrosopumilus* and *Nitrospira*). Nevertheless, the weak relationship between the relatively complex microorganisms involved in denitrification, DNRA, and responses to salinity depend on two factors. On the one hand, because of the diverse and complex functional bacteria involved in denitrification and DNRA, it is difficult to determine the relationships between the total bacterial community and dominant bacteria. On the other hand, the responses of microorganisms to the environment generally change after adaptation, which is related to the sensitivity of the microorganisms [49]. ANRA bacteria gradually adapted to high-salinity environments (Fig. 4), which promoted the generation of NH_4^+-N through ANRA. This is in line with the abundance of functional enzymes (aNiR), gene (*nirA*), and the main genera involved in ANRA, which gradually increased with increasing salinity (Figs. 2–4). Overall, various environmental factors influenced microbial communities, which helped to quantify the regulatory role of nitrogen-cycling bacteria in specific nitrogen conversion processes.

4.4. Implication

As a river used to irrigate agricultural areas in arid and semi-arid regions of China, the concentrations of NO_3^--N and TN in Qingshui River increased significantly with salinity. Ultimately, these concentrations far exceeded the standard water quality (2 mg/L of TN) for the surface water of China (GB 3838-2002). Except for the impact of surface runoff, the abundance of nitrogen-cycling functional genes and genera varied according to the selective pressure imposed by salinity. Overall, this research provides strong evidence that salinity changes the inorganic nitrogen transformation process in semi-arid rivers dominated by agricultural irrigation (Fig. 6). Under LS, the enrichment of DNRA-bacteria

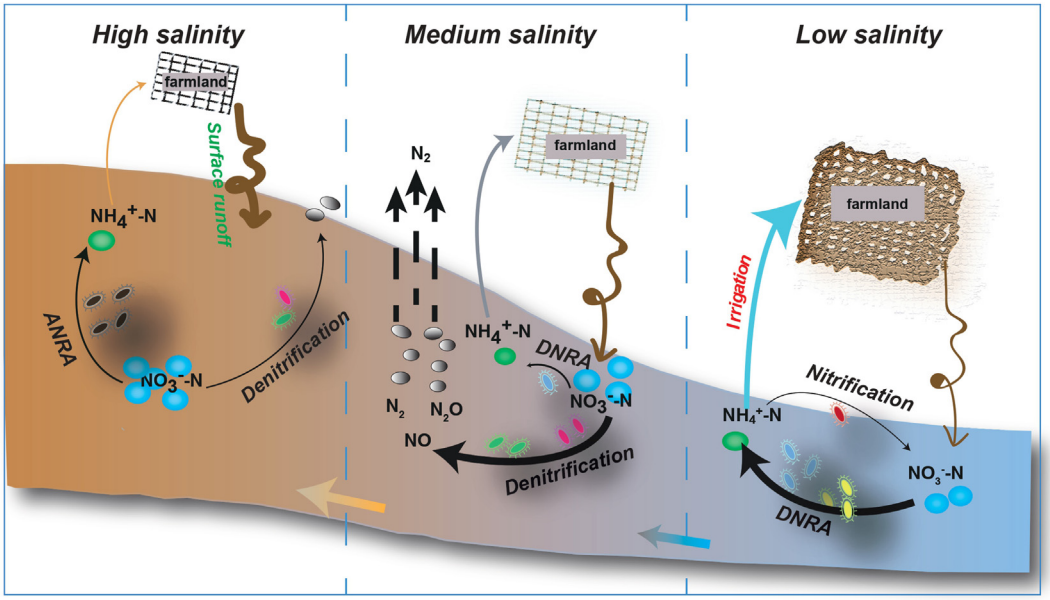

Fig. 6. Conceptual model of NO_3^--N conversion pathway in rivers used to irrigate agricultural areas in semi-arid regions under different salinity levels.

promoted the reduction of NO_3^--N to NH_4^+-N, thus supplementing the nitrogen required for crop growth in farmland. Under MS, the abundance of denitrifying bacteria was higher than that of DNRA bacteria, which allowed more nitrogen to escape from the water in the form of gas. Under HS, the selection pressure of salinity relatively promoted the growth of ANRA bacteria, and specific denitrifying bacteria could tolerate high salt stress. However, because of the decrease in resource availability caused by high salinity, the nitrogen output from irrigation or denitrification cannot be offset by input from surface runoff in farmland, resulting in a high nitrogen load. Furthermore, the quantitative effect of environmental factors (such as salinity and inorganic nitrogen) on different nitrogen-functional bacteria also offers reference value for microbial regulation to reduce nitrogen concentration.

5. Conclusion

By integrating multiple in-situ measurement data and meta-analysis, it was found that the salinity of rivers in arid and semi-arid regions of China is increasing. Moreover, elevated salinity of rivers increased the content of TOC (35%), TN (23%), and NO_2^--N (63%), though it reduced the concentration of NH_4^+-N (82%) and NO_3^--N (11%). To further evaluate the effects of different salinity levels on the mechanisms of inorganic nitrogen transformation pathways, the Qingshui River with a long-term salinity gradient was examined. The genes and enzymes involved in denitrification (such as *narG* and NAR) and DNRA [such as *narG*, *nirB*, NAR, and NiR (NADH)] were more abundant compared with those involved in ANRA regardless of the salinity level. This result indicates that traditional denitrification and DNRA are the primary processes of NO_3^--N reduction in rivers. Except for the dominant gene *narG* (whose abundance increased with increasing salinity), denitrification genes were enriched under MS, whereas the abundance of DNRA genes gradually decreased with increasing salinity. Additionally, the denitrifying bacteria differed in their correlations with salinity because of the diversity of functions and species; DNRA bacteria (such as *Hydrogenophaga* and *Curvibacter* carrying *nirB*) were significantly inhibited by salinity. Furthermore, functional bacteria mostly maintained network relationships through synergism under the three salinity levels; in particular, a higher proportion of positive correction was obtained under MS. Moreover, even though nitrifying and ANRA bacteria had low abundances, there were significant positive correlations with TN and negative correlations with NO_3^--N. Given that the increasingly severe freshwater salinization affects the form and concentration of nitrogen, it is critical to recognize that shifts in nitrogen transformation processes caused by salinity may improve water quality and reduce agriculture irrigation risks. This result helps regulate the enrichment of nitrogen-cycling bacteria to reduce nitrogen concentration.

CRediT authorship contribution statement

Q.Q.P.: conceptualization, data curation, methodology, writing–original draft, writing–review and editing. D.W.: data curation, methodology, writing–original draft, Writing–review and editing, formal analysis. Z.W.J.: data curation, investigation, formal analysis. M.A.: formal analysis, and writing–review and editing. L.X., X.Z., and F.Q.P.: methodology, investigation, formal analysis. P.S.: methodology, supervision, writing–review and editing. L.M.W.: conceptualization, project administration, funding acquisition, supervision, writing–review and editing. L.Z.M.: methodology, supervision, writing–review and editing. J.H.: conceptualization, methodology, supervision. P.Y., F.H., and B.X.: methodology, investigation.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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