

Original Research

Nitrogen Migration, Transformation, and Microbial Community Characteristics in the Hyporheic Zone of the Hun River Bank in Tiexi District, Shenyang, under Dam Regulation of the Dahuofang Reservoir

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Abstract

Dam regulation directly affects the intensity of hydraulic exchange and redox conditions in the hyporheic zone by changing river flow and water level, thereby influencing the biogeochemical processes of nitrogen. However, the quantitative mechanism of spatial succession of microbial communities in this process remains unclear. This study takes the Mojiabao section of the Hun River in Shenyang, China, as the study area. During the dry season when the dam releases water, three monitoring wells (W1, W2, W3) were set up along the direction perpendicular to the riverbank to collect groundwater samples. While measuring changes in nitrogen trioxides, we explored the spatial distribution characteristics of nitrogen transformation and microbial communities through high-throughput sequencing and PCR amplification of 16S rRNA genes, combined with alpha and beta diversity analyses. The results showed that the dominant phyla were Proteobacteria, Bacteroidetes, and Firmicutes; at the class level, γ -Proteobacteria were the dominant class; at the genus level, the nearshore W1 well was enriched with *Rhodospirillum rubrum* (aerobic nitrification), while the offshore W3 well was enriched with *Aeromonas* and *Pseudomonas* (denitrification). For every 50 m increase in vertical distance from the riverbank, the Shannon index increased by 0.5-0.8, with a significant increase in species richness and denitrification capacity. The aerobic-anaerobic gradient created by dam releases promotes the succession of microbial communities from nearshore γ -Proteobacteria (nitrification-dominant) to offshore α -Proteobacteria and Bacteroidetes (simultaneous nitrification and denitrification). This study elucidates the succession phenomenon of microbial communities in the subsurface flow

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zone under hydrodynamic control, and the microbial diversity indicators can be used in the analysis and assessment system of river ecological conditions.

Keywords: hyporheic zone, dam regulation, nitrogen transformation, nitrification and denitrification, microbial community characteristics

Introduction

As a hub between surface water and groundwater, the hyporheic zone plays a key role in hydraulic exchange between Hun River water and groundwater in the nearshore zone and in water quality improvement through the metabolic processes of its microbial community. The regulation of dams directly affects the hydraulic exchange intensity and redox conditions of the subsurface flow zone by changing the river flow and water level, thereby regulating the biogeochemical processes of nitrogen [1]. Wang et al. reviewed the influence of water exchange in the subsurface flow zone on nitrogen migration and transformation under the influence of agriculture, and pointed out that the intensity of hydraulic exchange is the key environmental factor regulating the biogeochemical behavior of nitrogen. The effect of the redox gradient on microbial community assembly in the hyporheic zone was revealed, and it was found that environmental filtering regulates bacterial community dynamics more significantly than geographical distance, and nitrogen species such as dissolved organic nitrogen and nitrate exert key deterministic selection pressures in community succession [2]. Shen et al. systematically explored the microbially mediated nitrogen biogeochemical processes in the subsurface flow zone [3]. Meng Xiangtao used Shifosi Reservoir as the study area and analyzed the biogeochemical behavior and prevention and control measures for nitrogen pollution in sediments. In addition, some progress has been made in numerical simulation studies of nitrogen migration and transformation in the hyporheic zone [4].

There are many studies on nitrogen transformation in the subsurface flow zone under natural flow conditions, but systematic supplementary research is needed on the periodic water storage caused by dams, i.e., the regulation of water release and how it affects the spatial succession of microbial communities and functions. Dams act on rivers in terms of flow rate, water level and even hydraulic residence time, and can directly regulate the intensity of water exchange and redox state in the subsurface flow zone, thereby controlling the microbial processes related to the nitrogen cycle, but the quantitative relationship underlying this control still needs to be clarified. Especially during the dam release period, there are still gaps in the redox zonation and functional differentiation of microorganisms in the subsurface flow zone. This study takes the release of water from Dahuofang Reservoir at Mojiabao in the middle and lower reaches of the Hun River during the dry season as an example, and uses high-throughput

16S rRNA gene sequencing to investigate the microbial composition of three monitoring wells (W1, W2 and W3) along a direction perpendicular to the riverbank. The succession pattern from nitrification to denitrification under the aerobic-anaerobic gradient is characterized based on alpha and beta diversity analyses, reflecting the microbial response associated with nitrogen transformation under dam regulation [5, 6].

Materials and Methods

Layout of Regional Monitoring Sections and Sampling Wells and Geological Lithology

Based on the geographical location and surrounding environment of the sampling wells in the study area, a monitoring profile I-I' was set up perpendicular to the direction of river flow. Existing observation wells on the monitoring profile, perpendicular to the Hun River bank, were selected as sampling wells, as shown in Fig. 1. The distance between the observation well groups was relatively uniform, and the environmental characteristics and hydraulic change gradients were obvious. The subsequent sample collection and testing work was conducted to combine the microbial characteristics of the subsurface flow zone with the hydraulic exchange characteristics of river water and groundwater under conditions of riverside groundwater extraction and to infer the location of the subsurface flow zone where nitrogen biogeochemical behavior occurs [7].

The study area was characterized based on existing mapping data from high-density groundwater sampling observation well groups in the nearshore zone [8]. The study area is located in the Mojiabao section of the middle and lower reaches of the Hun River in Shenyang City, China, on a temperate alluvial plain. Its stratigraphic lithology is mainly composed of Quaternary loose sediments, primarily silt, fine sand, and clayey silt, with local thin layers of clay. The aquifer thickness is approximately 15-25 m, with a permeability coefficient of 1.2-3.5 m/d, and groundwater flows from northeast to southwest. During the dry season, dam releases cause river water infiltration, forming a significant hydraulic gradient and redox gradient along the vertical direction of the riverbank. A low-permeability clay layer exists at a depth of approximately 20 m, limiting vertical mixing and facilitating the formation of horizontal aerobic-anaerobic zonation.

Existing well houses on the monitoring section I-I' were used to set up points where extraction was stopped

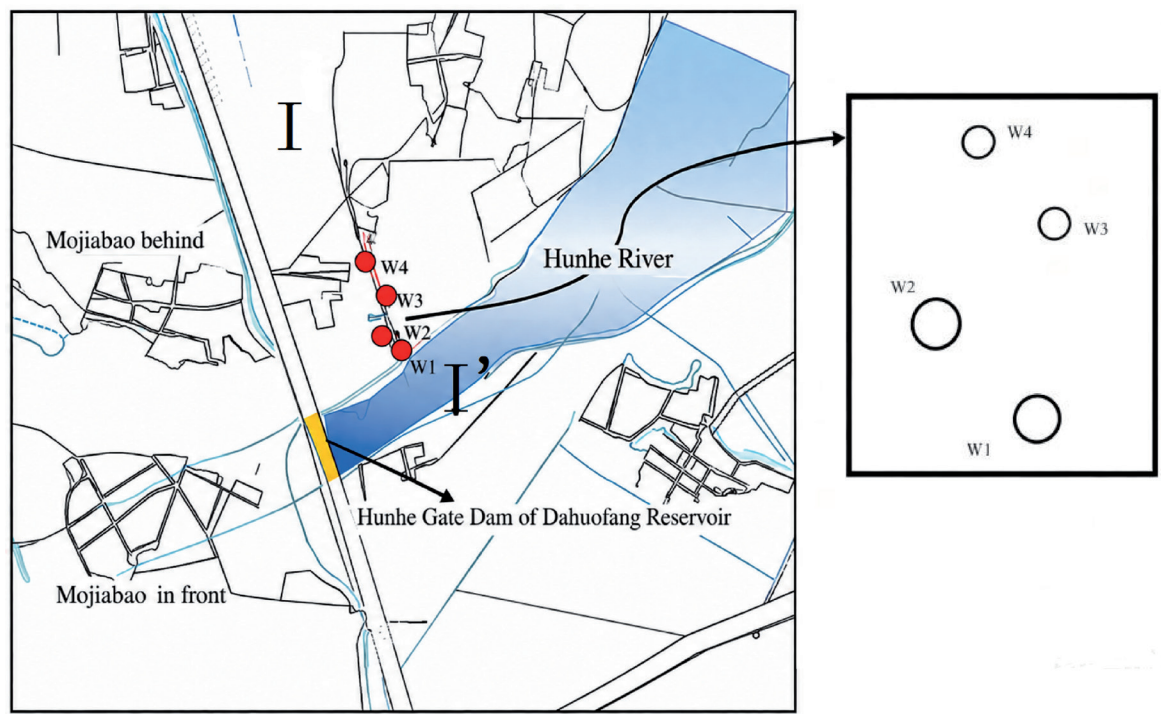


Fig. 1. Hyporheic zone monitoring section and sampling well distribution map in the study area.

[9]. Four groundwater sampling monitoring points at different straight-line distances from the Hun River were selected in the well houses, namely W1-W4 from near to far. Since W3 and W4 are close to each other and far from the Hunhe River, W1, W2 and W3 were selected as representative well locations for study [10].

Sample Collection and Processing

The water samples were taken from the study area along a direction perpendicular to the riverbank at the Liguabao water source. A representative complete hydrological year, 2021-2022, was selected from the past five years. Due to the dam’s water storage and relatively weak hydraulic exchange during the wet season, April 2021, when the hydraulic exchange during the dam’s water release was strong, was selected for analysis [11]. First, wells W1 to W3 at the I-I’ monitoring section were flushed. After pumping water for 30 min, groundwater samples were collected using a water pump. Larger impurities such as plant and animal remain and stone particles were removed on site. 500 ml of water was taken from each well and placed into a sterilized brown narrow-mouthed bottle of the same capacity (to prevent gas interference, no headspace was left), and it was tightly sealed with tin foil to prevent external contamination. Four samples were collected from each

of the three wells to determine the range, and a total of 12 groundwater samples were collected [12].

The collected groundwater samples were sealed in self-sealing bags and then covered with dry ice for low-temperature sealing and storage. They were refrigerated in foam boxes and transported back to the laboratory. The water samples were stored in foam boxes filled with dry ice packs and sent to Tianjin Novogene Technology Co., Ltd. for high-throughput microbial genome sequencing [13]. The distribution and sampling depths of groundwater sampling points W1-W3 are shown in Fig. 1 and Table 1.

Data Processing and Analysis

The raw data obtained from sequencing are first spliced and filtered to obtain effective data. Then, based on the effective data, noise reduction is performed using DADA2 to filter out sequences with an abundance of less than 5 [14], thereby obtaining the final ASVs [15]. Species annotation is performed on the representative sequence for each ASV to obtain the corresponding species information and abundance distribution based on the species. At the same time, abundance and alpha diversity analyses are performed on the ASVs to obtain species abundance within the sample and the information on common and unique ASVs between different samples

Table 1. Groundwater location and depth.

Drill hole number	W1	W2	W3
Sampling depth (m) in April 2023	6.62	10.75	11.07

Table 2. Quality control statistics for high-throughput sequencing data preprocessing.

Sample Name	Raw PE (#)	Combined (#)	Qualified (#)	Q20	Q30	GC%	Effective%
W1	88,572	80,474	79,501	99.22	96.96	53.15	77.82
W2	81,539	80,976	80,408	99.36	97.29	53.86	88.46
W3	81,105	76,356	75,443	99.22	96.99	52.90	83.96

or groups. At the same time, multiple sequence ratios are performed on the ASVs and displayed through PCoA dimensionality reduction analysis to explore the differences in community structure between different samples or groups [16].

The amplicon annotation results can be used to predict the function of microbial communities in biological samples using PICRUSt2 software [17]. The raw data for each sample are obtained by splitting according to the barcode. After removing the barcode and primers, the R1 and R2 sequence data are spliced using FLASH software [18]. The statistical results obtained at each step of data processing and shown in the Table 2 all met the sequencing quality control standards, and the number of effective sequences met the requirements for subsequent diversity analysis [19].

NH₄⁺-N in water samples was determined by Nessler's reagent spectrophotometry (HJ 535-2009), NO₃⁻-N by ultraviolet spectrophotometry (HJ/T 346-2007), NO₂⁻-N by N-(1-naphthyl)-ethylenediamine spectrophotometry (GB 7493-87), and TN by alkaline potassium persulfate digestion ultraviolet spectrophotometry (HJ 636-2012) [20].

Results and Discussion

Analysis of the Distribution Characteristics of Microbial Species and the Biogeochemical Mechanisms of Nitrogen

Distribution Characteristics of Microbial Species

(1) Noise Reduction and Species Annotation

The DADA2 method does not cluster based on similarity and is used for noise reduction; it only performs deduplication, which is equivalent to clustering with 100% similarity [20]. Each deduplicated sequence generated after DADA2 noise reduction is called an ASV (Amplicon Sequence Variant), corresponding to an OTU representative sequence or feature sequence, and the abundance table of these sequences in the samples, corresponding to the OTU table, is called the feature table [21]. Replacing OTUs with ASVs improves the accuracy, comprehensiveness, and reproducibility of marker gene data analysis [22]. A prerequisite for maintaining the normal functioning and cycling of an ecosystem is rich species diversity. The classify-sklearn algorithm of QIIME2 [23, 24] is used, and a pre-trained Naive Bayes classifier is applied to annotate each ASV

[25]. From the ASV annotation results and the feature table of each sample, species abundance tables at the kingdom, phylum, class, order, family, genus, and species levels can be obtained. The abundance table with annotation information is the core content of amplicon analysis. Based on the groundwater conditions and experimental objectives of the Mojiabao water source area under dam regulation, one or more species of interest can be selected from the abundance tables at the phylum, class, and genus levels in this paper. Further research can be conducted by combining the species composition and analysis of differences among samples from different groups with cluster analysis results [26].

(2) Species Relative Abundance and Ternary Phase Diagram

The species relative abundance bar chart is generated based on the species annotation results at different classification levels, so as to intuitively view the species composition and proportions within each groundwater sample in the subsurface flow zone at different classification levels. The following analysis uses the QIIME2 plugin to visualize the species composition at the representative phylum, class, and genus levels [27].

To explore the differences in dominant species among the three well sites at each classification level, the top 10 species with the highest average abundance at each classification level are selected and presented together with the simultaneously generated ternary plot (ternary phase diagram), which facilitates intuitive observation of the differences in dominant species among the three samples at different classification levels [28]. The ternaryplot command in the vcd package of R software is used to perform ternary phase diagram analysis. The ternary phase diagrams at the phylum, class, and genus levels are described below [29].

Phylum-Level Abundance of Microorganisms

Comparative analysis of species composition among groups at the phylum level: Figs 2 and 3 and Table 3 show the proportions of species at the phylum level at the three well sites W1, W2 and W3 during the dry season of 2021.

At the phylum level, the dominant microbial species found in the groundwater of the subsurface flow monitoring section mainly included Proteobacteria, Bacteroidetes, and Firmicutes. Other dominant species included Actinobacteria, Verrucomicrobia, Myxomycota, Planctomycetota, and Cyanobacteria [30].

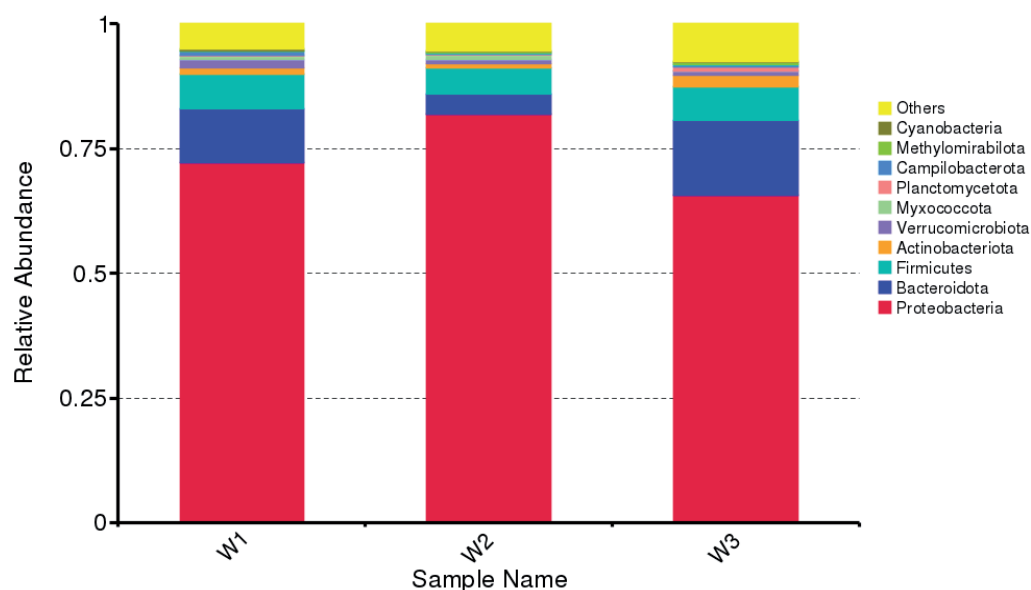


Fig. 2. Relative abundance of microbial species composition at the phylum level.

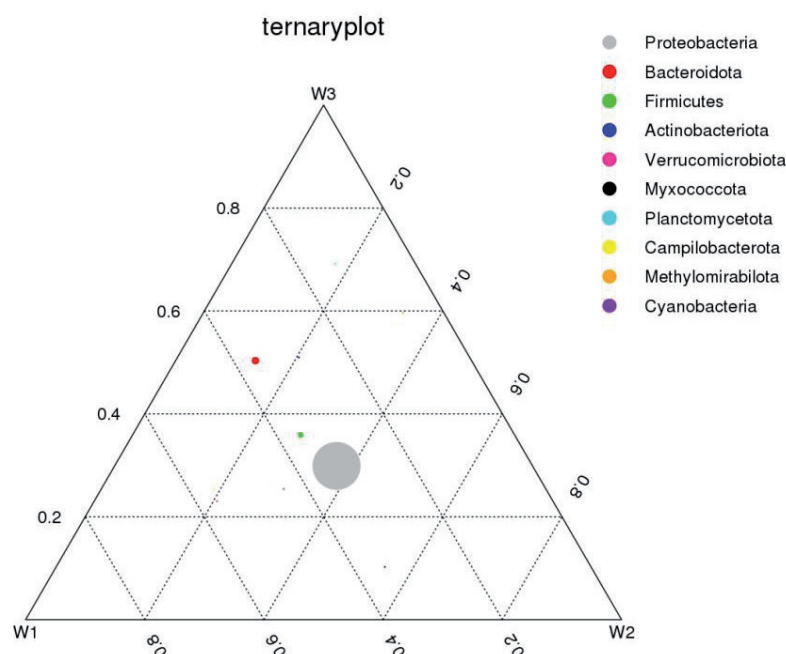


Fig. 3. Gate-level ternary phase diagram.

It is known that the dominant bacterial species in the groundwater of this study area are mainly Proteobacteria, Bacteroidetes, and Firmicutes [31].

(1) Proteobacteria

Most species of Proteobacteria are facultative or obligate anaerobes, and their metabolism is mainly chemoautotrophic and chemoheterotrophic, with only a few exceptions. Species of the genus *Pseudomonas* within the phylum Proteobacteria, which appear in large numbers in the subsurface zone, have a functional synergistic relationship with electrochemically active bacteria (*Shewanella oneidensis* MR-1), and can also serve as a design source for selecting highly

efficient anode bacteria for artificial desalination devices [32].

Proteobacteria are mainly facultative or obligate anaerobes, and their denitrifying microorganisms play a key role in nitrogen transformation. The contents of Proteobacteria at points W1, W2 and W3 were 72.311%, 81.886% and 65.710% respectively, with the highest content at point W2, indicating that denitrification was most intense at this point [33].

(2) Bacteroidetes

Bacteroidetes are composed of anaerobic or aerobic rod-shaped bacteria and are widely distributed in subsurface sediments and soils. The Bacteroidetes

Table 3. Proportion of species composition at the phylum level.

Taxonomy	W1	W2	W3
Proteobacteria	72.311%	81.886%	65.710%
Bacteroidetes	10.739%	3.971%	14.906%
Firmicutes	6.860%	5.386%	6.855%
Actinobacteria	1.283%	0.912%	2.282%
Verrucomicrobia	1.819%	0.666%	0.743%
Myxomycota	0.539%	0.860%	0.160%
Campylobacterota	0.840%	0.285%	0.383%
Planctomycetota	0.164%	0.212%	0.864%
Methylomirabilota	0.489%	0.238%	0.423%
Cyanobacteria	0.316%	0.220%	0.002%
Others	0.051%	0.054%	0.075%

content in W1 (10.739%) and W3 (14.906%) was much higher than that in W2 (3.971%), indicating that W1 and W3 well sites had a richer organic matter degradation environment [34].

(3) Firmicutes

Firmicutes can produce endospores to resist extreme environments. The sampling wells were in a partially anaerobic environment. The contents of Firmicutes at points W1, W2 and W3 were 6.860%, 5.386% and 6.855% respectively [35], which are related to the fermentation process of organic matter in the wells [36].

Besides the dominant species, the next most abundant microorganisms in the groundwater of the subsurface flow zone wells were Actinobacteria, Verrucomicrobia, Myxomycota, and Planctomycetota.

(1) Actinobacteria

Actinomycetes are aerobic saprophytes that can promote the decomposition of animal and plant remains and the ammonification of organic nitrogen [37]. The abundance of actinomycetes in W1 (1.283%) and W3 (2.282%) was higher than that in W2 (0.912%), indicating that W1 and W3 had a stronger potential for degrading organic matter and ammonifying organic nitrogen at the well sites [38].

(2) Verrucomicrobia

Verruciformis is mainly distributed in aquatic and soil environments. The highest content (1.819%) among the three wells was found in W1, which may be related to the higher organic matter input in the nearshore zone [39].

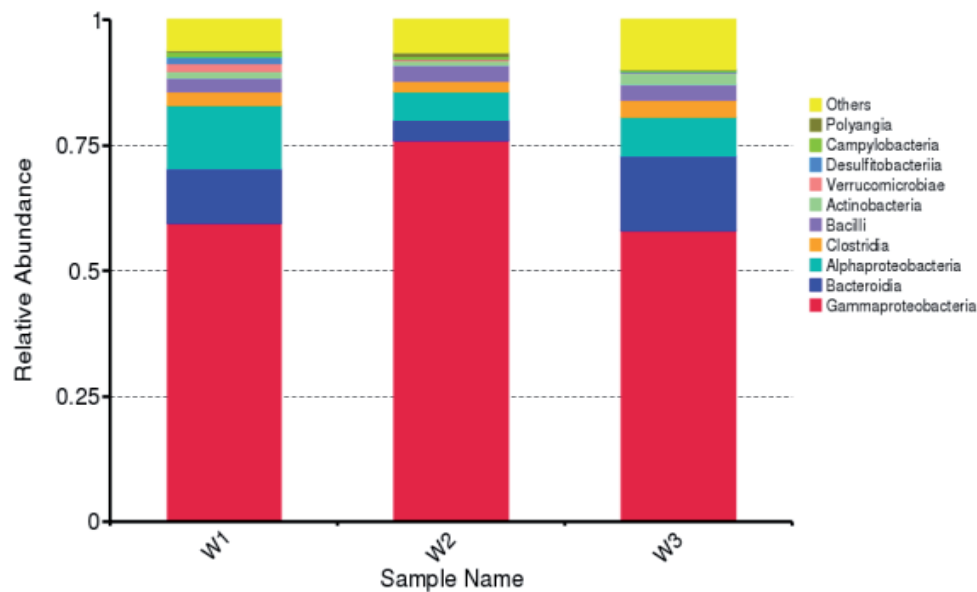


Fig. 4. Proportion of microbial species composition at the class level.

(3) Myxomycota

Myxomycetes are eukaryotic saprophytic microorganisms that can decompose decaying wood and debris. The contents of W1 (0.539%) and W2 (0.860%) were higher than those of W3 (0.160%), which is related to the larger wellhead diameter and the greater amount of decaying wood falling into the well.

(4) Planctomycetota

Planctomycetota is a small phylum of aquatic bacteria, usually found in brackish water, fresh water or seawater, and is an obligate aerobic bacterium. The content in W3 was the highest among the three wells, which means that the subsurface flow zone in this study area is an environment conducive to nitrification [40].

(5) Cyanobacteria

Cyanobacteria have nitrogen-fixing capabilities [41]. The content of W1 was the highest (0.316%), while that of W3 was the lowest (0.002%). This is because W1 has strong hydrodynamic interaction and good light conditions near the shore, which is conducive to the growth of cyanobacteria, while the anaerobic environment of W3 is not conducive to its metabolism.

Microbial Class-Level Abundance

Comparative analysis of species composition among groups at the class level: Figs 4 and 5 and Table 4 show the species composition and proportions at the class level for well sites W1, W2 and W3 during the dry season.

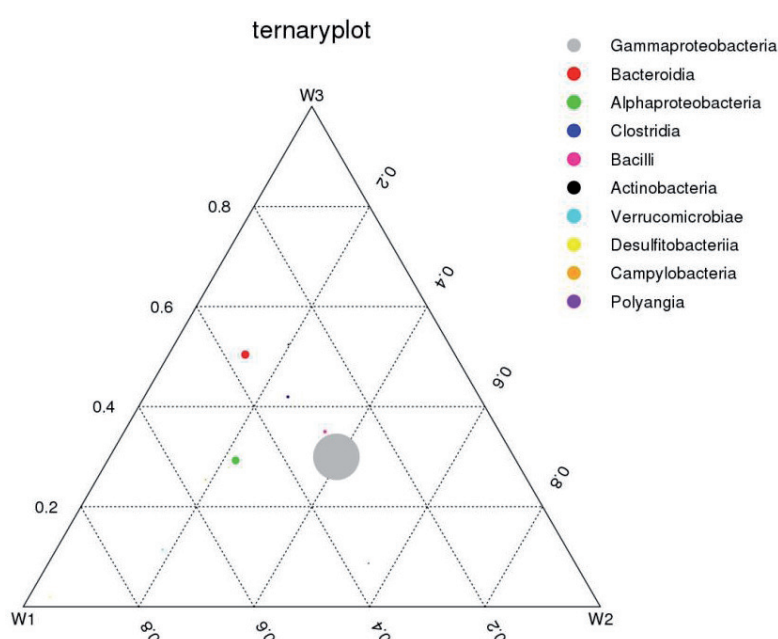


Fig. 5. Level ternary phase diagram.

Table 4. Proportion of species composition at the class level.

Taxonomy	W1	W2	W3
Gammaproteobacteria	0.59544	0.76039	0.58026
Bacteroidia	0.10733	0.03921	0.14885
Alphaproteobacteria	0.12757	0.05823	0.07684
Clostridia	0.02728	0.02047	0.03451
Bacilli	0.02788	0.03210	0.03228
Actinobacteria	0.01098	0.00779	0.02065
Verrucomicrobiae	0.01790	0.00471	0.00292
Desulfotobacteriia	0.01197	0.00047	0.00024
Campylobacteria	0.00840	0.00285	0.00383
Polyangia	0.00531	0.00822	0.00129
Others	0.05993	0.06555	0.09832

As shown in the figure, at the class level, through horizontal comparative analysis of the charts, it can be found that the dominant microbial species in the groundwater of the subsurface flow monitoring section mainly include Gammaproteobacteria, Bacteroidia, and Alphaproteobacteria. Secondly, there are Clostridia, Bacilli, and a small number of Actinobacteria and Verrucomicrobiota.

(1) Gammaproteobacteria

They can be widely distributed in various ecosystems, such as soil, freshwater lakes and rivers, oceans and salt lakes. They include free-living bacteria, biofilm-forming bacteria, and symbionts with completely different metabolisms in different genera. There are aerobic and anaerobic (obligatory or facultative) organisms, chemoautotrophic and organic autotrophic organisms, photoautotrophic organisms, and heterotrophic organisms. Gamma-Proteobacteria also exhibit extensive diversity, metabolic diversity, and functional redundancy in sediments, and they are responsible for key processes in organic carbon turnover and nitrogen and sulfur cycling. Gamma-Proteobacteria constitute the majority of the content in wells W1, W2, and W3, with abundances of 59.544%, 76.039%, and 58.023%, respectively. This indicates that the nitrogen and organic matter content in W2 is higher than in W1 and W3, indirectly confirming that nitrogen in well W2 is undergoing simultaneous nitrification and denitrification [42].

(2) Bacteroidia

They can survive in high ammonia nitrogen environments and play an indispensable role in the anaerobic denitrification process of food waste. The Bacteroidia content in W1 (10.733%) and W3 (14.885%) is significantly higher than that in W2 (3.921%), indicating that there is a large amount of undegraded residue in the groundwater environment of wells W1 and W3. This residue may come from animal feces and other pollution sources, and denitrification is more complete in well W2. The reaction distribution pattern is consistent with the abundance composition of Bacteroidetes analyzed previously at the phylum level [43].

(3) Alphaproteobacteria

They have denitrification capabilities. In wells W1, W2, and W3, the contents of Alphaproteobacteria were 12.757%, 5.823%, and 7.684%, respectively. Similar to Bacteroidia, the groundwater in wells W1 and W3 had more denitrifying bacteria than that in W2. The anaerobic denitrification potential in the edge area of the subsurface flow zone was stronger than that in W2.

Besides the dominant species, the microorganisms with higher abundances in the subsurface flow zone groundwater were as follows.

(1) Clostridia

Clostridia are anaerobic microorganisms. As one of the main microorganisms that degrade the pesticide pentachlorophenol, they are key microorganisms for

pentachlorophenol dechlorination and play a major role in the anaerobic methanogenesis process in the digester. This indicates that the groundwater in the well not only contains certain alkanes and aromatic hydrocarbons, but also a large number of methanogenic bacteria. Although the presence of methanogenic bacteria will degrade a certain amount of organic matter pollution in the groundwater, a large amount of methane will also be produced in the entire wetland, leading to the greenhouse effect and posing a certain risk of atmospheric pollution [44].

(2) Bacilli

Bacilli are a class of bacteria, including the orders Bacilli and Lactobacilli, which include the pathogen *Bacillus anthracis* (the cause of anthrax).

(3) Actinobacteria

The distribution pattern is similar to that of Actinobacteria. It can be seen that in well W1 (1.098%) and well W3 (2.065%), compared with well W2 (0.779%), there is a small amount of soil and a few dead branches falling from the well wall and from outside, which gradually form bottom sediment in the groundwater at the bottom of the well. Parasitic bacteria are generally anaerobic bacteria, and actinomycetes mainly promote the decay of animal and plant remains [45]. Well W1 is located near the edge of the infiltration subsurface flow zone of the Hun River, while Well W3 is located relatively close to the infiltration zone of the Xi River in the north. It receives various organic pollutants from the Hun River and the Xi River, indicating that Well W1 and Well W3 have stronger potential for degrading organic matter and ammonifying organic nitrogen.

(4) Verrucomicrobiota

Similar to the distribution level of Verrucomicrobiota in the previous section, it is mainly found in aquatic and soil environments, or in human excrement. It includes some ectosymbiotic bacteria of protozoa and endosymbiotic bacteria in the gametes of nematodes. Similarly, due to the small amounts of soil and dead branches falling from the well wall and from outside, bottom sediment gradually forms at the bottom of the well. Among the three sampling wells, W1 (1.790%) has more sediment in the surface sediment of groundwater [46].

Microbial Genus-Level Abundance

Comparative analysis of species composition among groups at the microbial genus level: Figs 6 and 7 and Table 5 show the species composition and proportions at the genus level in well sites W1, W2 and W3 during the dry season.

As shown in the figure, at the genus level, the dominant microbial species in the three groundwater well locations at the subsurface flow monitoring section are different, with other complex genera also being dominant. The abundance of other dominant species can be seen in the ASV annotation results bar chart [47]. Well location W1 mainly includes *Rhodospirillum rubrum*

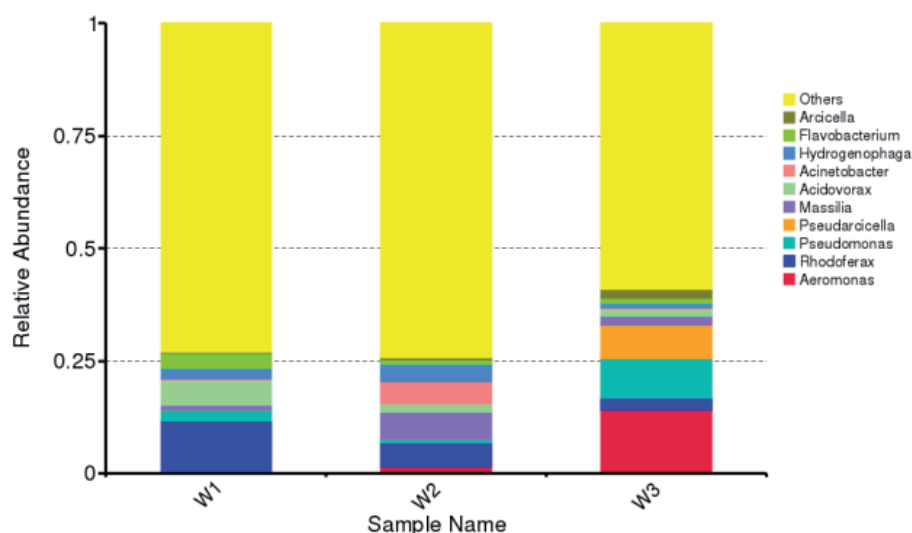


Fig. 6. Proportion of microbial species richness at the genus level.

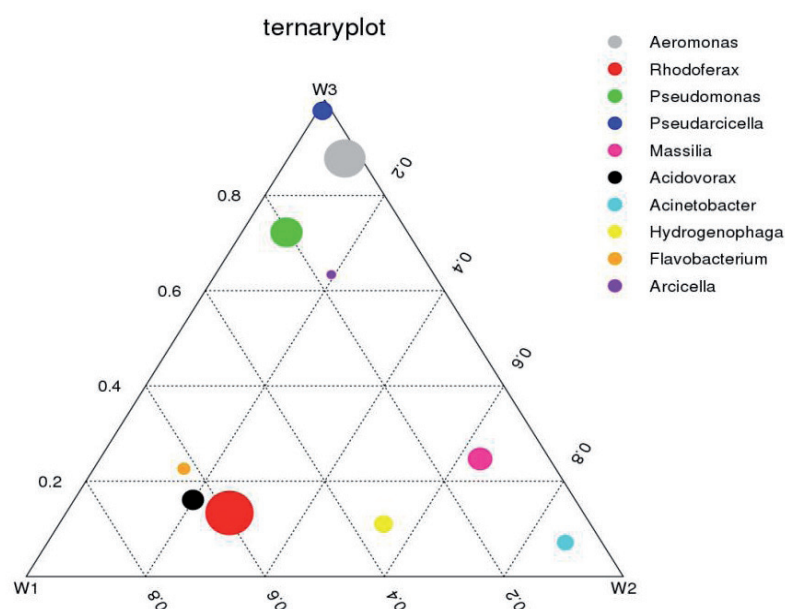


Fig. 7. A horizontal ternary phase diagram.

(content 11.156%) and *Acidovorax* (content 5.435%) of the family Comamonadaceae, class Betaproteobacteria; well location W2 mainly includes *Massilia* (content 5.971%) and Gammaproteobacteria, both of the family Oxalobacteraceae, class Betaproteobacteria. The dominant bacterial genera in well W3 include *Acinetobacter* (5.249%) of the family Moraxellaceae in the order Pseudomonadales. Well W3 primarily contains *Aeromonas* (14.191%) of the order Aeromonadales in the class Gammaproteobacteria and *Pseudomonas* (8.811%) of the family Pseudomonadaceae in the order Pseudomonadales.

The dominant bacterial genera in wells W1 and W3 belong to the classes Betaproteobacteria and Gammaproteobacteria, respectively. The distribution patterns at the phylum and class levels are consistent.

Proteobacteria include aerobic and anaerobic (obligatory or facultative) organisms, as well as chemoautotrophs, chemoorganoautotrophs, photoautotrophs, and heterotrophs [48]. Gamma-proteobacteria also exhibit extensive diversity, metabolic diversity, and functional redundancy in sediments, playing a dominant role in the key processes of organic carbon turnover and nitrogen and sulfur cycling. Gamma-proteobacteria are predominant in wells W1, W2, and W3, while the dominant bacterial species in well W2 include both Beta-proteobacteria and Gamma-proteobacteria, indicating that well W2 represents an intermediate stage in the nitrogen nitrification process. Its main functional bacteria nitrify NH_4^+ in groundwater into NO_2^- , and simultaneously complete denitrification, i.e., nitrate reduction from NO_3^- to NO_2^- [49].

Table 5. Proportion of species composition at the genus level.

Taxonomy	W1	W2	W3
<i>Aeromonas</i>	0.00446	0.01536	0.14191
<i>Rhodoferrax</i>	0.11156	0.05151	0.02511
<i>Pseudomonas</i>	0.02476	0.00912	0.08811
<i>Pseudarcicella</i>	0.00112	0.00057	0.07332
<i>Massilia</i>	0.01091	0.05971	0.02314
<i>Acidovorax</i>	0.05435	0.01689	0.01366
<i>Acinetobacter</i>	0.00371	0.05249	0.00432
<i>Hydrogenophaga</i>	0.02436	0.03821	0.00775
<i>Flavobacterium</i>	0.02995	0.00726	0.01088
<i>Arcicella</i>	0.00586	0.00661	0.02158
Others	0.72894	0.74226	0.59021

The important biogeochemical processes of nitrogen infiltration into the subsurface flow zone are determined to some extent by the redox sequence guided by microorganisms. Combining abundance bar charts and ternary phase diagrams, Proteobacteria and Firmicutes are the dominant species of denitrifying bacteria in anaerobic environments. Therefore, the denitrification that occurs before the river infiltrates into the detection wells W1, W2, and W3 is particularly strong. Since Bacteroidetes and Actinobacteria are the dominant species, they have a stronger potential to degrade organic matter into ammonium nitrogen in an aerobic environment between wells W1 and W2, resulting in a relatively strong nitrification. The main reason for this phenomenon is that well W1 is located closest to the near-shore zone of the Hun River at the monitoring section. During the dry season, the Hun River water infiltrates into the subsurface flow zone from the monitoring section. River water with a large amount of dissolved oxygen and high ammonia nitrogen enters the subsurface flow zone to carry out nitrification and form nitrate nitrogen. When it infiltrates into the anaerobic environment of the study area, nitrification and denitrification gradually occur simultaneously, leading to redox reactions.

Analysis of Nitrogen Migration, Transformation, and Biogeochemical Mechanisms

Analysis of the Spatial Distribution and Migration/Transformation Mechanism of Nitrogen in the Subsurface Zone of the Study Area

Looking at the concentrations from W1 to W3, the $\text{NH}_4^+\text{-N}$ concentrations decrease progressively (W1: 0.35~0.85 mg/L, W2: 0.12~0.40 mg/L, W3: 0.05~0.20 mg/L), indicating that ammonia nitrogen is gradually converted and utilized from W1 to W3. The

$\text{NO}_3^-\text{-N}$ concentration first increases and then decreases (W1: 1.5~3.2 mg/L, reaching a peak of 3.8~6.5 mg/L at W2, and decreasing to 1.2~2.8 mg/L at W3), while the $\text{NO}_2^-\text{-N}$ concentration remains at a low level (0.015~0.050 mg/L).

W1 is dominated by nitrification (a high-DO (3.5~5.0 mg/L), high-ORP (+80~+150 mV) environment is conducive to ammonia-oxidizing bacteria oxidizing NH_4^+ to NO_2^- , and further oxidizing to NO_3^-). W2 is the nitrification-denitrification transition zone (nitrate nitrogen accumulation reaches its peak here, while denitrification begins, DO drops to 1.5~3.0 mg/L, ORP drops to 0~+80 mV). W3 is dominated by denitrification (a low-DO (0.5~1.5 mg/L), low-ORP (-50~-20 mV) environment is conducive to denitrifying bacteria reducing NO_3^- to N_2 , so the NO_3^- concentration decreases significantly).

Analysis of Nitrogen Biogeochemical Mechanisms and Microbial Community Responses under Environmental Gradients

(1) Environmental Gradient-Driven Microbial Community Succession

The release of water from the dam during the dry season leads to the infiltration of high-DO river water, forming an aerobic-anoxic-anaerobic gradient along the groundwater flow direction. This redox gradient is a key environmental factor driving the microbial community formation pattern in the subsurface flow zone. Studies have shown that environmental filtration may be a more significant regulator of bacterial community dynamics than geographical distance. The gradients at each point in this study are as follows.

The concentrations of nitrogen forms and environmental factors in the groundwater of different wells are shown in the table below.

Table 6. Nitrogen species concentrations and environmental factor ranges in groundwater from each monitoring well (April 2021).

Well location	NH ₄ ⁺ -N (mg/L)	NO ₃ ⁻ -N (mg/L)	NO ₂ ⁻ -N (mg/L)	TN (mg/L)	DO (mg/L)	ORP (mV)
W1	0.35~0.85	1.5~3.2	0.015~0.036	2.8~5.6	3.5~5.0	+80~+150
W2	0.12~0.40	3.8~6.5	0.025~0.050	4.2~7.8	1.5~3.0	0~+80
W3	0.05~0.20	1.2~2.8	0.030~0.045	2.0~4.5	0.5~1.5	-50~-20

W1 (nearshore, aerobic): High DO level (approximately 4.2 mg/L), with γ -Proteobacteria accounting for 59.5%. Ammonia-oxidizing bacteria (AOB) nitrify NH₄⁺ to NO₂⁻, then gradually oxidize it to NO₃⁻. *Rhodoferrax* (11.2%), an enriched species in the nearshore zone, demonstrates the feasibility of aerobic nitrification in this region.

W2 (Mid-section, facultative): DO levels have decreased to a moderate level. The abundance of γ -Proteobacteria is the highest (76.0%), and β -Proteobacteria are also present. Therefore, this area can be considered a transition zone characterized by simultaneous nitrification and denitrification. The strength of hydraulic exchange directly affects nitrogen metabolism and the composition of the microbial community.

W3 (offshore, anaerobic): The DO level under anaerobic conditions is approximately 0.8 mg/L. Alphaproteobacteria and Bacteroidetes gradually become the dominant bacterial groups (7.7% and 14.9%, respectively), carrying denitrification genes that reduce

NO₃⁻ to N₂. The abundant presence of *Aeromonas* (14.2%) and *Pseudomonas* (8.8%) in this system indicates a strong denitrification process. The water level changes caused by dams promote nitrate removal through nutrient transport and microbial community proliferation; however, excessively high shear stress can damage the biofilm and weaken this effect.

(2) Driving Effect of Redox Gradient on Nitrogen Transformation Pathways

DO and ORP play a decisive and stimulating role in the switching of nitrogen transformation pathways in the undercurrent zone. In this study, DO decreased from 4.2 mg/L in W1 to 0.8 mg/L in W3, and ORP decreased from +120 mV to -35 mV, showing a clear redox gradient. The well section with high DO and high ORP (W1) provided suitable growth conditions for nitrifying bacteria, which could promote NH₄⁺→NO₃⁻, while the well section with low DO and low ORP (W3) activated denitrifying bacteria, catalyzing NO₃⁻→N₂.

The intensity of hydraulic exchange (controlled by dam release) determined the width and slope of

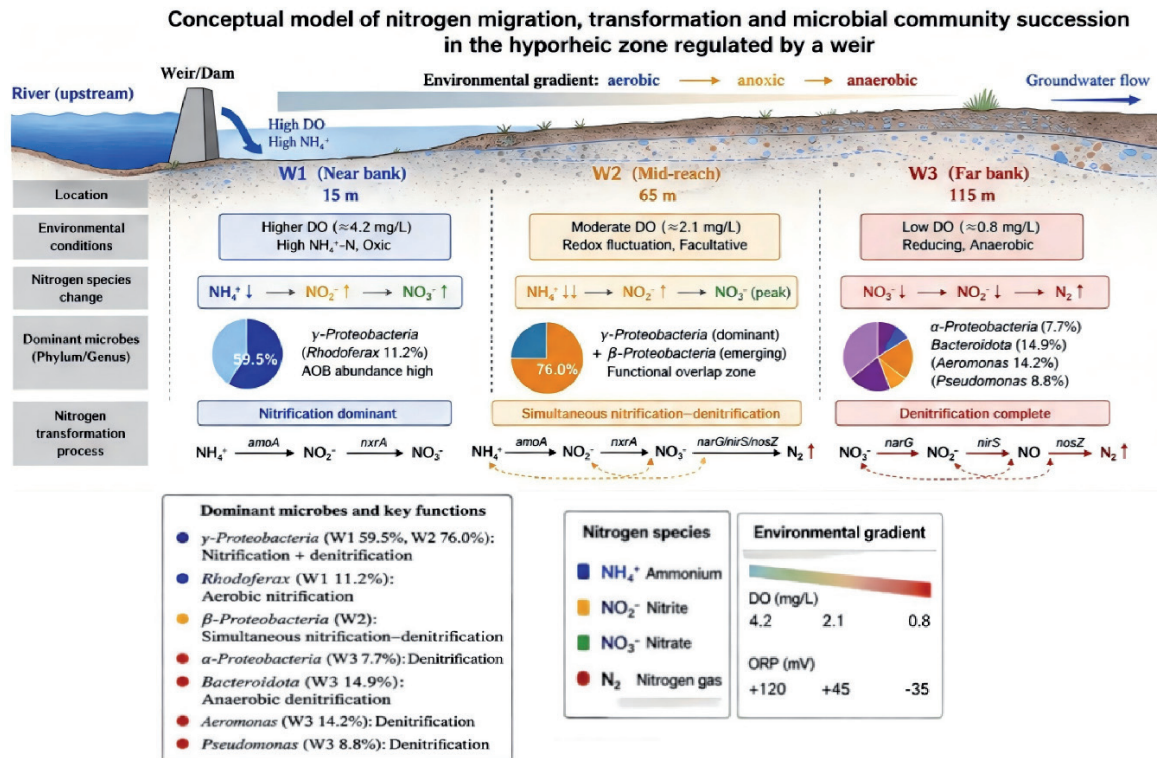


Fig. 8. Conceptual model of nitrogen migration and transformation and microbial community succession in the subsurface flow zone under dam regulation.

this gradient. Moderate release during the dry season enhanced river infiltration, allowing the aerobic-anaerobic transition zone to fully develop between W1 and W3, providing spatial conditions for nitrification-denitrification coupling. The Fig. 8 illustrates the relationships among the environmental gradient, microbial succession, and nitrogen function.

(3) Response Mechanism of Dominant Microorganisms to the Nitrogen Transformation Process

As can be seen from the above, the responses of different microbial groups to the nitrogen transformation process reflect clear functional differentiation, as shown in the Table 7.

(4) Redundancy Analysis of Microbial Community and Environmental Factors (RDA)

To quantitatively reveal the coupling relationship between microbial communities and environmental factors, redundancy analysis (RDA) was performed using the 10 most abundant species (*Rhodoferrax*, *Acidovorax*, *Massilia*, *Acinetobacter*, *Aeromonas*, *Pseudomonas*, *Pseudarcicella*, *Hydrogenophaga*, *Flavobacterium*, and *Arcicella*) at the genus level as response variables and environmental factors (distance from the riverbank, DO, ORP, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$) as explanatory variables (see Fig. 9).

The first axis of the RDA explained 62.3% of the community variation, and the second axis explained 26.8%, together explaining 89.1%. Monte Carlo

permutation tests (999 permutations) showed that distance from the riverbank ($p = 0.018$) and dissolved oxygen (DO) ($p = 0.024$) were the most significant factors contributing to changes in microbial community structure, with ORP ($p = 0.067$) and $\text{NO}_3^-\text{-N}$ ($p = 0.083$) showing similar significance. *Rhodoferrax* and *Acidovorax* were dominant under high DO and $\text{NH}_4^+\text{-N}$ conditions, indicating their suitability for aerobic nitrification environments, while *Aeromonas* and *Pseudomonas* showed synchronous changes under low DO and high $\text{NO}_3^-\text{-N}$ conditions, indicating their dominance in anaerobic denitrification. The RDA results further demonstrate that the functional zoning from aerobic nitrification to anaerobic denitrification is regulated by environmental gradients (DO, ORP). During the dry season, the dissolved oxygen (DO) content in the subsurface flow zone of the Hun River is 3.5-5.0 mg/L near the shore, decreasing to 0.5-1.5 mg/L towards the middle reaches. The DO gradient is the main driver of microbial succession and can be considered the basis for the formation of the microbial community.

The paper discusses the spatial distribution of microbial communities (at the phylum, class, and genus levels) and their relationship with nitrogen speciation based on 16S rRNA amplicon sequencing. Quantitative analysis of the abundance of functional genes (such as *amoA*, *nirK*, *nirS*, and *nosZ*) and nitrogen transformation rates (nitrification and denitrification)

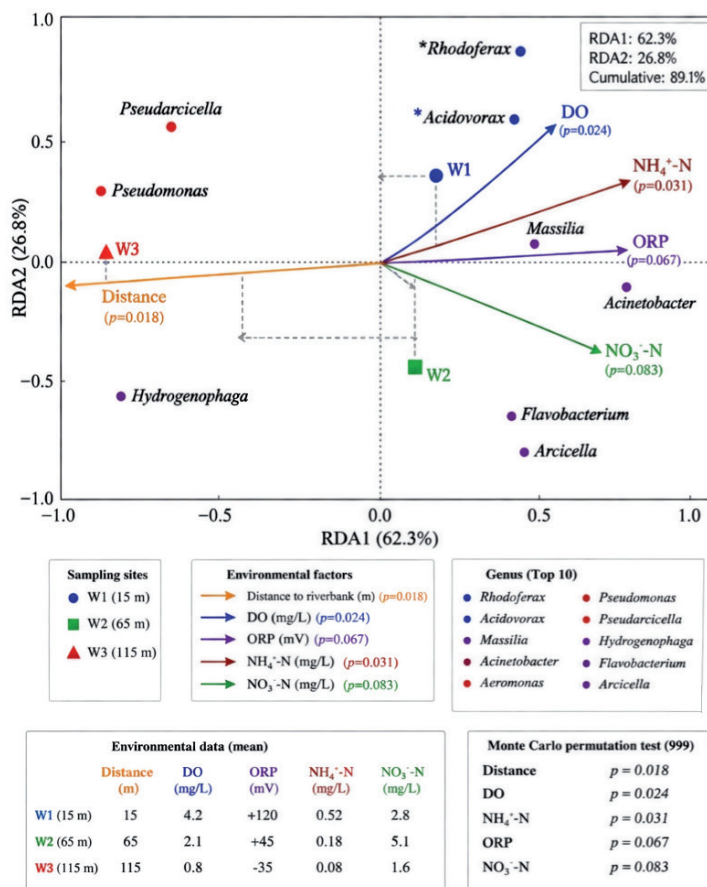


Fig. 9. Redundancy analysis (RDA) of genus-level microbial communities and environmental factors.

Table 7. Response mechanisms and functional differentiation of different microbial groups to nitrogen transformation.

Microbial taxa	Nitrogen transformation function	Response mechanism
Gamma-Proteobacteria (59.5% in W1, 76.0% in W2)	Nitrification + Denitrification	Under aerobic conditions, $\text{NH}_4^+ \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^-$ is catalyzed; under facultative conditions, NO_3^- reduction is initiated
<i>Rhodotorula</i> genus (11.2% in W1)	Aerobic nitrification	The oxidation of NH_4^+ dominates in nearshore high-DO environments
Beta-Proteobacteria (present in W2)	Simultaneous nitrification-denitrification	Simultaneous oxidation of NH_4^+ and reduction of NO_3^- occur in the transition zone
Alphaproteobacteria (7.7% in W3)	Denitrification	NO_3^- is reduced to N_2 under anaerobic conditions
Bacteroidetes (14.9% in W3)	Anaerobic denitrification	In anaerobic environments, it dominates the complete reduction of $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{N}_2$
<i>Aeromonas</i> spp. (14.2% in W3)	Denitrification	Anaerobic respiration occurs under hypoxic conditions, with NO_3^- acting as the electron acceptor
<i>Pseudomonas</i> (8.8% in W3)	Denitrification	A facultative anaerobe that reduces NO_3^- under low-DO conditions.

should be conducted through metagenomics or isotope tracing experiments; this approach will constitute a future research direction for this project.

Alpha Polymorphism Analysis

A traditional and efficient method for analyzing the diversity of microbial communities within a sample is alpha diversity analysis [50]. Diversity is further divided into the number of species, i.e., richness, and the evenness of the distribution of individuals in the community. Both are determined by this method. Chaol is often used to estimate the total number of species. The larger the value, the more species there are. It is one of the commonly used indices for estimating the total number of species in ecology [51].

(1) Alpha Diversity Index

The Alpha diversity index statistical Table presents the rarefaction curve data from the species diversity analysis. The alpha diversity indices (observed_otus, shannon, simpson, chaol, goods_coverage, dominance, and pielou_e) of groundwater from well sites W1 (nearshore zone) to W3 are statistically analyzed and shown in Table 8.

“chaol” in ecology estimates the total number of species in a community sample; the “chaol” index is positively correlated with low-abundance species in the community. As shown in the table, well W3, which is farther from the riverbank, has more low-abundance

species in its community. “dominance” can be seen as the probability that two randomly selected sequences come from the same sample; a larger index reflects better species evenness. Well W2 in the study area shows good species evenness with some randomness. “observed_otus”: the number of species directly observed; a larger index indicates more observed species. Similar to “chaol”, well W3 has relatively more observable species. “pielou_e”: evenness index. The more evenly the species are distributed, the larger the “pielou_e”. Well W1 has a more even distribution of species compared to the other two wells. “shannon”: the total number of taxa in the sample and their proportion. The higher the community diversity, the more even the species distribution, and the larger the total number of taxa represented by the Shannon index. The Simpson index represents the diversity and evenness of species distribution within the community. The better the species evenness, the larger the Simpson index. Compared with the other two wells, W1 has a more even species distribution. The reason for this is that during the dry season, the dam controls the release of water, and the microbial species in the groundwater containing infiltrated Hunhe River water in the W1 well, which is closest to the riverbank, are relatively evenly distributed.

(2) Species Accumulation Box Plot

The analysis of the increase in species diversity with increasing sample size is used to judge whether the sample size is sufficient and to estimate species richness.

Table 8. Alpha diversity index statistics.

Sample_Name	chaol	dominance	observed_otus	pielou_e	shannon	simpson
W1	790.5	0.029	789	0.742	7.145	0.971
W2	837.375	0.066	837	0.656	6.367	0.934
W3	889.429	0.035	889	0.698	6.842	0.965

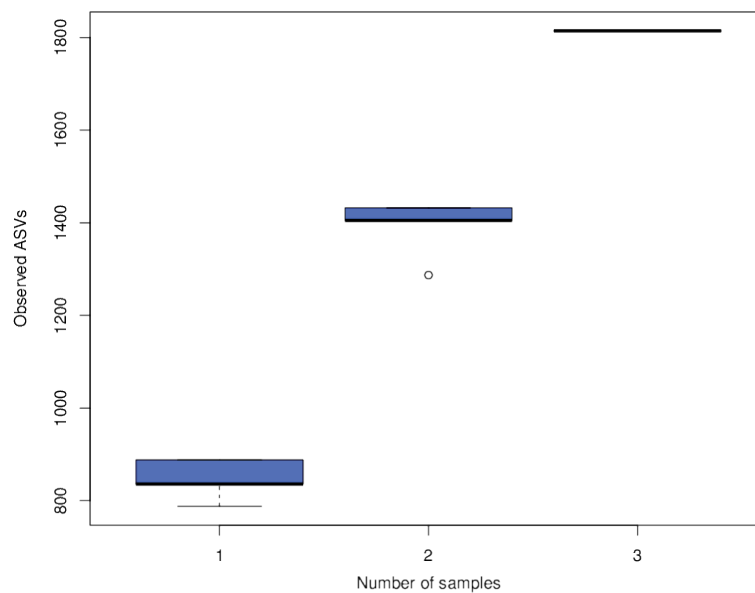


Fig. 10. Alpha species accumulation box plot.

A species accumulation box plot can be used to describe this.

As shown in Fig. 10 above, compared to other wells in the study area, well W1 showed a large number of species in its groundwater community. In well W3, the species distribution in the groundwater community did not change significantly with increasing sample size, while the species distribution in well W2 was relatively uniform.

By comparing the alpha diversity index with the cumulative species box plot, it can be seen that at the I-I' monitoring section, well W1, the closest to the Hun

River, had the most even species distribution compared to the other two wells. Well W2 showed relatively good community evenness, while well W3, the farthest from the riverbank, had relatively fewer observable species, indicating that nitrification and denitrification were relatively complete.

Beta Polymorphism Analysis

Beta Diversity is a comparative analysis of the microbial community composition of different samples. Differences between groups were analyzed using

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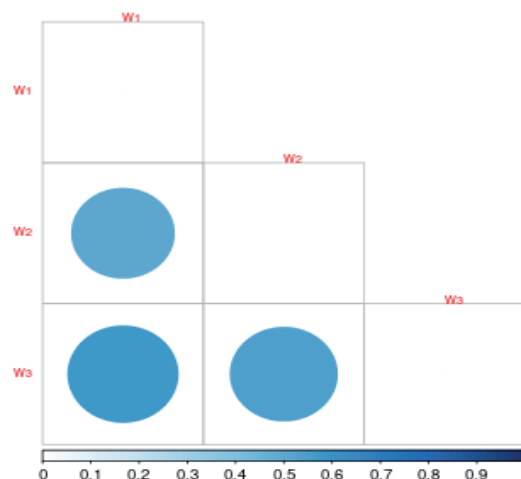


Fig. 11. Beta diversity index Unweighted UniFrac distance matrix heatmap.

the Beta Diversity Index, and multivariate statistical methods such as Principal Component Analysis (PCA), Principal Coordinates Analysis (PCoA), and Non-Metric Multi-Dimensional Scaling (NMDS) were employed to identify differences between different samples (groups).

(1) Distance Matrix Heatmap

In the comparison of microbial community composition, the four indicators – Weighted UniFrac distance, Unweighted UniFrac distance, Jaccard distance, and Bray-Curtis distance – correspond to the dissimilarity coefficients between different samples in the Beta Diversity analysis. The smaller the value, the smaller the difference in species diversity between the two samples. The heatmap plotted using the Unweighted UniFrac distance is shown in Fig. 11 below.

The size and color of the circles in the grid represent the dissimilarity coefficients between two samples. Larger circles correspond to darker colors, indicating greater differences between the two samples; conversely, smaller circles correspond to lighter colors, indicating smaller differences. As shown in the figure, the species differences among the three wells are relatively uniform. The composition and abundance of the microbial community in the subsurface flow zone are similar. The microbial community structure and abundance of samples from wells W1 and W3 show the greatest differences. Since W1 is the subsurface flow zone sample closest to the riverbank and W3 is the furthest from the riverbank, it is judged that the microorganisms gradually undergo nitrification and denitrification reactions with increasing distance from the riverbank in this section, resulting in relatively large differences in community structure and abundance.

(2) Multivariate Statistical Methods

Principal component analysis (PCA), principal coordinates analysis (PCoA), and non-metric multidimensional scaling (NMDS) are shown in Figs 12, 13, and 14.

According to the above figure, there is very little difference in the community composition among the three groups of samples from wells W1 to W3. The values of PCoA on the vertical axis are 32.14% and 42.02%, and the value of PCA on the vertical axis is 44.66%. These data indicate the probability that the factors affecting the differences in the microbial community structure in the subsurface flow zone cause the differences in the microbial community shown in the figure, or indirectly indicate the contribution rate of other environmental factors in the subsurface flow zone to the differences in the microbial community. The relative abundance of the microbial community is affected by dissolved oxygen, hydrodynamic conditions, etc., and the corresponding values do not reach 50%, indicating that the probability of differences in the microbial community caused by environmental factors is small, thus indicating that the composition of the bacterial community among different sample groups has a certain similarity.

The data analysis reveals that the differences in water quality and physicochemical composition of the subsurface flow zone among the three well sampling points in the study area are small. Further studies can be conducted on areas with obvious differences in bacterial species to reflect the effects of species diversity in the subsurface flow zone. It is speculated that the reason for the high degree of similarity of bacterial species composition among different sample groups

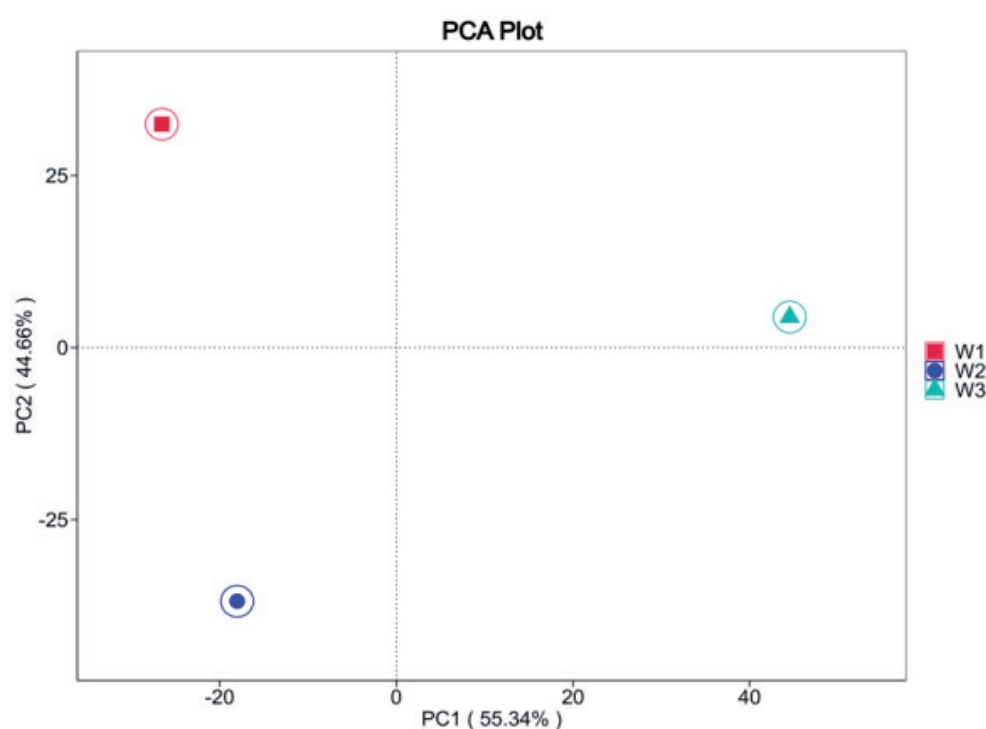


Fig. 12. PCA plot.

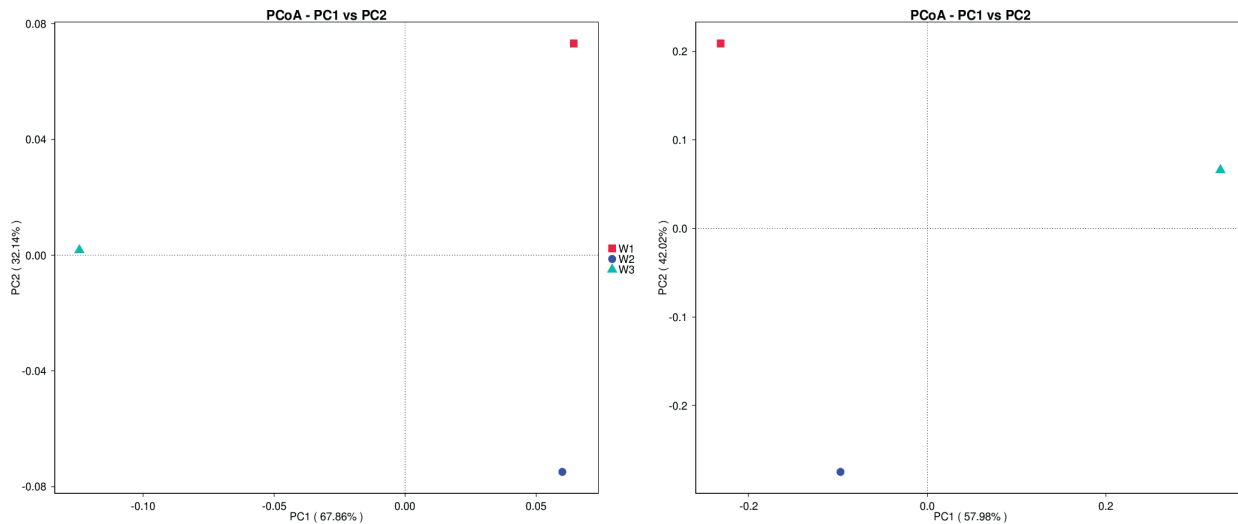


Fig. 13. Two-dimensional PCoA (left: based on Weighted UniFrac distance; right: based on Unweighted UniFrac distance).

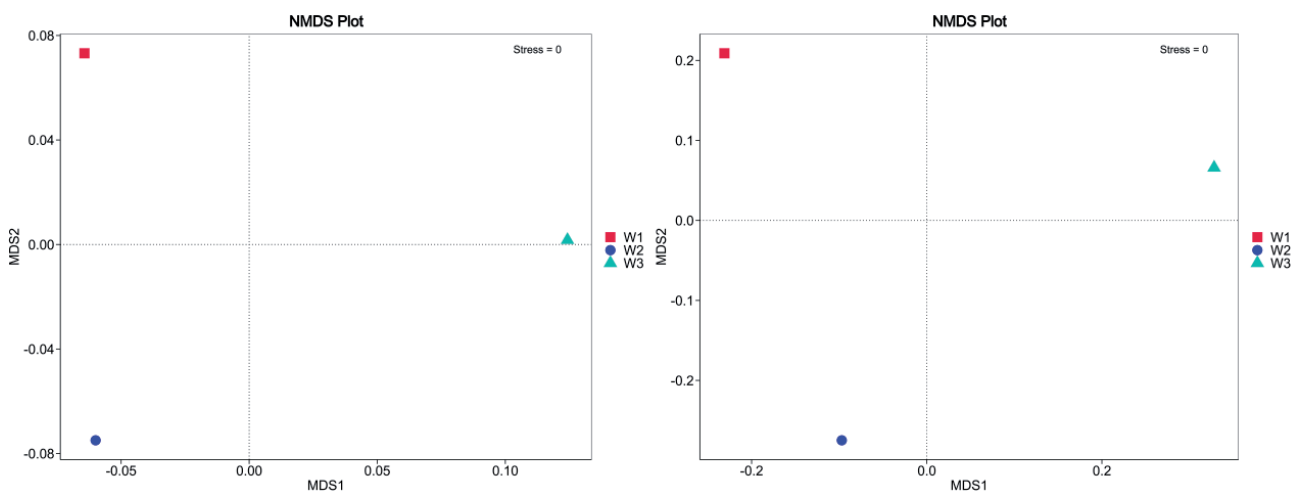


Fig. 14. NMDS (left: based on Weighted UniFrac distance; right: based on Unweighted UniFrac distance).

is that the Liguabao water source is spatially homogeneous, and the differences between the points in the W area where mining has stopped are not significant, so the differences in bacterial species composition among different sample groups are not significant. In the figure, W3 is far away from W1 and W2, and the groundwater microbial community in well W3 is significantly different from those in wells W1 and W2.

Conclusions

(1) During the dry season, the release of water from dams leads to the infiltration of highly oxygenated river water, creating an aerobic-anoxic-anaerobic gradient along the groundwater flow. This drives the succession of the microbial community from nearshore γ -Proteobacteria (aerobic nitrification) to offshore α -Proteobacteria and Bacteroidetes (facultative/anaerobic denitrification). The dominant phyla in the

subsurface flow zone are Proteobacteria, Bacteroidetes, and Firmicutes.

(2) The spatial distribution of nitrogen forms is as follows: W1 is dominated by nitrification ($\text{NH}_4^+ \rightarrow \text{NO}_3^-$), W2 is a transition zone between nitrification and denitrification (nitrate nitrogen accumulation reaches its peak), and W3 is dominated by denitrification ($\text{NO}_3^- \rightarrow \text{N}_2$). Well W3, farthest from the riverbank, has the highest species richness (Chao1=889.4), indicating the most complete nitrification and denitrification reactions; well W2 has the highest abundance of Proteobacteria (81.9%), indicating strong denitrification.

(3) If the vertical distance from the riverbank increases by 50 m, the Shannon index can rise by 0.5 to 0.8, indicating that the denitrification effect is directly proportional to this distance. RDA indicates that the vertical distance from the riverbank and dissolved oxygen (DO) are the main environmental factors influencing the composition of the microbial community ($p < 0.05$).

(4) Based on the current understanding of gradient patterns, the preliminary design considers extending the width of the riverbank ecological buffer zone to 80-100 m. Furthermore, microbial diversity indicators such as the Shannon and Chao1 indices will be incorporated into the river health assessment system, but their universality needs further demonstration.

(5) The limitations of this study are that the sampling only covered a single dry season and did not cover the dynamic impact of the wet season and interannual hydrological fluctuations on the microbial community; the study area was limited to temperate alluvial rivers, and the applicability of the conclusions to other climatic zones and geological backgrounds needs further verification.

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Data Availability Statement

The data presented in this study are available from the authors upon request.

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Conflicts of Interest

All authors declare no conflicts of interest.

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