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Rhizosphere Soil Microbial Survival States and N-Related Process during Riparian Plant Dormancy: Influences of Plant Locations and Plant Species

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Abstract: The plant dormancy period in the riparian zone affects the activity of microorganisms and their related nitrogen (N) process, which necessitates an investigation of the influence of the dormancy period on the microbial community. This study sampled two groups of soils (ashore and offshore soils) of two typical plants (*Acorus calamus*, *Canna indica*) in rhizosphere soils and bulk soils during the dormancy period to study the microbial communities. The results suggested that in ashore soils, especially in *Canna indica* soils, there was a lower abundance of N-related genes (4.79×10^6 copies/g) due to relatively competitive ecological niche competition because of possible sufficient substrate. Therefore, microbial communities still play a major role in the removal of N-related nutrients during plants' dormancy period. In addition, the results also showed that during the plant dormancy period, the cell necrosis processes accounted for relatively lower proportions (15.75%, 7.54%, 21.46%, and 5.23% in ashore and offshore *Canna indica* and ashore and offshore *Acorus calamus*, respectively), suggesting an unexpected fairly strong microbial survival ability in the dormancy period compared to the commonly expected weak microbial state. This high microbial vitality provides us insight into the restoration of riparian soils during the plant dormancy period.

Keywords: dormancy period; microbial livability; nitrogen removal; wetness and dryness; microbial functional gene

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1. Introduction

The riparian zone refers to the riverbed between the high and low water levels of the river, and the area above the highwater level until the influence of the river water completely disappears [1]. The riparian zone is the key intersection of the water and terrestrial ecosystems [2]. The riparian zone plays an important role, especially in the current situation of water pollution control in China, to control pollution and restore ecosystems [3–5]. It also embodies rich biodiversity and unique edge effects, which render the riparian habitats dynamic, complex, and diverse, where the environmental effects are significant [6]. The environmental effects of the riparian zone are regulated by the interplay of internal factors, including soil, vegetation, microorganisms, and other biological factors [7–9]. Moreover, the environmental effects of the riparian zone are also affected by external factors, such as seasonal changes in temperature [10], various precipitation patterns [11], and changes in the hydrology and water quality of rivers [10–13].

Soil microorganisms mediate various biological processes and regulate the soil environment where the microorganisms reside [14]. In particular, they play a vital role in nitrogen(N)-related processes, such as denitrification [15], anaerobic ammonium oxidation

(anammox) [16], and dissimilatory nitrate reduction to ammonium (DNRA) [17]. In the soil system, rhizosphere forms a unique microenvironment with high soil microbial activity and nutrient turnover rate due to its rhizosphere effect [18], which provides important growth substances and surface for microorganisms [19]. In return, the microbial community accelerates nutrient cycling and plant growth [20]. The biogeochemical cycle of N is closely related to the ecological restoration of rivers. In brief, microorganisms within the rhizosphere have a positive effect on the N cycle. For instance, the abundance of AOA-*amoA* (a key enzyme in the limiting step of nitrification in the oxidation of ammonia nitrogen to nitrite) in the rhizospheres of aquatic plants was higher than in the bulk soils, meaning more active N cycling in the plant rhizosphere [21]. The higher bacterial diversity and N-fixing bacteria found in the rhizosphere soil can be explored further regarding their role in N cycling in environment management [22]. Microorganisms in soils of different hydrological conditions have distinct performances and activity due to variations in factors such as the water content, availability in substrates, and oxygen [23,24].

In riparian zones, plants play an important role in the process of nutrient cycling [25]. Differences in hydrological conditions can significantly affect the growth state of plants [26] and, consequently, the activity and function of rhizosphere microorganisms [27]. Previous studies involving plants and microorganisms usually exposed them to optimal experimental conditions for their rapid growth [28,29]. In addition, the plant growth period is more commonly employed for studies related to the N removal mechanism involving rhizosphere microorganisms of aquatic plants [29,30]. There is a lack of research on the dormant period of plants in the natural environment. For instance, during the winter period, harsh environmental factors, such as low temperature and drought, may severely impact on the plants and microorganisms in rhizosphere soils [31,32]. There are also some studies on the seasonal changes of soil bacteria communities in *Suaeda* wetland, the anaerobic ammonium oxidizing bacterial community in mangrove sediments, and rhizosphere soil microbial abundances under different vegetation types [33–35]. Few studies have reported the survival states of microbes and the microbial activities during plant dormancy in the winter. In this study, we chose two naturally growing plants (*Acorus calamus* and *Canna indica*) in the riparian zone, which are typically and commonly used in the ecological restoration of the riparian zone in China [36,37]. The two plants chosen in our study play an important role in riparian zone restoration during their growing periods, but there is little knowledge about the role during their dormancy period. When plants are in their dormancy period, the species, structure, and nitrogen cycling function of microorganisms in rhizosphere soil may change, which is worth studying. In addition, considering the significant effects of the hydrological conditions on the biogeochemical properties and processes [2,38], the rhizosphere's effect in different hydrological zones during the dormant period is worthy of being studied.

In view of this, our study aimed (1) to compare the soil microbial community structure of two macrophytes' (*Acorus calamus* vs. *Canna indica*) rhizosphere in various hydrological zones (ashore vs. offshore) during the plant dormancy period; (2) to investigate the changes in the functional genes in three N-related pathways within rhizosphere microorganisms; (3) to explore how environmental factors affect the structure and functional genes of rhizosphere microorganisms; and (4) to illuminate the roles of microbial survival states in ecological restoration during the plant dormancy period by flow cytometry analysis.

2. Materials and Methods

2.1. Study Area and Description

This study was conducted in the riparian zone of a tributary of Wangyu River, called Gushiqiao River, that belongs to Taihu Lake Basin, which is located in Wuxi City, Jiangsu Province, China (31°27′27″ N, 120°29′51″ E, and 21.5 m a.s.l.), representing a typical river with riparian geographic characteristics (Figure 1). The water level varied between 1.55 and 1.82 m, and the average flow rate was 0.03 m s^{-1} [39]. The annual mean rainfall was 1121 mm, mainly between May and September [40]. A typical subtropical monsoon climate

influenced the study area in four distinctive seasons. The mean annual temperature was 18.6 °C, with the highest temperature of 38 °C occurring in July and the lowest temperature of 0 °C occurring in January. We conducted our sampling on 3 January 2022. The average temperature on the sampling day was approximately 3 °C.

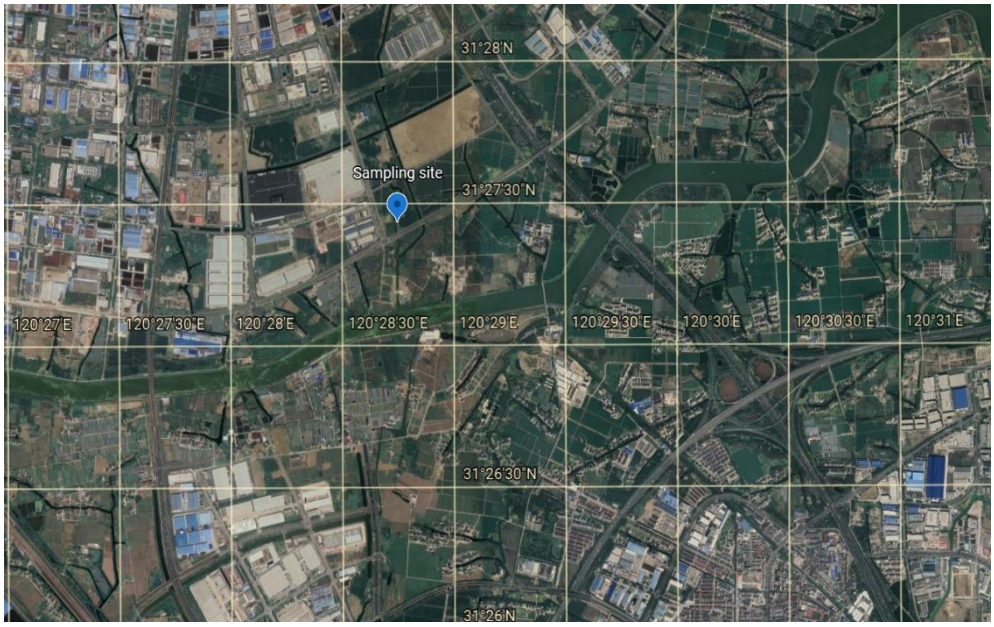

Figure 1. Sampling site in the riparian zone of a tributary of Wangyu River.

The riparian zone is vital to riverbank restoration, and the study area was included in the restoration project Water Diversion from Yangtze River to Taihu Lake. The previously bare riparian zone, since 2019, has been planted with several species to rehabilitate the riverbank. All plants naturally grow on the riverbank after plantation without any anthropogenic activities. In this study, we chose two typical riparian plants (*Acorus calamus* and *Canna indica*). These species are semi-aquatic plants, whose habitat is relatively far from the river rather than in the river channel [41]. *Acorus calamus* and *Canna indica* were evenly scattered on the riverbank in our study site.

2.2. Sample Collection

We collected soil samples from two hydrological zones, i.e., ashore and offshore, to investigate the effects of the hydrological conditions. The offshore samples were collected at the locations where roots were able to contact the water from the adjacent river while the ashore soils were sampled at least 3 m away from the river where roots were in the opposite condition compared to ashore soils. Three plots (1 m × 1 m) were randomly selected in the rhizosphere of both *Acorus calamus* and *Canna indica* in each hydrological zone [42]. The rhizosphere soils were acquired in accordance with the protocol described [9]. Briefly, the entire plant was removed from the soil and gently shaken to remove soil not tightly attached to the roots, Then, the roots were washed in 2 L of physiological solution (9 g L^{-1} NaCl) to obtain the more tightly attached rhizosphere interface soil. The resulting water-soil mixture was vortexed for 5 min and centrifuged at 4000 rpm (2683.2× *g*) for 5 min; the supernatant was discarded. The remaining soil fraction was merged with the soil obtained by shaking, which together constituted the rhizosphere soil. Three bulk soils (0–20 cm) were also randomly sampled in comparison with the rhizosphere soils in both the ashore and offshore zones. In the ashore zone, we sampled three *Acorus calamus* plant and three *Canna indica* plants in each plot, then randomly collected three subgroups of rhizosphere soils from each plant. Then, each plant's rhizosphere soil that was from the same species was combined into one sample soil, obtaining three samples for each species. We used the same method in the offshore zone. For bulk soils, we randomly collected three samples in

each hydrological zone. All these samples were kept on ice and brought to the laboratory to conduct physical and chemical experiments.

2.3. Soil Physiochemical Analysis

Soil water content (SWC) was measured as gravimetric weight loss after drying at 105 °C for 24 h [43]. Soils for physiochemical analysis were passed through a 2-mm sieve to remove stones and plant fragments. Approximately 10 g of fresh soil was mixed with 2 M KCl solution (50 mL) and then was shaken for 1 h, followed by filtration using a 0.45-μm filter. Then, the soil ammonium (NH_4^+-N) and nitrate (NO_3^--N) in the KCl extracts were measured by continuous flow analysis technology (Skalar San, Netherlands). In short, NH_4^+-N reacted with hypochlorite (ClO^-) in alkaline solution and formed chloramine, which reacted with salicylate under certain conditions and presented a blue-green color (ISO 11732). Nitrate-N was first reduced to nitrite (NO_2^--N), then reacted with the acid chromogenic agent to produce a red dye, and the obtained result was the content of NO_2^--N and NO_3^--N in the original extracts. The same steps were carried out on a separate subsample of the soil [15].

2.4. DNA Extraction, Quantitative PCR, and Illumina MiSeq Sequencing

Total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (D5625-01) (Omega Bio-Tek, Norcross, GA, USA) and stored at −20 °C prior to further analysis. The quantity and quality of extracted DNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively.

In this study, the reaction mixtures were: 2×SYBR real-time PCR premixture, primer F (10 μ M) 0.4 μL, primer R (10 μM) 0.4 μL, DNA template 1 μL, and ddH2O 14.75 μL. The PCR conditions were 95 °C pre denaturation for 5 min, followed by 40 cycles of 95 °C denaturation, and 56 °C for the *nirS* gene. Negative controls with no template DNA, but all other reaction mixtures were added in parallel to exclude the possibility of contamination. The standard curve coefficients of the variation and efficiencies were as follows: *narG* (R2 = 0.9986, efficiency = 91.64%), *nirS* (R2 = 0.9971, efficiency = 92.35%), *nirK* (R2 = 0.9949, efficiency = 92.70%), *hzsB* (R2 = 0.9969, efficiency = 88.57%), and *nrfA* (R2 = 0.9953, efficiency = 90.57%) and the dissolution curves were a single peak.

For high-throughput sequencing, the barcode primer set 338F (5′-ACTCCTACGGGAG GCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the V3-V4 region of the 16S rRNA gene [44]. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 250 bp sequencing was performed using the Illlumina MiSeq platform with a MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

2.5. Flow Cytometry Analysis

We used multicolor fluorescence FCM combined with a dual-staining procedure to distinguish the different physiologic status of bacteria. By staining cells with a combination of fluorescein annexin V-FITC and PI, it is possible to distinguish and quantitatively analyze non-apoptotic cells (Annexin V-FITC negative/PI negative), early apoptotic cells (Annexin V-FITC positive/PI negative), late apoptotic/necrotic cells (Annexin V-FITC positive/PI positive), and dead cells (Annexin V-FITC negative/PI positive) through flow cytometry and fluorescence microscope.

To test the physiologic status of soil microorganisms, the soil and PBS buffer (1:1 ratios) were centrifuged at 1000× *g* for 10 min at 4 °C after aspirating the supernatan along with passing through 0.45 mm and 0.22 mm membranes in turn. The following procedure was conducted according to the manual protocol of the Annexin V-FITC Apoptosis Detection Kit (Beyotime). In brief, the cell deposit was added to 195 μL Annexin V-FITC buffer with

10 µL PI for the single-staining procedure and 5 µL Annexin V-FITC with 10 µL PI for the dual-staining procedure. The left supernatant was used as a blank control. Then, the cells showed four different cell populations marked as follows: PI positively and FITC negatively stained cells showing dead cells (upper left), FITC and PI double-stained cells showing late apoptosis (upper right), double-negative cells showing the live cell population (lower left), and FITC positively and PI negatively stained cells showing early apoptosis (lower right).

2.6. Data Analyses

Sequence data analyses were mainly performed using QIIME2 and R packages. ASV-level alpha diversity indices, such as the Chao1 richness estimator, Shannon diversity index, and Simpson index, were calculated using the ASV table in QIIME2, and visualized as box plots. Beta diversity analysis was performed to investigate the structural variation of microbial communities across samples using Bray–Curtis metrics [45]. The taxonomy compositions and abundances were visualized using MEGAN [46] and GraPhlAn [47]. A Venn diagram was generated to visualize the shared and unique ASVs among samples or groups using R package "VennDiagram", based on the occurrence of ASVs across samples regardless of their relative abundance [48]. Microbial functions were predicted by PICRUSt2 (phylogenetic investigation of communities by reconstruction of unobserved states) [49] on the MetaCyc and KEGG databases.

The means and standard deviations of the soil physicochemical properties and abundance of N-related functional gene abundances were calculated (n = 3). The effects of hydrologic zones (ashore and offshore), plant species (*Acorus calamus*, *Canna indica*, and no plant (i.e., bulk soil)), and their interactions on soil biogeochemical properties were firstly analyzed by a one-way ANOVA. Duncan's multiple range test was applied for multiple comparisons of the means. Statistical analyses of the data were performed using Origin 2018.

3. Results and Discussion

3.1. Soil Physiochemical Properties

From Figure 2a, it is shown that the moisture content of offshore plants' soil was significantly higher than the ashore ones. The rhizosphere soil of *Acorus calamus* contained a higher moisture content compared to that of the *Canna indica* and bulk soils. This result may be caused by the differences in the root morphology between the two species. For instance, *Acorus calamus* is a perennial plant with a creeping and extensively branched rhizome [50]. Its roots are inclined to gather high-moisture soil. While the soil attached to *Canna indica* roots, which were about 2–5 mm in diameter with numerous root hairs, contained lower moisture due to the higher transpiration rate within this morphology at later growth stages [51–53]. Compared with bulk soil, the rhizosphere of *Acorus calamus* and *Canna indica* consisted of a higher moisture content in terms of offshore soil, respectively. This occurred because of the effects of the rhizosphere on root water uptake compared with bulk soil [54]. In the case of rhizosphere soils, with the exception of offshore *Acorus calamus*, and ashore bulk soil, the concentration of NH_4^+-N was significantly higher than other forms of nitrogen (Figure 2b), in which the content of ashore *Canna indica* and ashore *Acorus calamus* was higher than that of offshore *Canna indica* and offshore *Acorus calamus*. The discrepancy may be attributed to lower soil moisture in the ashore *Canna indica* and ashore *Acorus calamus* soils. One study [55] found that nitrification was slow at a soil moisture level of almost 20% or less, resulting the accumulation of NH_4^+-N. Nitrification refers to the process of ammonia oxidation into nitrate in two steps with the participation of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria. In the first step, ammonia-oxidizing bacteria oxidize NH_4^+ to NO_2^-. Ammonia-oxidizing bacteria use NH_4^+ as the only energy and CO_2 as the only carbon source. Lower soil moisture may lead to a decrease in the amount of CO_2 dissolved, causing the first step of restrained nitrification, resulting in the accumulation of NH_4^+. The NO_3^--N concentration was especially highest among

the three nitrogen forms in offshore *Acorus calamus* (Figure 2c). Regarding the NO_2^--N concentration, it was the significantly lowest index among the rhizosphere and bulk soils (Figure 2d).

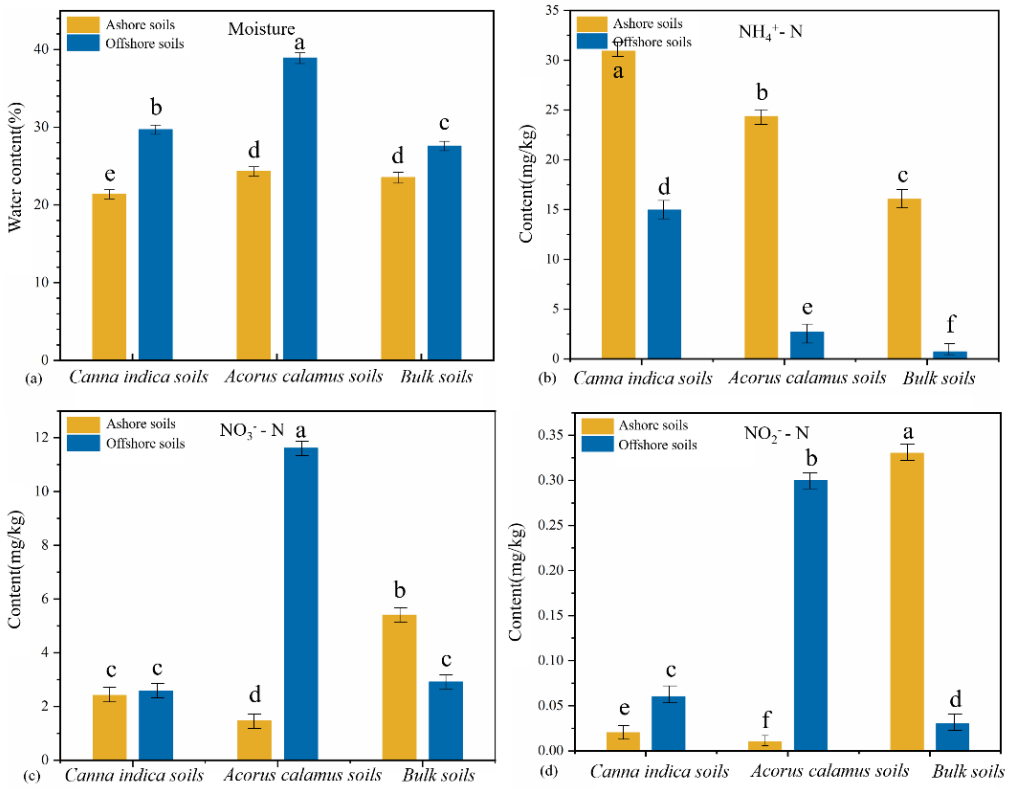

Figure 2. Physiochemical properties of *Acorus calamus* and *Canna indica* rhizosphere and bulk soil. (**a**) Soil moisture content among rhizosphere and bulk soils; (**b**) Ammonium (NH_4^+-N) concentration; (**c**) nitrate (NO_3^--N) concentration; (**d**) nitrite (NO_2^--N) concentration. Letters (a, b, c, d, e and f) above the bars were based on Waller-Duncan analysis at significance level of 0.05.

3.2. Soil Microbial Community Structures and Diversity

The alpha diversity values of the groups of the two plants' rhizosphere soils and bulk soils were measured using the Chao1 index, Shannon diversity index, and Simpson index (offshore < ashore). In Figure 3a, these values reveal a reduction in the bacterial diversity of the offshore groups of all soils as compared to the ashore groups of the soil samples. In general, the lowered moisture content of the soil limited substrate diffusion to microbial cells [56] and thus inhibited the activity of bacteria [57], which could lead to a reduction in the bacterial diversity in ashore soils, containing a lower moisture content; however, the result from this study contradicted this explanation. The reason that this happened may be the cause of the dormant period of plants, i.e., the later growth stage. During the dormant period of plants, which occurs in winter, when nutrient conditions in the rhizosphere are limited and microorganisms have to obtain massive organic substrates from plants [58], microorganisms in ashore soils can utilize senescent roots as a source of nutrient substrates instead of leaching nutrients in the offshore soils, resulting from the cleansing effect of the rivers. This explains why the bacterial diversity in the ashore soils was higher than in the offshore soils. The group of ashore samples has significantly more operational taxonomic units (OTUs) than the offshore samples as indicated by the rarefaction curves of the detected bacterial OTUs (Figure 3b). Specifically, ashore *Acorus calamus* had similar OTUs to offshore *Acorus calamus* while ashore *Canna indica* had significantly higher OTUs compared to offshore *Canna indica*, indicating that the hydrological zones had little effect on the total microbial OTUs in the rhizosphere soils of *Acorus calamus* but significantly affected that of *Canna indica*, which is likely attributed to the root morphology discrepancy. *Canna*

indica roots had numerous thin root hairs, ranging from 2–5 mm in diameter, which senesce and become nutrient substrates for microorganisms to compensate for the nutrient shortage within the soil during plant dormancy (i.e., the winter season of our study). The plants on the offshore side may be influenced by the flowing river and the scrubbing effect may take away the scarce nutrients, causing offshore *Canna indica* to have less microorganisms than ashore *Canna indica*. Additionally, A-Ci (ashore *Canna indica*) had the most OTUs while A-Ac (ashore *Acorus calamus*) had the lowest ones when excluding bulk soil. This indicates that *Canna indica* in the ashore environment may have greater microbial diversity compared to that of *Acorus calamus* during the plant dormancy period due to the different root morphology.

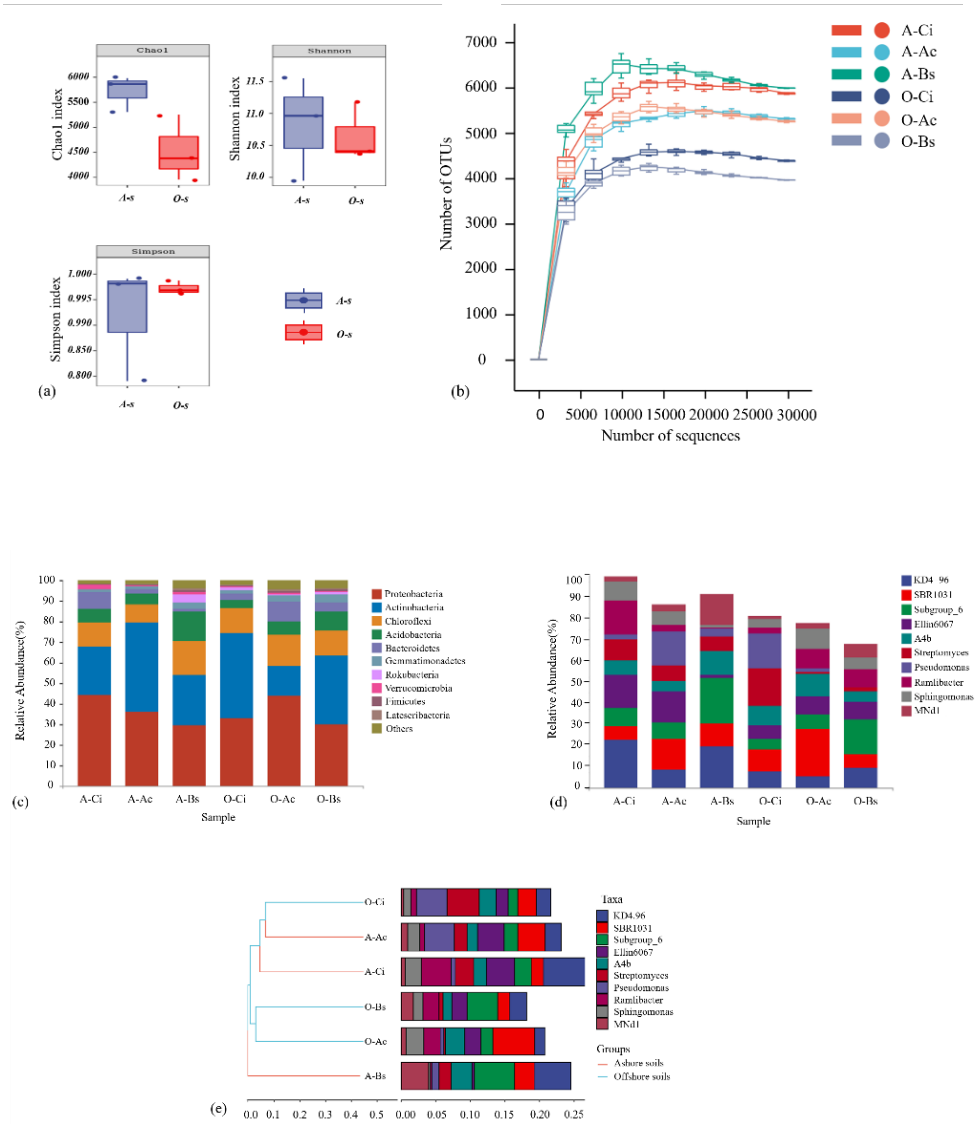

Figure 3. Soil microbial community structures and diversity. (**a**) Relative α-diversity for ashore and offshore soils; (**b**) rarefaction curves of detected bacterial operational taxonomic units (OTUs); (**c**) taxonomic composition analysis at the phylum level; and (**d**) taxonomic composition analysis at the genus level. O refers to the offshore soils while A refers to the ashore soils. At the same time, A-Ci, A-Ac, A-Bs, O-Ci, O-Ac, and O-Bs, respectively, refer to Ashore-*Canna indica*, Ashore-*Acorus calamus*, Ashore-bulk soil, Offshore-*Canna indica*, Offshore-*Acorus calamus*, and Offshore-bulk soil; (**e**) hierarchical clustering analysis of different samples, A-Ci, A-Ac, A-Bs, O-Ci, O-Ac, and O-Bs, respectively, refer to Ashore-*Canna indica*, Ashore-*Acorus calamus*, Ashore-bulk soil, Offshore-*Canna indica*, Offshore-*Acorus calamus*, and Offshore-bulk soil.

In Figure 3c, insight is provided into the structural diversity of the rhizosphere microbial communities in ashore and offshore soils. The six most abundant bacterial phyla in the six samples collected from ashore and offshore soils were *Proteobacteria* (mean 36.1%; A-Ci 44.4%; A-Ac 36%; A-Bs 29.5%; O-Ci 33.1%; O-Ac 43.8%; O-Bs 29.9%), *Actinobacteria* (mean 30.1%; A-Ci 23.5%; A-Ac 43.4%; A-Bs 24.2%; O-Ci 41.1%; O-Ac 14.4%; O-Bs 33.7%), *Chloroflexi* (mean 12.8%; A-Ci 11.5%; A-Ac 9%; A-Bs 16.8%; O-Ci 12.2%; O-Ac 15.5%; O-Bs 11.9%), *Acidobacteria* (mean 7.5%; A-Ci 6.7%; A-Ac 4.8%; A-Bs 14.0%; O-Ci 4.0%; O-Ac 6.3%; O-Bs 9.3%), *Bacteroidetes* (mean 4.8%; A-Ci 7.9%; A-Ac 2.3%; A-Bs 1.7%; O-Ci 3.0%; O-Ac 9.5%; O-Bs 4.4%), and *Gemmatimonadetes* (mean 2.6%; A-Ci 1.7%; A-Ac 1.5%; A-Bs 2.9%; O-Ci 2.0%; O-Ac 3.4%; O-Bs 3.9%). At the class level, *α*, *γ*, and *δ-Proteobacteria*, *Actinobacteria*, *Anaerolineae*, *Bacteroidia*, *KD4-96*, *Subgroup_6*, *Acidimicrobiia*, *Thermoleophilia*, and *Chloroflexia* were detected with >1% abundance in the rhizosphere sediments (Figure 3d).

A total of 423 bacterial genera were detected in the rhizosphere and bulk communities of *Acorus calamus* and *Canna indica*. Of these, the five most abundant genera (≥1%) were *KD4-96* (3.3%), *SBR1031* (3.2%), *Subgroup_6* (3.0%), *Ellin6067* (2.4%), *A4b* (2.1%), *Streptomyces* (2.0%), *Pseudomonas* (1.8%), *Ramlibacter* (1.8%), and *Sphingomonas* (1.6%).

In Figure 3e, there are clear clusters among samples. Sample A-Ac and sample O-Ci clustered together at the genera level. This result can explain that the rhizosphere microbes of samples A-AC and O-CI were similar in genetic lineage. Similarly, sample O-Ac and sample O-Bs clustered together at the genera level. However, sample A-Ci and sample A-Bs were apparently away from other groups of samples. This result may explain the rhizosphere microbes' independent composition and structure.

3.3. Functional Gene Abundance of N-Reduction Pathways

Five kinds of N-related functional genes: *narG*, *nirS*, *nirK*, *hzsB*, and *nrfA* (*narG* gene was the assimilatory nitrate reduction target gene, *nirS* and *nirK* were denitrification target genes, *hzsB* was the anammox target gene, and *nrfA* was the dissimilatory nitrate reduction to ammonium target gene), were qualified with qPCR (Figure 4). In general, *nirK* occupies the most gene copies of the five, followed by *nirS*, *narG*, *hzsB*, and *nrfA* except for the bulk soil. Several studies have shown that bacteria possessing the *nirS* gene prefer to be in an environment with a low oxygen concentration, but *nirK* gene carriers are not sensitive to oxygen [59,60]. Especially, the oxygen is transferred to the rhizosphere to support the life of microorganisms [61,62]. There is plenty of oxygen in the microenvironment of plant roots. Additionally, some studies also showed that *nirK*-denitrifying bacteria played a dominant role in soil denitrifying communities in the riparian zone [63–65]. This probably explained the higher proportion of *nirK* genes in the studied soils. In *Canna indica* rhizosphere soils, the average abundance of *nirK* gene copies of offshore soil was 7.49 × 106 copies per gram of dry weight sediment, which was significantly higher than 3.10 × 106 copies in ashore soil. This appearance was also found in the *Acorus calamus* soils. One study explained that the proportion of bacteria possessing *nirK* genes was higher in areas prone to periodic flooding [66], such as in the transitional areas and river–riparian interface. Additionally, the copies of the *nirS* gene, *narG* gene, and *hzsB* gene were much higher in the offshore soils except for the *nrfA* gene in ashore soils, which were twice the copies of offshore soils. From Figure 1b, the soil NH_4^+-N content in ashore *Canna indica* was lower than offshore *Canna indica*. In the DNRA process [67], NO_3^--N is gradually reduced to NH_4^+-N under the action of microorganism possessing *nrfA* genes, which is in accordance with the result of the two-fold higher *nrfA* genes copies in ashore soil. In the anammox process, NO is also reduced to N_2 with NH_4^+ as the electron substrate, used by the microorganism for processing *hzsB* genes. Hence, the higher *hzsB* genes in offshore soils were consistent with the reduced NH_4^+-N content in offshore soils.

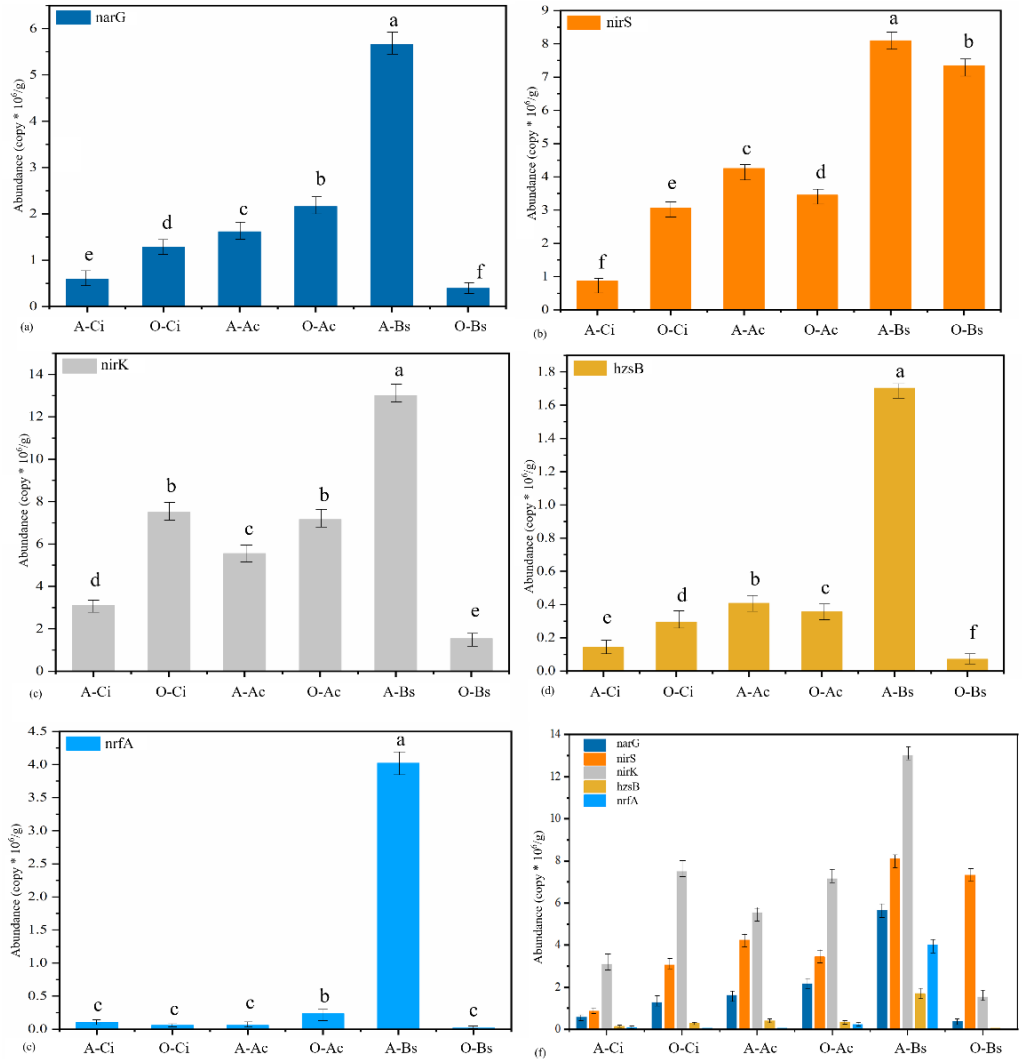

Figure 4. Five N-related relative functional genes' abundance. (**a**) *narG* gene abundance among rhizosphere and bulk soils. (**b**) *nirS* gene abundance; (**c**) *nirK* gene abundance; (**d**) *hzsB* gene abundance; (**e**) *nrfA* gene abundance; (**f**) five functional genes' abundance in total. Letters (a, b, c, d, e and f) above the bars were based on Waller-Duncan analysis at significance level of 0.05.

In *Acorus calamus* soils, the *nirK*, *narG*, *nrfA*, and *hzsB* gene copies were significantly higher in the offshore soils. In contrast, for the *nirS* gene copies, the ashore soils had more copies than the offshore soils. One study suggested that the different *nir* genes in soil do not exhibit similar behaviors with some discrepancy [64], as some organisms possessing different nitrite reductases prefer different niches, maintaining two nitrite reductase systems of equivalent function but dissimilar structure over a long time [68]. In offshore soils, the *nirK* gene copies of *Acorus calamus* were 7.16 × 10^6 copies, which is almost the same as the copies of *Canna indica*, and the *nirS* gene copies and *hzsB* gene copies of *Acorus calamus*. *Acorus calamus* had significantly more copies of *nrfA* genes and *narG* genes than *Canna indica*. There was much higher NO_3^--N and NO_2^--N in the offshore *Acorus calamus* soils, providing sufficient substate nutrients for the first step in denitrification, Anammox, and the DNRA process. The higher *narG* genes within it also showed that it facilitated the transformation from NO_3^--N to NO_2^--N. The much higher *nrfA* genes helped the second step in the DNRA process, producing more NH_4^+-N substrate in theory; however, from Figure 1b, there was a lower NH_4^+-N content, probably due to the relatively strong Anammox process consuming NH_4^+-N by the microorganism containing *hzsB* genes. In ashore soils, in general, the gene copies of *narG*, *nirS*, *nirK*, and *hzsB* in *Acorus calamus* were significantly higher than in *Canna indica*, except that the *nrfA* gene copies in *Canna indica*

soils were twice the copies of *Acorus calamus* soils. In the later growth stage of the plant or in the winter, the fine roots age and become decaying roots [69]. One study showed that microorganisms absorb more carbon sources from decaying roots than the root exudates of a living plant [70]. The *Canna indica* roots contained numerous fine root hairs with a diameter of about 2–5 mm. Under the presence of decaying roots, its rhizosphere microbiome was enriched with more processes, with the metabolism of carbohydrate and membrane transporters [71]. In this scenario, some microorganisms have overlapped niches, though in great diversity, which may cause intense competition between microorganisms, thus leading to a decrease in N-related functional genes.

3.4. Cell Apoptosis Analysis

In Figure 5c, it shows that most bacteria underwent apoptosis and necrosis processes in the rhizosphere of ashore *Acorus calamus*. In winter, the weather was usually dry, which was not suitable for the reproduction and growth of microorganisms, especially when the nutrients were also scarce. Meanwhile, the differences in the quantity and quality of the rhizodeposition, soil moisture content, and root architecture were consistent with the differences in the population densities [72]. The lower soil moisture content in ashore *Acorus calamus* soil may contribute to cell apoptosis and necrosis. In terms of the proportion of all samples, the proportion of cell necrosis processes in ashore *Acorus calamus* soils and cell apoptosis processes was the largest among all samples. The proportion of cell necrosis is two to three times that of offshore *Canna indica* and offshore *Acorus calamus*. Although, in an adverse situation, in its rhizosphere soil, the number of bacteria apoptosis processes was almost three times that of necrosis processes. In theory, apoptosis is defined as a controlled type of cell death that can be induced by a variety of physiologic and pharmacological agents [73] while necrosis is the end result of a bioenergetic catastrophe resulting from ATP depletion to a level incompatible with cell survival [74]. The much higher apoptosis process explained that, although in an unfavorable environment, most bacteria underwent naturally physiologic processes during the plant dormancy period and part of them may have experienced necrosis processes on account of the environmental stresses leading to cell survival failure.

In Figure 5a, it illustrates that, in the rhizosphere of ashore *Canna indica*, most of the bacteria underwent apoptosis and necrosis processes, as shown in Figure 5c. In terms of the sample proportion, the proportion of cell necrosis was only less than that of ashore *Acorus calamus* and the proportion of apoptosis was also only less than that of ashore *Acorus calamus*, which is in alignment with the role of decaying roots in supplying carbon substrate nutrients for microorganisms. The proportion of cell necrosis was two to three times of that of offshore *Canna indica* and offshore *Acorus calamus*, almost the same as ashore *Acorus calamus*. In rhizosphere soil, the number of bacterial apoptosis processes was almost three times higher than the number of necrosis processes. This meant that the necrosis of bacteria was not aimed at supporting the rhizosphere effect of the plants, which would provide some nutrients and relatively suitable habitats for plants, but rather to help microorganisms survive in the environment during winter.

In contrast, Figure 5b,d shows that only part of the bacteria went through apoptosis and necrosis processes in the rhizosphere soil of offshore *Canna indica* and offshore *Acorus calamus*. In offshore *Canna indica*, the number of apoptosis processes and necrosis processes was not high. Additionally, the number of bacterial necrosis processes was almost the same as offshore *Acorus calamus*. In offshore *Acorus calamus*, the number of bacteria apoptosis processes was much greater than that of necrosis processes. Additionally, the number of bacteria apoptosis processes was much higher than that of offshore *Canna indica*.

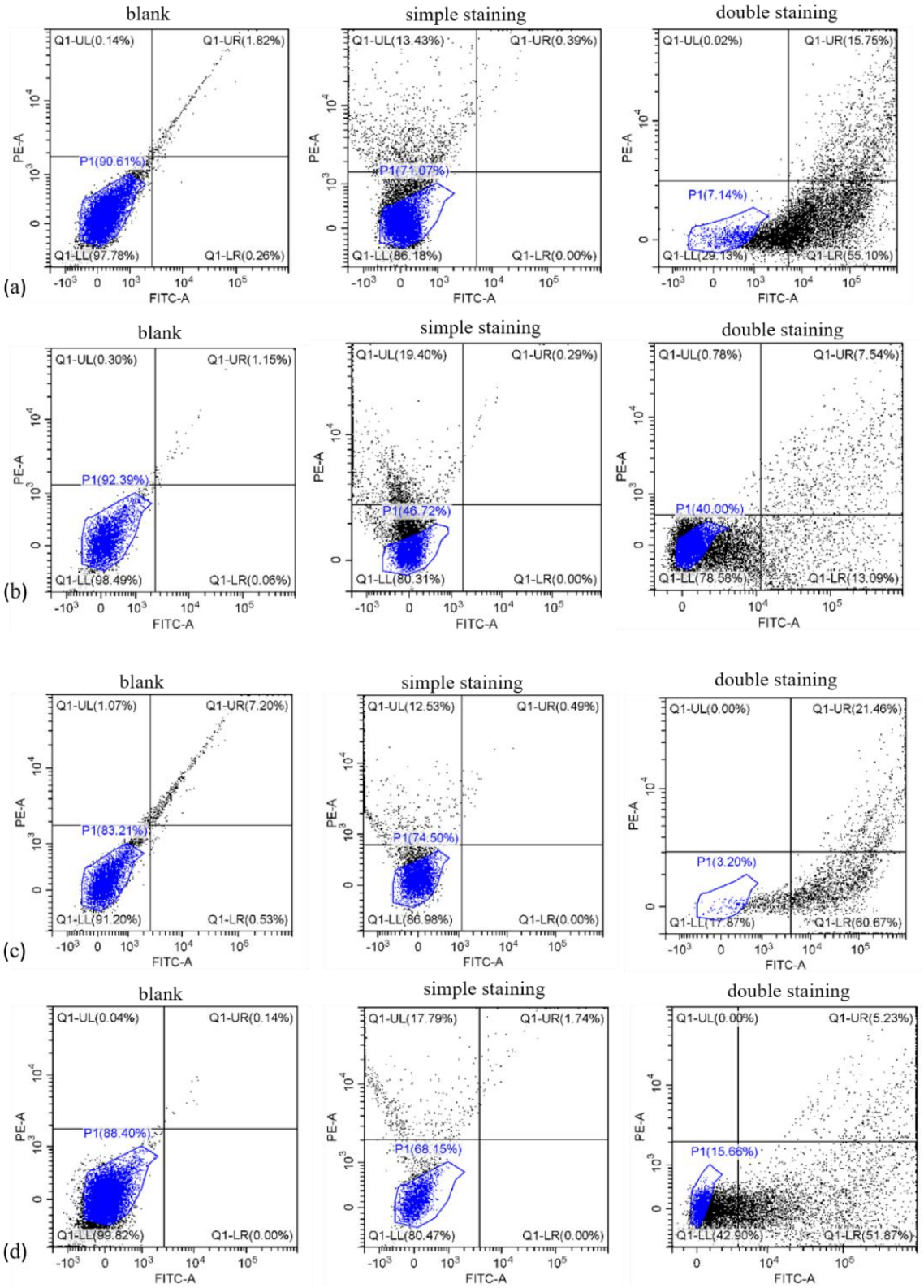

Figure 5. Cell apoptosis analysis for (**a**) ashore *Canna indica*, (**b**) offshore *Canna indica*, (**c**) ashore *Acorus calamus*, and (**d**) offshore *Acorus calamus*. Each of the sectors in the presented diagrams (**a**–**d**) stands for: PI positively and FITC negatively stained cells showing dead cells (upper left), FITC and PI double−stained cells showing late apoptosis (upper right), double−negative cells showing the live cell population (lower left), and FITC positively and PI negatively stained cells showing early apoptosis (lower right).

4. Conclusions

Plants species and their geographic location have a substantial effect on the diversity and structure of microorganisms and their associated populations in plant rhizosphere soils. Especially, during plants' dormancy period, the outer environment and plants' soil micro-environment also have a crucial influence on microbes. To investigate the potential effect of plants' dormancy period on microbes, we used the high-throughput sequencing method along with flow cytometry analysis to explore the microorganisms' physiologic status and its community composition and function. The results showed that the ashore rhizosphere soils, especially in *Canna indica* soils, had great microbial diversity. However, there were relatively fewer N-related genes, which may be due to competition for an ecological niche driven by more available substrates. This indicates that microorganisms removed less nitrogen. The results also showed that the process of cell necrosis made up a relatively lower proportion during plants' dormancy period. This meant that the expected process of mass necrosis of bacteria did not occur. It is most likely that some of the bacteria went through this process during the late period of plant growth.

In conclusion, microbial communities within the riparian plant soils during plants' dormancy period still play a major role in the removal of N-related nutrient and maintain unexpected vitality, which provides insight for the management of riparian zone soils in the winter during plants' dormancy period. It can guide managers through a better planting strategy, instructing them to plant the species *Acorus calamus* and *Canna indica* to improve the environment in the riparian zone throughout the year.

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