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Research Article

Seasonal Succession of Bacterial Communities in the Land-Based Container with Recycling Water Aquaculture System

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The land-based container with recycling water aquaculture system (C-RAS) has gained popularity in China due to its water conservation and pollution reduction capabilities. However, there is a dearth of theoretical research on this subject. In this study, water from Koi-cultured container and three-stage eco-ponds was sampled over three seasons and then the bacterial communities were analyzed. The findings of this study indicate that the implementation of three-stage eco-ponds significantly enhances water quality. Specifically, during the summer season, eco-ponds exhibit the highest removal efficiencies of total nitrogen and total phosphorus in comparison to other seasons. During winter, the bacterial communities' operational taxonomic units, diversity, and richness experience a substantial decline. The system was dominated by *Proteobacteria*, *Bacteroidota*, and *Actinobacteriota* phyla. The lower relative abundance of *Actinobacteriota* and *Cyanobacteria* elucidated the reason for the lower off-flavor compounds in container-cultured fish compared to pond-cultured fish. The bacterial community was significantly influenced by water temperature and pH, as revealed by canonical correspondence analysis. These findings establish a fundamental basis for the development of a healthy and sustainable C-RAS technology.

1. Introduction

Since January 2021, the Yangtze River basin, including the Huaihe River basin, and other significant bodies of water have implemented a comprehensive fishing ban. This has resulted in a significant reduction in fishery production space, aquaculture becomes an alternative to meet the growing demand for fish. However, high-density aquaculture is accompanied by excessive use of water resources, aquaculture emissions, feed waste, and increased costs, making the aquaculture industry now facing the double challenge of improving quality and green development [1, 2]. In recent years, extreme weather events have had a negative impact on aquaculture. Therefore, it is imperative to develop new aquaculture systems to mitigate these challenges. One such system that has gained traction in China is the land-based container with recycling water aquaculture system (C-RAS). This system

offers several advantages, such as water conservation, temperature regulation, reduced disease and drug usage, and removal of heavy metals [3]. The C-RAS system included an intensive recirculated water culture system made up of land-based water pushing containers, wastewater treatment equipment (multistage eco-ponds), a water circulating connection system, and other auxiliary facilities [4].

The C-RAS integrated factory combines recirculating aquaculture and pond engineered recirculating aquaculture, and has been recognized as one of the 10 major leading agricultural technologies by the Ministry of Agriculture and Rural Areas for three consecutive years (2018–2020). This technology exhibits promising potential for practical application. The majority of current research on the C-RAS pertains to the quality of aquatic products. Jia et al. [4] conducted a comparative analysis of the volatile substances, mineral elements, nutritional components, muscle
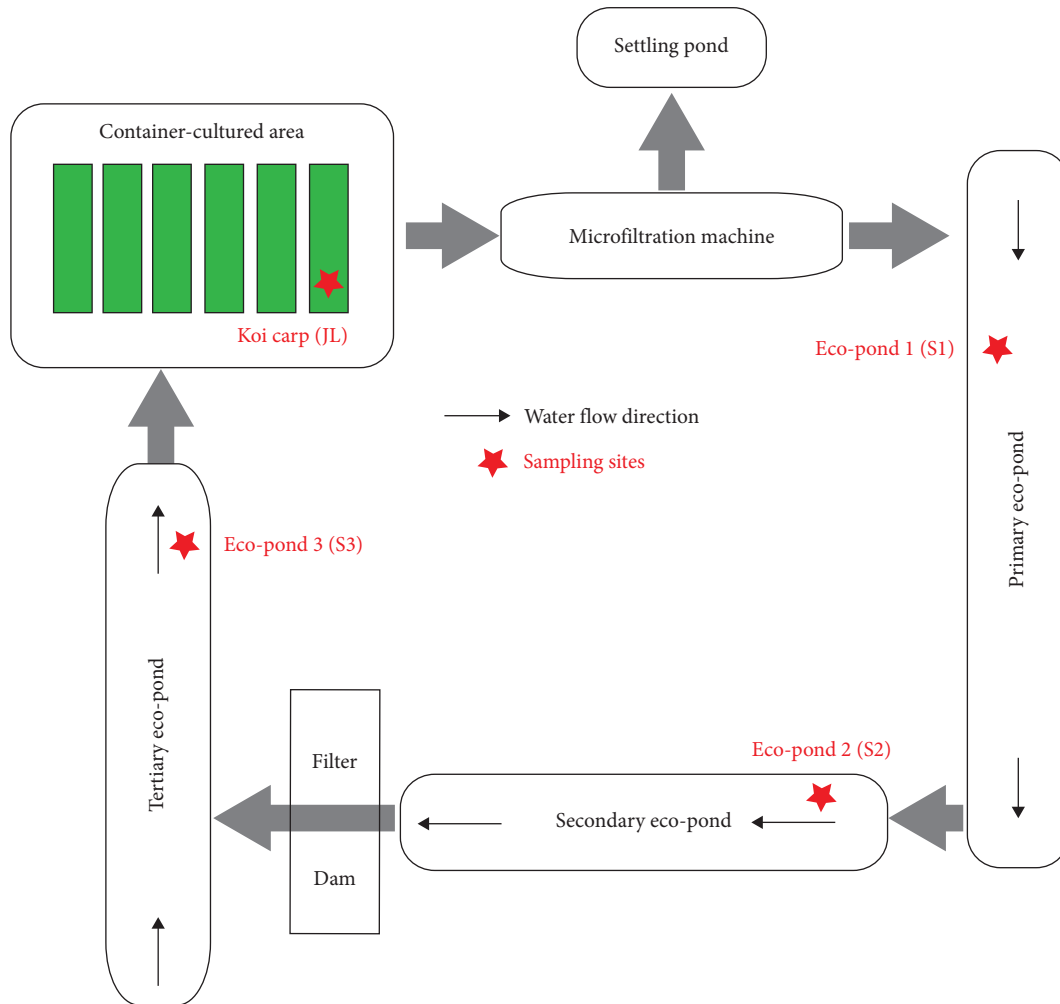
FIGURE 1: Layout of the C-RAS system and the distribution of sampling locations in the present study. JL denoted the breeding Koi containers, while S1, S2, and S3 represented the three distinct eco-ponds.

characteristics, and morphological characteristics of large-mouth bass cultured in container and traditional pond culture systems. Similarly, Zhao et al. [5] investigated and analyzed the water quality conditions and phytoplankton community structure characteristics in container aquaculture water bodies and multistage eco-ponds. However, to date, no studies have explored the bacterial community composition in C-RAS.

The role of microorganisms in pond ecosystems is noteworthy, as they facilitate nutrient cycling, regulate water quality, combat pathogens, preserve host health, and augment biodiversity [6]. Microbial population density and diversity in the water body affect the contaminant removal rate in different units of the ecologically engineered pond system [7]. Consequently, it is imperative to conduct research and enhance the water treatment performance of this system by comprehending intricate microbial communities.

The current investigation utilized high-throughput sequencing of bacterial 16S rRNA genes to evaluate bacterial communities in the water of container and multistage eco-ponds over a 1-year period. The study aimed to achieve three

objectives: (1) assess the efficacy of the system's various units in nutrient removal across different seasons, (2) determine the seasonal progression of bacterial communities in the different units, and (3) elucidate the correlation between bacterial communities and wastewater purification efficiency.

2. Materials and Methods

2.1. The C-RAS Design. The experiments were carried out at Youjiliangzhuang Agro-Technology Co., Ltd., situated in Taihe County of Fuyang City, Anhui Province, China. The experimental system comprised a container breeding area, a microfiltration machine, a settling pond, a primary eco-pond (eco-pond 1), a secondary eco-pond (eco-pond 2), a filter dam, and a tertiary eco-pond (eco-pond 3) (Figure 1). One container body housed approximately 7,000 Koi carp (*Cyprinus rubrofuscus*). Following solid–liquid separation by the microfiltration machine, the container culture wastewater was discharged into the eco-pond 1. The eco-pond 1 exhibited a waterfall at intervals of 50 m and was adorned with a variety of flora, including lotus, water lilies, and iris. The eco-pond 2 was furnished with ecological floating beds

and featured the cultivation of water hyacinths and other floating plants. A dam was erected between the eco-pond 2 and eco-pond 3 to enhance the filtration of impurities. The eco-pond 3 was utilized for the cultivation of silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Hypophthalmichthys nobilis*).

2.2. Sampling and Water Quality Parameters Analysis.
Figure 1 displays the sampling locations. The samples were obtained thrice during autumn (October 2021), winter (February 2022), and summer (July 2022). JL denoted the breeding Koi containers, while S1, S2, and S3 represented the three distinct eco-ponds. On-site measurements of pH, dissolved oxygen (DO), and temperature were conducted using a multi-parameter water quality checker (Professional Plus, YSI, USA). Chemical analysis of all water samples was performed within 24 hr of collection. A polycarbonate membrane (0.22 μm) was used to filter 100 mL of water samples and filters were stored at $-80°C$ refrigerator until DNA extraction and Illumina sequencing. The filtered water was used for chemical analysis, including total nitrogen (TN), total phosphorus (TP), total ammonia nitrogen (TAN), nitrite-nitrogen (NO_2^-- N), and nitrate-nitrogen (NO_3^--N), which were tested with an ultraviolet–visible spectrophotometer following the standard method [8]. All analyses were performed in triplicate. TAN exists in two forms, which are the ammonium ion (NH_4^+-N) and the ammonia molecule (NH3-N). NH3-N had higher toxicity effect on aquatic organisms between the two forms of ammonia [9]. The NH3-N form can be calculated following the Henderson–Hasselbach relationship [10].

$$pKa = 0.09018 + \left(\frac{2,729.92}{273.2 + T} \right), \tag{1}$$

$$NH3\text{-}N = \frac{Tan \times 1}{(1 + 10^{pKa-pH})}, \tag{2}$$

where pKa is the acid dissociation constant and T is the water temperature in °C.

2.3. DNA Extraction, Sequencing, and Data Processing.
The genomic DNA from water samples was extracted by using the E. Z.N.A. Water DNA kit (Omega, USA). The extracted DNA was amplified with the primers 338F and 806R in the V3–V4 hypervariable region of 16S rRNA genes [11]. Sequencing was conducted on an Illumina MiSeq platform, which constructed by Majorbio BioPharm Technology Co. Ltd. (Shanghai, China). Details about DNA extraction, PCR amplification, sequencing, and data processing are provided in the Supplementary Materials. The sequencing data obtained in this study were deposited in the NCBI database with a BioProject accession number PRJNA976078.

2.4. Statistical Analysis.
After assembling and quality filtering, the high-quality reads were clustered into operational taxonomic units (OTUs) using USEARCH 7.0 (https://drive 5.com/uparse/) at a similarity level of 97% [12]. For each representative sequence, the Ribosomal Database Project (https://rdp.cme.msu.edu/) by Bayesian classifier was used for the taxonomic analysis based on the Silva Database (https://www.arb-silva.de) at 70% threshold. All statistical analyses based on the OTUs information were carried out with an online software package (Majorbio cloud, https://cloud.majorbio.com/) developed by Majorbio BioPharm Technology Co. Ltd. Mothur software was used to analyze α diversity, including the Sobs, Shannon, Chao1, and Simpson [13]. At the OTU level, the structural variation of bacterial communities across samples was visualized using the nonmetric multidimensional scaling (NMDS) based on the Bray–Curtis dissimilarity matrix. Canonical correspondence analysis (CCA) was performed to assess the effects of physicochemical factors on the bacterial communities [14]. Spearman's rank correlation was calculated to estimate the relationship between the environmental factors and relative abundances of genera [15].

3. Results and Discussion

3.1. Effect of Ecological Ponds in Treating Wastewater in Different Seasons.
The study aimed to determine the seasonal fluctuations of the primary parameters of water bodies at designated sampling sites (Tables 1 and S1). The results, presented in Table 1, indicate a gradual reduction of physicochemical indicators in the direction of water flow. The pH values of most water samples collected from eco-ponds ranged from 7.75 to 8.52, indicating slight to moderate alkalinity. Nitrogen and phosphorus are the key parameters for assessing eutrophication in aquatic environments, and are also the most prevalent pollutants generated by aquaculture [16]. Both NH3-N and nitrite could be harmful to aquatic organisms. In order to safeguard fishery resources against the deleterious effects of NH3-N and NO_2^-- N, the Chinese government has established a threshold of 0.020 mg/L NH3-N and 0.12 mg/L NO_2^-- N for fisheries [17]. As demonstrated in Table 1, the concentration of NO_2^-- N and NH3-N in the effluent was found to be below the prescribed limit. Consequently, the treated tail water can be reused for fisheries. The highest removal efficiencies of TN and TP in summer (68.17%, 83.04%) compared to other seasons (31.7%, 81.97%; 64.11%, 82.33%), suggesting that temperature is a crucial factor in the removal of nitrogen and phosphorus contaminants in multistage eco-ponds. This result is in accord with the findings of Dong et al. [18], who also reported that summer temperatures favored enhanced microbial activity in constructed wetlands. Moreover, the removal rate of nitrogen and phosphorus contaminants in eco-pond 1 was higher than that in the eco-pond 2 and eco-pond 3 during summer and winter sampling periods. But in autumn, eco-pond 2 showed higher pollutant removal capacity.

3.2. Microbiota Diversity in the C-RAS

3.2.1. α Diversity.
A total of 1,639,952 valid bacterial 16S rRNA gene sequences were obtained from the 24 samples. In order to compare the samples at the same sequencing depth, 32,437 sequences were selected for each sample by random sampling. Figure S1 demonstrates that the sequencing depth was adequate for evaluating the bacterial communities. At the same sequence depth, the observed species and

TABLE 1: Water quality indicators of the multistage eco-ponds and Koi containers in different seasons.

| Season	Sampling sites	Sample ID	TN (mg/L)	TP (mg/L)	TAN (mg/L)	NH3-N (mg/L)	NO_3^--N (mg/L)	NO_2^--N (mg/L)	Removal efficiency (%) TN	TP
Autumn	Koi container	AJL	2.789	0.294	0.507	0.012	0.064	0.066		
	Eco-pond 1	AS1	2.911	0.253	1.274	0.03	0.569	0.256	31.7	81.97
	Eco-pond 2	AS2	1.977	0.098	0.68	0.026	0.689	0.125		
	Eco-pond 3	AS3	1.904	0.053	0.513	0.015	0.421	0.066		
Winter	Koi container	WJL	3.385	0.266	0.975	0.03	0.161	0.266		
	Eco-pond 1	WS1	1.685	0.154	0.62	0.014	0.408	0.068	64.11	82.33
	Eco-pond 2	WS2	1.628	0.061	0.251	0.009	0.285	0.02		
	Eco-pond 3	WS3	1.215	0.047	0.097	0.004	0.092	0.001		
Summer	Koi container	SuJL	4.42	0.283	1.413	0.034	0.127	0.283		
	Eco-pond 1	SuS1	2.312	0.123	0.805	0.019	0.554	0.049	68.17	83.04
	Eco-pond 2	SuS2	2.07	0.064	0.484	0.077	0.181	0.012		
	Eco-pond 3	SuS3	1.407	0.048	0.14	0.018	0.488	0.008		
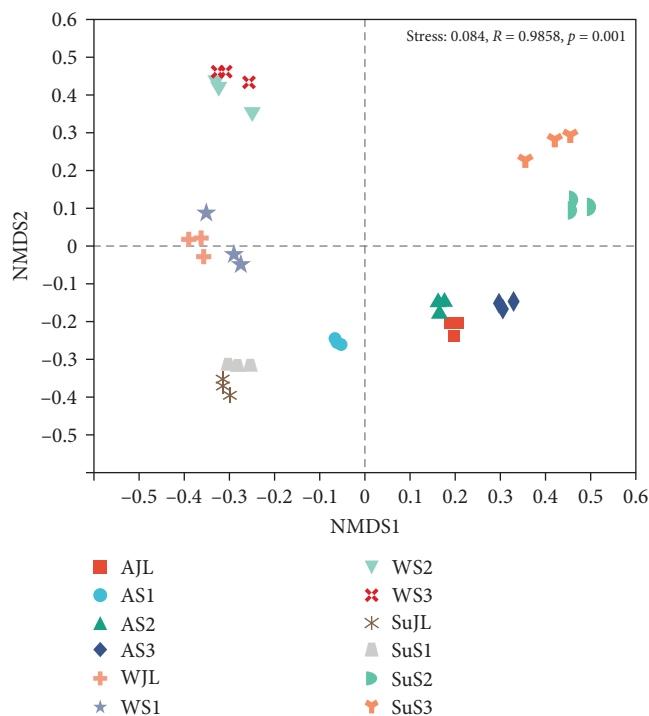
FIGURE 2: Nonmetric multidimensional scaling (NMDS) analysis for the visualization of β diversity in the C-RAS. A, autumn; W, winter; Su, summer. JL, water from containers for breeding Koi; S1, water from the primary eco-pond (eco-pond 1); S2, water from the secondary eco-pond (eco-pond 2); S3, water from the tertiary eco-pond (eco-pond 3).

Chao1 richness estimator ranged from 248 to 1,721 and from 398.50 to 2,639.35, respectively in these samples (Table S2). The Shannon diversity and Simpson diversity indices ranged from 1.53668 to 5.13561 and from 0.01782 to 0.58709, respectively. The autumn season in the eco-pond 1 exhibited the greatest bacterial richness (Chao1) and diversity (Shannon), with significant seasonal variations in these indices suggesting that season is a crucial factor influencing the α diversity of eco-ponds.

3.2.2. β Diversity. β diversity analysis was conducted to examine the differences in bacterial composition among samples, and NMDS analysis based on Bray–Curtis distances was utilized to present the results (Figure 2). Generally, the proximity of two samples on the plot indicates a greater similarity in their compositions. During the autumn season, the multistage eco-ponds exhibited a higher degree of similarity among samples in comparison to other seasons. Specifically, samples obtained from the eco-pond 2 and eco-pond 3 were found to be clustered together, indicating a similarity in bacterial community composition. The NMDS analysis further supported this finding, as samples from the same eco-pond were observed to cluster together, thereby establishing the reliability and reproducibility of the sequencing results.

3.3. Bacterial Community Structure and Composition in the C-RAS. To further investigate the bacterial communities, statistical analysis was conducted on 4,996 OTUs at the phylum level, revealing the identification of 50 phyla among the classifiable sequences. The dominated bacterial phyla (relative

abundance > 1%) account for 97.63%–99.91% of the total community abundance. Among them, *Proteobacteria* was the most abundant members (52.57%), followed by *Bacteroidota* (23.83%), *Actinobacteriota* (12.99%), *Cyanobacteria* (8.57%), and *Verrucomicrobiota* (1.34%) (Figure 3). The rest of the 37 phyla (relative abundance+ < 1% in each sample) were defined as "other phyla" accounting for 0.09%–2.37%. Some bacteria in eco-ponds had a distinct altering rule for their abundance with regard to different seasons. For example, the phylum of *Proteobacteria*, usually had the lowest abundance in summer and the highest abundance in winter, which was similar to a previous report on Lake Taihu [19]. On the contrary, *Bacteroidota* tended to have the lowest abundance in winter, while *Actinobacteriota* tended to have the highest abundance in summer. *Proteobacteria* was the predominant phylum in the eco-ponds, previous studies reported that *Proteobacteria* are closely associated with nitrogen removal, biological phosphorus removal, and organic matter degradation [20, 21]. Therefore, the higher nitrogen and phosphorus contaminants removal efficiency of eco-ponds in winter was presumably due to the high abundance of *Proteobacteria*. The quality of fish in aquaculture systems is significantly diminished by the presence of off-flavor, thereby negatively impacting the economic efficiency of the fishery [22]. Off-flavor is caused by a variety of bacterial communities, such as cyanobacteria, streptomycetes, and myxobacteria [23]. Liu et al. [24] observed that container-cultured carp had lower relative contents of off-flavor compounds compared to pond-cultured carp, but did not provide an environmental explanation for the observed
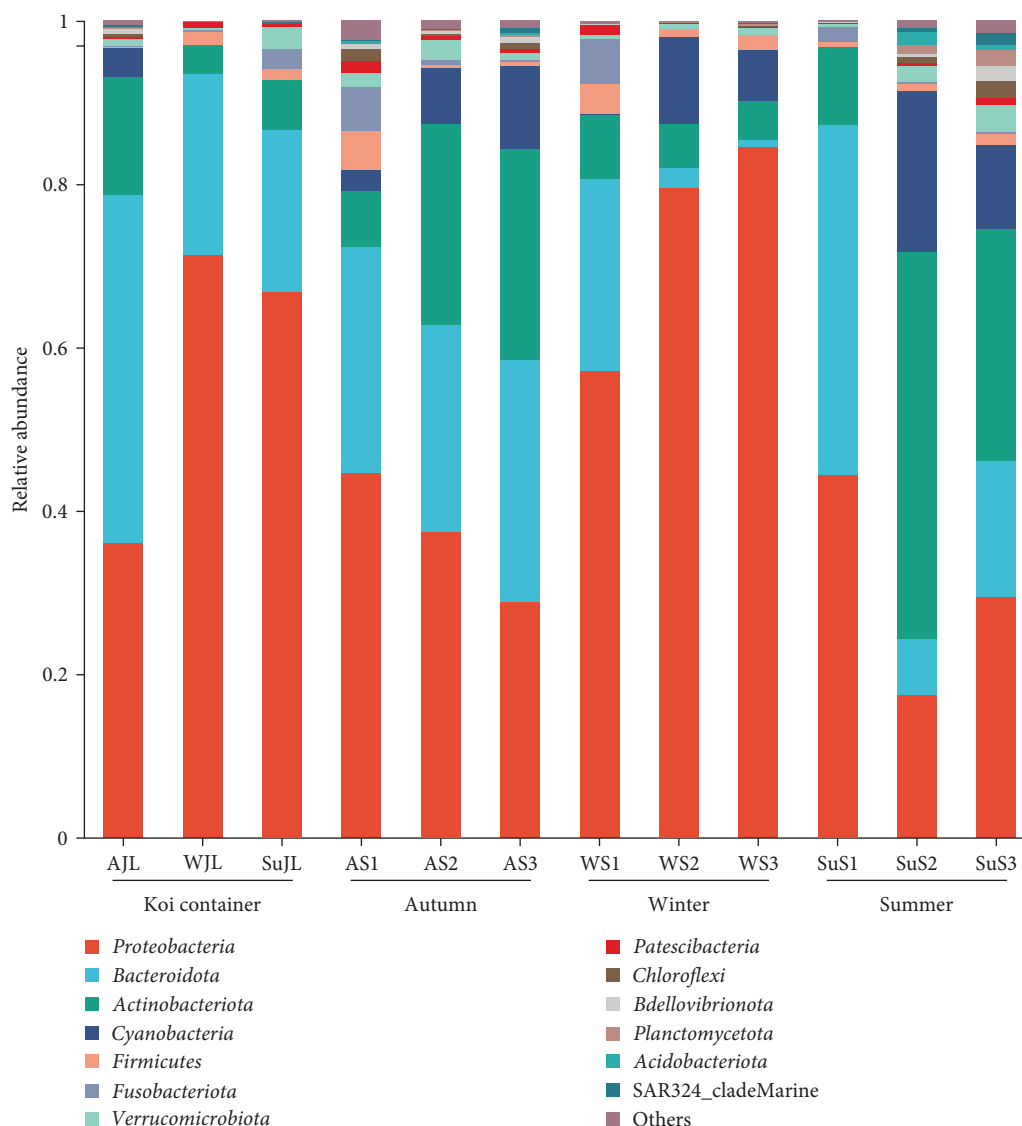
FIGURE 3: Analysis of the relative abundance at the phylum level of the bacterial community in the C-RAS in different seasons.

differences. In the present study, the relative abundance of *Actinobacteriota* and *Cyanobacteria* in Koi containers was observably lower than in eco-ponds (Figure 3), which might explain why container-cultured fish have the less off-flavor compounds.

3.4. Effects of Water Quality Parameters on Bacterial Community Composition. CCA was conducted to investigate the correlations between bacterial communities and environmental factors of the water samples at the OTU level (Figure 4). According to variance inflation factors with 999 Monte Carlo permutations, seven important environmental factors, water temperature (T), DO, pH, TP, TN, NO_2^-- N, and NO_3^--N were selected in the CCA plot. The interpretation rates of the first two axes of CCA sequencing were 19.51% and 16.85%, and the cumulative interpretation rate was 36.36% (Figure 4). In the ordination plot, the length of an environment factors arrow indicated the strength of the

correlation between bacterial community structure and environment factors. Of the aforementioned environmental factors, water temperature ($r^2 = 0.8927$; $p = 0.001$) emerged as the primary determinant of bacterial community dynamics, although other factors also exerted discernible effects. Extensive research has established water temperature as a key driver of bacterial community structure in aquatic ecosystems, owing to its pivotal role in regulating microbial growth and survival [25, 26]. In addition, pH ($r^2 = 0.8739$; $p = 0.001$) also showed a significant impact on the bacterial community variance by CCA. Previous studies have found a significant association between pH and bacterial community variance in wastewater treatment systems [27].

Spearman's correlation test was further conducted to examine the association between the 10 most prevalent genera and environmental factors (Figure 5). The findings revealed a statistically significant negative correlation between *Acinetobacter* and water temperature ($p < 0.001$). *Acinetobacter* strains are known
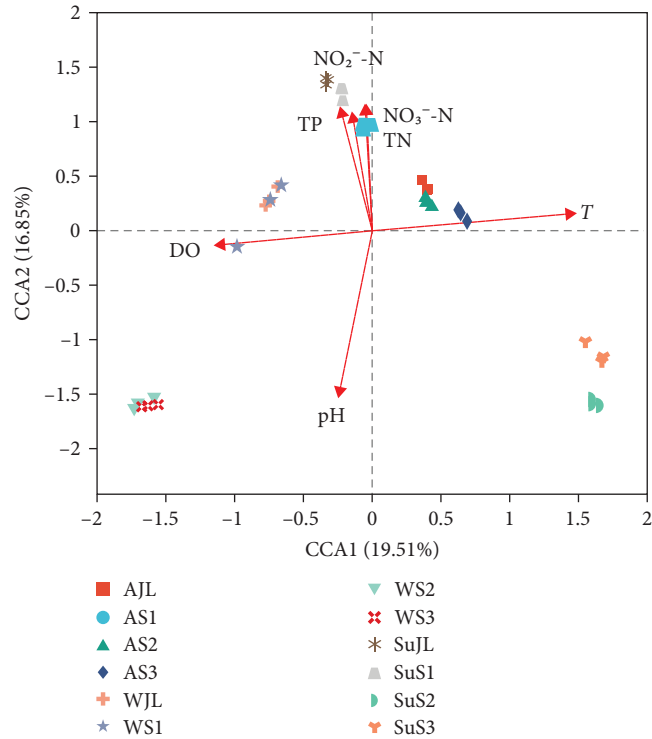
FIGURE 4: Canonical correspondence analysis (CCA) biplot showing bacterial compositions from different samples in relation to environmental variables. DO, dissolved oxygen; TP, total phosphorus; TN, total nitrogen; $NO_2^- - N$, nitrite-nitrogen; $NO_3^- -N$, nitrate-nitrogen; T, water temperature; pH, in the water.
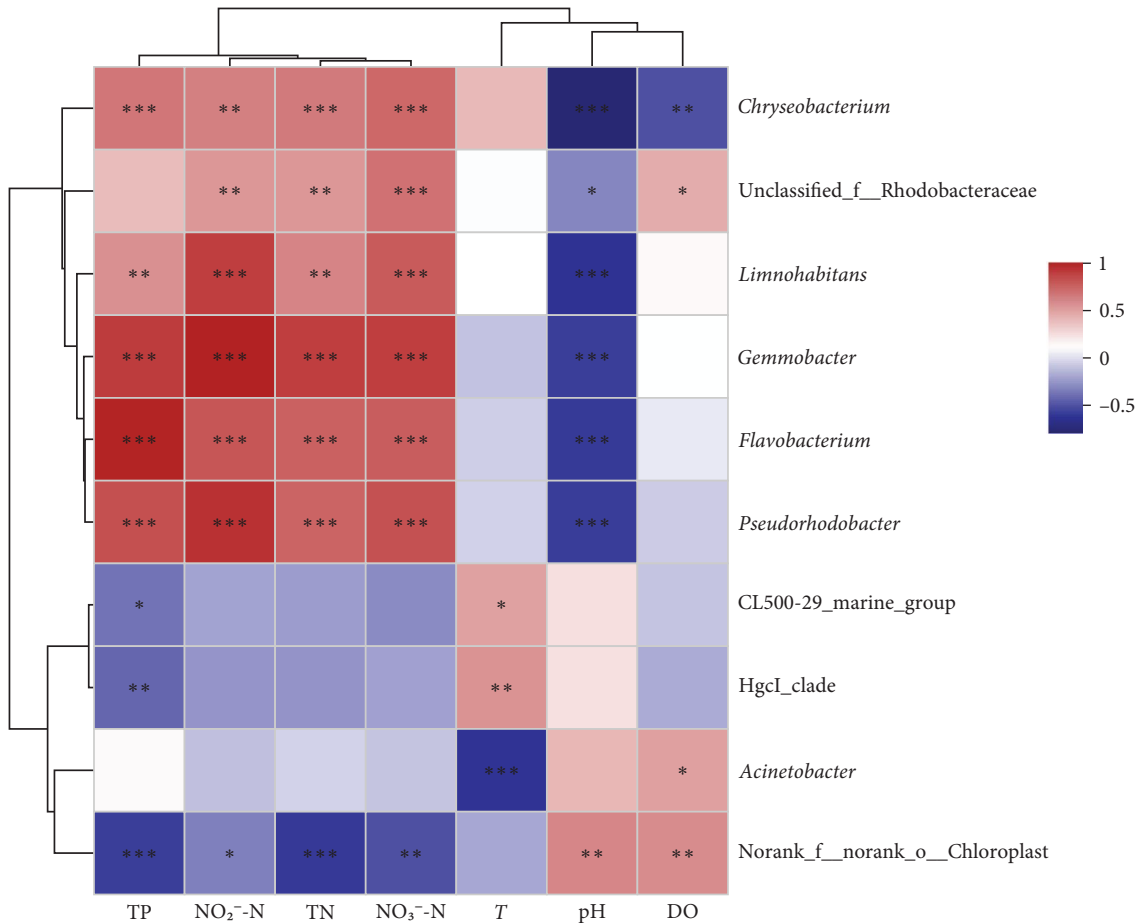

FIGURE 5: A Spearman correlation plot presenting the relationship between microbial abundance and water quality parameters. The relative abundances of the 10 most abundant genera are included. The color of the spots in the right panel represents the R-value for Spearman's correlation for the identified genera and water quality parameters ($^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$).

to thrive in low-temperature conditions and exhibit high nitrogen removal capacity [28]. *Flavobacterium*, *Chryseobacterium*, *Limnohabitans*, *Pseudorhodobacter*, and *Gemmobacter* were positively correlated with nitrogen and phosphorus nutrients ($p < 0.01$ or $p < 0.001$). This suggests that the high concentration of nitrogen and phosphorus nutrients may facilitate their growth. The abundant genera CL500-29_marine_group and hgcI_clade (belong to *Actinobacteriota* phylum) were positively related to water temperature and negatively related to nitrogen and phosphorus nutrients, which was similar to previous findings that the relative abundance of *Actinobacteriota* decreased with the increase of nutrient concentration [29].

4. Conclusions

This study revealed changes in the diversity and composition of bacterial communities in C-RAS over different seasons for the first time. Our results showed that *Proteobacteria*, *Bacteroidota*, and *Actinobacteriota* were the most predominant phyla in the container body and three-stage eco-ponds. The relative abundance of *Proteobacteria* was highest in winter, while the relative abundance of *Bacteroidota* was highest in autumn. The lower relative abundance of *Actinobacteriota* and *Cyanobacteria* in Koi containers explained why container-cultured fish have the less off-flavor compounds. CCA shows that temperature and pH are the principal factors that drive the dynamics of bacterial community diversity and relative abundance in the C-RAS.

Data Availability

All data generated or analyzed during this study are included in this published article. The 16S rRNA gene sequences obtained in this study have been submitted to the NCBI SRA database under accession numbers PRJNA976078.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Wenxuan Lu, Na Gao, and Jinliang Chen designed the experiments. Na Gao, Yize Wang, Yangyang Liang, and Ting Fang did the data curation and formal analysis. Na Gao wrote the original manuscript, Wenxuan Lu and Kai Cui reviewed and edited the manuscript. All authors have read and approved the manuscript.

Acknowledgments

Supplementary Materials

Material and Methods: details about DNA extraction, PCR amplification, sequencing, and data processing. Table S1: water quality indicators of the multistage eco-ponds and Koi containers in different seasons. Figure S1: rarefaction curves of operational taxonomic units of the 36 samples. A: autumn; W: winter; Su: summer. JL: water from Containers for breeding Koi; S1: water from the primary eco-pond (eco-pond 1); S2: water from the secondary eco-pond (eco-pond 2); S3: water from the tertiary eco-pond (eco-pond 3). Table S2: the seasonal α-diversity indices of the bacterial community of the multistage eco-ponds and Koi containers. (*Supplementary Materials*)

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