

## Enhanced aquaculture wastewater treatment using water spinach (*Ipomoea aquatica* Forsskal) and exogenous compound bacteria

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### ABSTRACT

Bioremediation technology has been widely recognized as a sustainable treatment method for purifying aquaculture wastewater. We established two types of ecological floating beds, one with water spinach (WS) only and another with water spinach combined with exogenous compound bacteria (WS-ECB) for the treatment of the Pacific white shrimp aquaculture wastewater of low-salinity. The exogenous compound bacteria used in this study was composed of *Rhodospseudomonas palustris* and *Bacillus subtilis* in a certain proportion. Microbial community dynamics were analyzed by using high-throughput sequencing of 16S rRNA and water quality was determined. Both two treatments were effective in the remediation of aquaculture wastewater. The removal efficiencies of COD<sub>Mn</sub>, TN and TP in the WS group were 34.33 %, 66.54 % and 73.01 %, respectively, and the removal efficiency of NH<sub>4</sub><sup>+</sup> in the WS-ECB group was 93.79 %. Furthermore, the addition of water spinach and exogenous compound bacteria increased the abundance of some bacteria that are involved in regulating water quality and altered the succession of microbial communities. The microbial communities were shaped by both stochastic processes (including limited dispersal) and deterministic processes (including environmental conditions and biotic interactions). Importantly, stochastic processes dominated the assembly of both water and rhizosphere microbial communities in different treatments, while deterministic processes contributed more to the rhizosphere microbial community in the WS-ECB than in the WS. Overall, our study emphasizes that plant and microbial remediation was a potential and effective approach to remove the nutrients of water, and clarifies the bacterial community dynamics and assembly mechanisms during aquaculture wastewater treatment processes.

### 1. Introduction

Presently, the global shrimp farming industry is developing rapidly. Pacific white shrimp (*Penaeus vannamei*) has become the first choice for low-salinity inland farming in many countries [1], but the increasing production of aquaculture waste has caused many environmental problems. Unconsumed feeds and feces of aquatic organisms in aquaculture water bodies generate large quantities of suspended particulate matters and N and P pollutants. Continued accumulation of the discharged wastewater in the environment can negatively impact the surrounding aquatic ecosystems [2–4]. Various methods have been applied to aquaculture wastewater treatment in order to reduce the concentration of eutrophic wastewater discharged into the aquatic environment.

Traditional physicochemical treatments including adsorption, advanced oxidation and membrane separation technologies have been widely used in aquaculture wastewater treatment [5]. In recent years, bioremediation treatments have been favored for their low cost, low energy consumption, and environmental friendliness, which utilize plants, bacteria, and their synergistic effects to remediate contaminated water.

Phytoremediation is the remediation of contaminated environments through adsorption and uptake by plants and the microorganisms associated with them, which has the advantage of being safe and sustainable [6]. A variety of artificial wetlands have been created to alleviate the eutrophication of water bodies, and impressive outcomes have been obtained [7,8]. Ecological floating beds (EFBs) is an ecological technique developed from artificial wetlands that improves water

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quality by cultivating aquatic or terrestrial plants on floating beds at the water surface [9,10]. Aquatic macrophytes can effectively reduce the concentration of pollutants in water, but some macrophytes, such as water hyacinth, may lead to species invasion during the restoration of water bodies [11–13]. In many studies, water spinach (*Ipomoea aquatica* Forsk.) has a superior ability to absorb nitrogen and phosphorus and other pollutants in water, which may be attributed to its developed root system [14]. Moreover, the by-product of water spinach produced by the planted floating-bed can be harvested at regular time intervals, which could achieve good economic benefits as vegetable and animal feed [15,16]. In aquaculture, microorganisms play a multifaceted role in maintaining water quality and managing waste [17]. *Bacillus* species have demonstrated great ability in the maintenance of water quality in aquaculture. On the one hand, it optimizes the rate of nitrification and denitrification to promote the purification of aquaculture water, while on the other hand, it contributes to the population of beneficial microbiota in the aquatic environment, thus promoting the decomposition of pollutants in the water [18]. Similarly, photosynthetic bacteria are capable of absorbing nitrite nitrogen and nitrate nitrogen, and the use of photosynthetic bacteria has been a common practice in aquaculture wastewater treatment [19]. Numerous studies back up the use of microorganisms in wastewater remediation. For instance, *Bacillus* regulates a variety of water quality parameters in wastewater from aquaculture, and a complex made of highly effective ammonia-oxidizing bacteria demonstrated good nitrogen and ammonia degradation in wastewater [20–22]. The synergistic remediation of plants and microorganisms in wastewater overcomes the limitations of using them alone. The strong root system of plants attracts microorganisms to colonize, and the microorganisms mineralize and decompose organic matter in the water to produce nutrients for plant absorption [23]. Exogenous compound bacteria (ECB) refers to the mixture of two or more non-antagonistic strains in a certain proportion [22]. In addition, ECB can expand the range of substrate utilization under specific environment and achieve a desirable treatment effect compared to a single microbial agent [24]. It has been reported that the removal of ammonia nitrogen and total nitrogen from wastewater was improved by the combination of *Bacillus subtilis* and *Rhodospseudomonas palustris* [25]. The growth of *B. subtilis* may contribute to the nitrogen fixation by *R. palustris* during the co-culture process of the two strains [26]. *R. palustris* and *B. subtilis* are also two environmental probiotics used in aquaculture wastewater. *R. palustris* can convert the small organic molecules into nutrients, but it can't decompose large molecular organic matter in aquaculture water [19]. While *B. subtilis* can secrete digestive enzymes to digest some organic matter [25,27]. In addition, *R. palustris* and *B. subtilis* are considered as potential plant growth-promoting bacteria and widely used as beneficial microbial inoculums of crops [28]. Our preliminary experiments also showed that ECB (including *R. palustris* and *B. subtilis*) could efficiently remove the pollutants by adding the ECB in the aquaculture wastewater (author's unpublished data). Therefore, it is promising to combine ECB with ecological floating beds to purify aquaculture wastewater. Current study on the remediation of wastewater by plants in combination with exogenous compound bacteria focuses on evaluating the removal of pollutants during the remediation process [29]. Biological processes involving microorganisms are also important steps in the removal of pollutants, and microbial intervention can accelerate nutrient cycling and facilitate the removal of nitrogen and phosphorus pollutants by plants [30]. Therefore, an in-depth understanding of the dynamics of the complex community composition of microorganisms and their interactions with environmental factors is imperative for the bioremediation of wastewater [31]. It is worth noting that due to the unstable effect of ECB, one of the main limitations for aquaculture wastewater treatment with ECB is to ensure their retention inside aquaculture systems [32]. In this study, we regularly added ECB to ensure the water restoration efficiency, but the cost should be taken into account in remediation of aquaculture wastewater.

In this study, water spinach was used as floating bed plant, and the

compound of *R. palustris* and *B. subtilis* was used as ECB to set up ecological floating bed for the treatment of low-salinity aquaculture wastewater. We determined water quality indicators and collected microbial samples of water and rhizosphere during a 20-day experimental period. 16S rRNA high-throughput sequencing was used to assess microbial community dynamics and community aggregation patterns in water and rhizosphere. We aimed to: (1) evaluate the effect of water spinach and water spinach combined with exogenous compound bacteria on the removal of nutrients in aquaculture wastewater; (2) explore the differences in composition and diversity of microbial communities in water and rhizosphere between different treatments; (3) analyze the community assembly patterns of water and rhizosphere microbial communities in different treatments and control groups. These results will provide some support for revealing the potential of plant and exogenous complex bacteria to repair aquaculture wastewater.

## 2. Materials and methods

### 2.1. Ecological floating beds description and the experiment design

This study was carried out at the Pilot Test Base of Ningbo University at Meishan, and wastewater was collected from the aquaculture systems of pacific white shrimp (*Penaeus vannamei*) with the salinity of 5‰–7‰. After 2 weeks of pre-culture in a plastic bucket containing aquaculture wastewater, water spinach with relatively uniform characteristics were selected for the experiment. The experimental device used nine transparent plastic boxes with the size of 45 × 31 × 27 cm. 20 L aquaculture wastewater was added to each plastic box. The experiment consisted of three groups with three replicates each: (1) Water spinach treatment group (WS); (2) Water spinach combined with exogenous compound bacteria treatment group (WS-ECB); (3) Control group (Control). A styrofoam floating bed with eight holes was put in each plastic box. About 80 g of water spinach were evenly transplanted to each styrofoam floating bed of the WS group and WS-ECB group. The water spinach was planted in each box with a planting density of 114 plants/m<sup>2</sup>. The experiment was conducted in a greenhouse to ensure equal light and temperature conditions for all boxes. Micro-aeration was performed for each treatment during the experiment. The aeration device used in the experiment is an electric aerator (flow rate 75 L/min). The rubber aeration hose, diverter and air stone were assembled and then connected to the electric aerator to ensure consistent micro-aeration conditions for each box. The water was replaced by 20 % every 2 days to simulate the flow and exchange of water bodies during the operation of aquaculture tail water treatment system. Water exchanges in this study resulted in the loss of ECB in the water. So, we added ECB to the WS-ECB group by 2 ‰ of water volume every 4 days to ensure the amount of exogenous complex bacteria in the water. The compound bacteria used in the experiment were *R. palustris* (OD<sub>600</sub> = 0.6) and *B. subtilis* (OD<sub>600</sub> = 1.2) in the exponential growth period, which were diluted 10 times and 100 times, respectively, and then compounded according to the volume ratio of 1: 3. The experiment lasted for 20 days to simulate the wastewater discharge cycle during shrimp farming.

### 2.2. Water sample treatment and analysis

500 mL water samples for the determination of physical and chemical indexes were collected on day 0 (D0), day 4 (D4), day 8 (D8), day 12 (D12), day 16 (D16) and day 20 (D20), respectively. Unfiltered water samples were used directly in the chemical oxygen demand (COD<sub>Mn</sub>), total nitrogen (TN) and total phosphorus (TP) analysis, and water samples for ammonia nitrogen (NH<sub>4</sub><sup>+</sup>-N), nitrate nitrogen (NO<sub>3</sub><sup>-</sup>-N), nitrite nitrogen (NO<sub>2</sub><sup>-</sup>-N) and active phosphate (PO<sub>4</sub><sup>3-</sup>-P) were filtered through 0.45 μm mixed cellulose ester membrane.

Water quality parameters except COD<sub>Mn</sub> were measured using an ultraviolet-visible spectrophotometer (UV-5500) at specific wavelengths. The COD<sub>Mn</sub> concentration was determined by the acidic

potassium permanganate oxidation method, and the specific reagents used in this method were sodium oxalate and potassium permanganate. TN and TP were determined by simultaneous digestion method, and the specific reagents used in this method were potassium persulfate, boric acid and sodium hydroxide.  $\text{NH}_4^+$ -N was determined by the sodium hypobromite oxidation method, and the specific reagents used in this method were potassium bromate, potassium bromide sulfonamide and N-1-naphthylethylenediamine dihydrochloride.  $\text{NO}_2^-$ -N was measured by the diazo-coupling spectrophotometry method, and the specific reagents used in this method were sulfonamide, hydrochloric acid and N-1-naphthylethylenediamine dihydrochloride.  $\text{NO}_3^-$ -N was measured by the zinc-cadmium reduction method, and the specific reagents used in this method were sulfonamide, N-1-naphthylethylenediamine dihydrochloride, chromic chloride and zinc foils. Meanwhile,  $\text{PO}_4^{3-}$ -P was measured by Mo—Sb anti spectrophotometric method, and the specific reagents used in this method were sulfuric acid, ammonium molybdate, antimony potassium tartrate and ascorbic acid. The unit of each parameter is mg/L. Pure water was used as the analysis blank, and a duplicate experiment was performed twice of per sample.

### 2.3. Sample collection, DNA extraction, PCR amplification and sequencing

Water and root microorganism samples were collected every 4 days. 100 mL water sample was filtered by a filter membrane with a pore size of 0.22  $\mu\text{m}$ . 1 g roots were collected with sterile scissors and tweezers and placed in a sterile centrifuge tube. The filter membranes of water samples were immediately transferred to a  $-80^\circ\text{C}$  freezer. And root samples in centrifuge tubes were temporarily stored in an incubator containing ice packs and then transferred to a  $-80^\circ\text{C}$  freezer within 1 h. Then the filter membranes and root samples were stored at  $-80^\circ\text{C}$  for subsequent microbial sequencing. Sterile water was used as negative control during sample processing and sequencing. The V3-V4 region of 16S rRNA was amplified and high-throughput sequencing was performed using Illumina Miseq high-throughput sequencing platform of Guangzhou Meige Gene Company. The primers used for amplification of the 16S rRNA gene were 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Primer pair 338F/806R can identify the taxa present effectively, but there are also some missing taxa. For example, the 16S rRNA genes of some specific taxa have poor complementarity with primers which will lead to underestimation of these taxa in a microbial community [33]. USEARCH version 11.0.667\_I8 was used to conduct subsequent bioinformatics processing on the sequencing data. To reduce the effects of the low-quality reads and chimeric sequences, the UNOISE3 algorithm (unoise\_alpha = 2, minsize = 8, according to default settings) was used to denoise. After quality filtering, the remaining sequences were clustered into zero-radius Operation Taxon Units (ZOTUs). The Ribosomal Database Project (RDP) Classifier algorithm was applied to classify and annotate the representative operation classification units [34]. All raw sequences from this study have been submitted to the NCBI Sequence Read Archive (SRA) database under the BioProject accession number PRJNA1056332 (BioSample accessions: SAMN39097944-SAMN39098024).

### 2.4. Statistics analysis

The low-abundance taxa (abundance < 0.01 %) were abandoned and then normalization of data was performed (11,523 OTUs in each sample). Subsequent analyses of  $\alpha$ -diversity and  $\beta$ -diversity were all performed based on this normalized data. Physical and chemical indexes of water body and  $\alpha$ -diversity index of bacterial community were pre-processed using Microsoft Excel 2016 and analyzed with SPSS (version 20.0). A one-way ANOVA and Tukey multiple comparisons test were conducted to compare the significant differences ( $P < 0.05$ ) among treatments.

In ecological studies, Bray-Curtis similarity matrix has been widely

employed to calculate  $\beta$  diversity of bacterial communities and is considered to be one of the most robust similarity coefficients [35,36]. A Bray-Curtis similarity matrix was constructed based on Hellinger's transformed microbial sequence matrix using the "vegan" package in R. Non-metric multidimensional scaling analysis (NMDS) was performed to visualize differences in the composition of the bacterial communities, and a permutation multivariate analysis of variance (PERMANOVA) was performed using the adonis() function in the "vegan" package to quantify the significant effects of sampling time and treatment on the variation of the bacterial communities. Linear discriminant analysis and effect size (LEfSe) was performed to identify the significant taxa. In this study, the alpha value for the non-parametric factorial Kruskal-Wallis sum-rank test was 0.05, and the threshold for the linear discriminant analysis (LDA) score for biomarkers was 4.5. The detrended correspondence analysis (DCA) supports the choice of redundancy analysis (RDA) to explore the correlation between environmental factors and microbial community structure. Prior to the RDA, forward selection for environmental factors was performed using ordistep(). And the vif.cca() function in the "vegan" package was used to remove environmental factors with strong multicollinearity [37]. Then RDA and Mantel test analyses were carried out to uncover the relationship and correlations between environmental factors and microbial community structure using "vegan" package in the R environment.

The beta nearest taxon index ( $\beta\text{NTI}$ ) was calculated using the "picante" package to quantify the relative contributions of stochastic and deterministic processes to the bacterial community assembly process. In addition, a neutral community model (NCM) was used to fit the relationship between the frequency of OTUs occurrence and its relative abundance in bacterial communities to assess the importance of neutral processes for bacterial community construction. The neutral model was performed by employing the "stats4", "minpack.lm", and "Hmisc" packages in R software.  $R^2$  was used to assess the fit of the model, and the community construction process was considered to conform to the neutral model when  $0 < R^2 < 1$ . Use the "ggplot2" package in R software to build the above graphs.

## 3. Results

### 3.1. Variation of physical-chemical parameters

After 20 days of experiment, the nutrient concentrations in the treated water were lower than initial levels, and the removal of  $\text{COD}_{\text{Mn}}$ , TN, TP, and  $\text{PO}_4^{3-}$  in WS and WS-ECB were higher than that of control group (Table 1,  $P < 0.05$ ). The removal rate of  $\text{COD}_{\text{Mn}}$  in WS was 34.11 %, which was twice that of control group. No significant difference in  $\text{NH}_4^+$  concentration was observed between the three groups after treatment. The concentration of ( $\text{NO}_3^- + \text{NO}_2^-$ )-N was lower than the initial concentration, but the removal rate of WS and WS-ECB was lower than control group (Table 1).

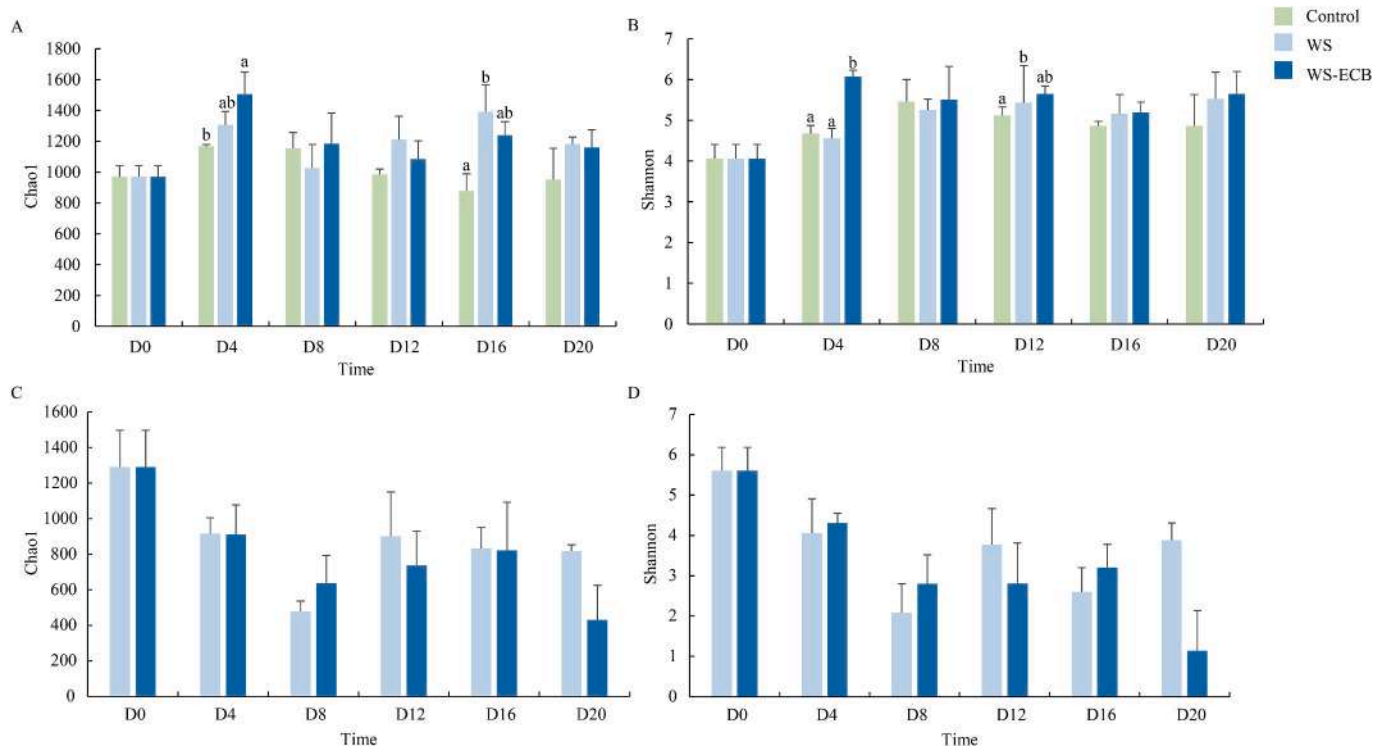
### 3.2. $\alpha$ -Diversity of microorganisms in water and rhizosphere

16S rRNA high-throughput sequencing was performed on water and rhizosphere microbial samples, and a total of 5110 OTUs were obtained. Chao1 index and Shannon index were used to evaluate community richness and diversity (Fig. 1). In the water samples (Fig. 1A, B), the Chao1 index of the WS-ECB group was significantly higher than that in the control group at D4 ( $P < 0.05$ ), and the Chao1 index of the WS group was significantly higher than that in the control group at D16 ( $P < 0.05$ ). With the progress of the experiment, the Shannon index of all water samples gradually increased, and the Shannon index of the WS-ECB group was significantly higher than that of control group and WS group at D4 ( $P < 0.05$ ), while the Shannon index of the WS group was significantly higher than control group at D12 ( $P < 0.05$ ). However, no significant difference in Chao1 index and Shannon index between different groups in rhizosphere samples during the whole experiment

**Table 1**  
Concentration of nutrients in aquaculture wastewater before and after treatment (Mean  $\pm$  SD, mg/L).

Parameters (mg/L)	Untreated	Control	WS	WS-ECB	Removal efficiency (%)		
					Control	WS	WS-ECB
COD <sub>Mn</sub>	19.00 $\pm$ 0.38 <sup>a</sup>	15.68 $\pm$ 0.38 <sup>b</sup>	12.52 $\pm$ 0.74 <sup>c</sup>	12.80 $\pm$ 0.49 <sup>c</sup>	17.47 %	34.11 %	32.63 %
TN	8.00 $\pm$ 0.17 <sup>a</sup>	3.95 $\pm$ 0.04 <sup>b</sup>	2.68 $\pm$ 0.15 <sup>d</sup>	3.03 $\pm$ 0.05 <sup>c</sup>	50.60 %	66.54 %	62.15 %
TP	2.46 $\pm$ 0.06 <sup>a</sup>	0.96 $\pm$ 0.07 <sup>b</sup>	0.66 $\pm$ 0.07 <sup>c</sup>	0.86 $\pm$ 0.04 <sup>c</sup>	60.8 %	73.01 %	65.12 %
(NO <sub>3</sub> <sup>-</sup> + NO <sub>2</sub> <sup>-</sup> )-N	1.32 $\pm$ 0.08 <sup>a</sup>	0.23 $\pm$ 0.01 <sup>c</sup>	0.29 $\pm$ 0.02 <sup>c</sup>	0.46 $\pm$ 0.04 <sup>b</sup>	82.58 %	78.03 %	65.15 %
PO <sub>4</sub> <sup>3-</sup> -P	0.71 $\pm$ 0.05 <sup>a</sup>	0.36 $\pm$ 0.02 <sup>b</sup>	0.14 $\pm$ 0.01 <sup>c</sup>	0.14 $\pm$ 0.01 <sup>c</sup>	49.46 %	80.69 %	80.28 %
NH <sub>4</sub> <sup>+</sup> -N	2.23 $\pm$ 0.08 <sup>a</sup>	0.38 $\pm$ 0.04 <sup>b</sup>	0.27 $\pm$ 0.02 <sup>b</sup>	0.14 $\pm$ 0.03 <sup>b</sup>	82.89 %	88.01 %	93.79 %

Note: Different letters indicate a significant difference between different treatments in the same row ( $P < 0.05$ ). Control: control group, WS: water spinach group, WS-ECB: water spinach - exogenous compound bacteria group.



**Fig. 1.**  $\alpha$ -Diversity index. (A) Chao1 index of water samples. (B) Shannon index of water samples. (C) Chao1 index of rhizosphere samples. (D) Shannon index of rhizosphere samples. The difference of different letters in the same period was significant ( $P < 0.05$ ). Control: control group, WS: water spinach group, WS-ECB: water spinach - exogenous compound bacteria group.

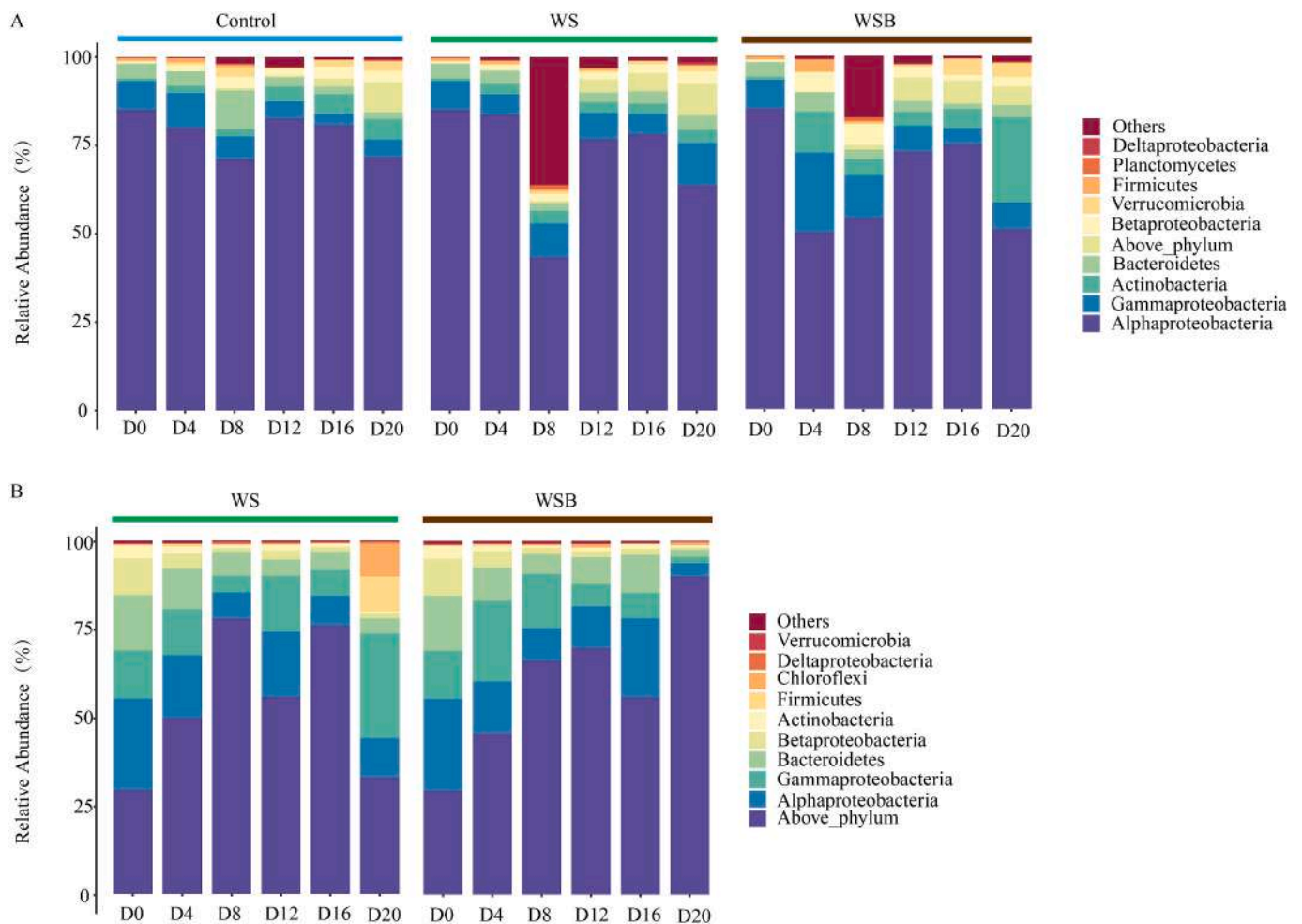
(Fig. 1C, D).

### 3.3. Dynamic of dominant bacterial in water and rhizosphere

The OTU was divided into phyla and class levels, and the proportion of water and rhizosphere samples varied between different treatments and different periods. In the water samples, Alphaproteobacteria accounted for the largest proportion in the three groups in all sampling periods, and the average relative abundance accounted for 78.62 % (Control), 71.85 % (WS) and 64.71 % (WS-ECB), respectively. The proportion of Alphaproteobacteria decreased to 43.37 % in WS at D8, while the proportion of Alphaproteobacteria in WS-ECB increased at D4 and D8 (D4: 50.11 %; D8: 54.17 %). The average relative abundance of Alphaproteobacteria in root samples during the whole experiment period was 14.77 % (WS) and 14.61 % (WS-ECB), respectively (Fig. 2A). In the root samples, the abundance of Gammaproteobacteria in WS first decreased and then increased to 29.52 % at the end of the experiment, and the abundance of Gammaproteobacteria in WS-ECB first increased and then decreased (Fig. 2B).

In terms of  $\beta$ -diversity of bacterial communities, multivariate

analysis of variance showed that sampling time and treatment methods had significant effects on bacterial community structure in water and rhizosphere. Sampling time explained the largest overall variation in bacterial community composition ( $R^2 = 0.514$ ,  $P < 0.05$ , PERMANOVA; Table 2), treatment methods also significantly affected bacterial community structure in water ( $R^2 = 0.095$ ,  $P < 0.05$ , PERMANOVA; Table 2). During the same time period, with a few exceptions, the sample is usually clustered together. For rhizosphere samples, the bacterial community structure was greatly affected by sampling time ( $R^2 = 0.286$ ,  $P < 0.05$ ; PERMANOVA; Table 2). In addition, we also noted significant synergistic effects of sampling time and treatment on bacterial community composition (Water:  $R^2 = 0.145$ ,  $P < 0.05$ ; Root:  $R^2 = 0.169$ ,  $P < 0.05$ ; PERMANOVA; Table 2). To further understand the dynamic process of bacterial community, NMDS ordination analysis was performed (Fig. 3). Based on the Bray-Curtis similarity between the microbial communities in water, the samples from the control group could be divided into 2 periods (period 1: day 0–4; period 2: day 8–20), whereas the samples from the WS group and the WS-ECB group were divided into the same 3 periods (period 1: day 0–4; period 2: day 8; period 3: day 12–20). PERMANOVA results showed no significant difference between



**Fig. 2.** Relative abundances of the dominant bacterial taxa at phylum/class levels. (A) Relative abundances of the dominant bacterial taxa in water. (B) Relative abundances of the dominant bacterial taxa in rhizosphere. The predominant phylum of Proteobacteria is grouped at the class level. Control: control group, WS: water spinach group, WS-ECB: water spinach - exogenous compound bacteria group.

**Table 2**  
Quantitative effects of sampling time and treatment on differences in bacterial community structure-PERMANOVA analysis based on adonis function.

	Time		Treatment		Time: Treatment	
	R <sup>2</sup>	P	R <sup>2</sup>	P	R <sup>2</sup>	P
Water	0.514	0.001	0.095	0.001	0.145	0.001
Root	0.286	0.001	0.026	0.295	0.169	0.013

the control group and the two treatment groups in period 1. The WS group ( $P = 0.0056$ ) and the WS-ECB group ( $P = 0.007$ ) were significantly different from the control group in period 2. The WS group was significantly different from the WS-ECB group ( $P = 0.007$ ) in period 3. Based on the Bray-Curtis similarity between the rhizosphere microbial communities, both the WS group (period 1: day 0–16, period 2: day 20) and the WS-ECB group (period 1: day 0, period 2: day 4–20) were divided into two periods. A significant difference ( $P < 0.05$ ) between the two groups was observed in period 2.

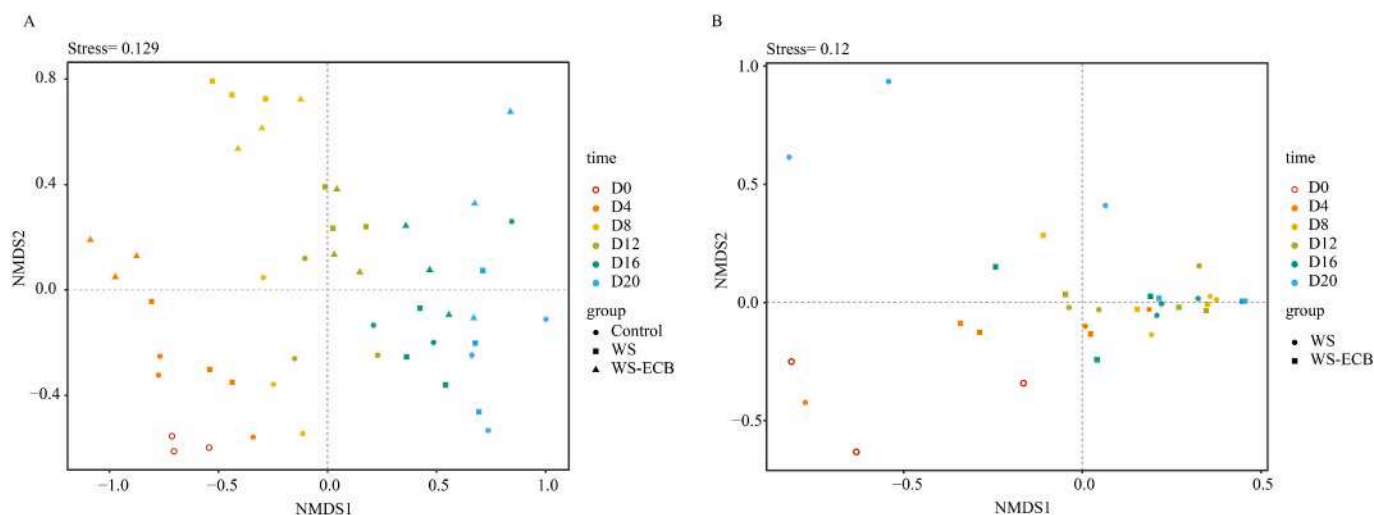
LefSe was used to analyze the distribution of taxa with statistical significance at genus level in different groups of water and rhizosphere samples (Fig. 4). In the water samples, *Lewinella*, *Halomona*, *Sphingobium*, *Hydrogenophaga*, *Caulobacter* and *Rhizobium* were the potential biomarkers of the WS group. *Reyranella* and *Mycobacterium* were potential biomarkers in WS-ECB group in water. Among rhizosphere microorganisms, *Sphingobium* was observed in the WS group, while no

marker genera were observed in the WS-ECB group, only *Oceanospirillales* were observed.

### 3.4. The importance of environmental factors and random processes in the formation of microbial community structure

$\beta$ NTI can be used to infer ecological processes, with  $|\beta\text{NTI}| \geq 2$  indicating that deterministic factors have a strong influence on the community, and  $|\beta\text{NTI}| < 2$  implying that stochastic processes dominate. The null model-based analysis showed that stochastic processes dominated the assembly of water and rhizosphere microbial communities in different groups. Moreover, the relative scale plots of selection and dispersion showed that the contribution of stochastic factors to the water microbial communities of the WS group and WS-ECB group was greater than that of the control group (Fig. 5A, C). The determinant contribution of rhizosphere microbial community was greater in the WS-ECB group than in the WSS group (Fig. 5B, C).

Redundancy analysis and Mantel test were used to further determine the relationship between physicochemical factors and microbial community structure. Prior to RDA analysis, forward selection of environmental factors is performed based on ordistep (). Seven environmental variables ( $\text{COD}_{\text{Mn}}$ , TN, TP,  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ ,  $\text{NO}_2^-\text{-N}$ ,  $\text{PO}_4^{3-}\text{-P}$ ) and two environmental variables ( $\text{COD}_{\text{Mn}}$ ,  $\text{NH}_4^+\text{-N}$ ) were included in the RDA analysis of water and rhizosphere microbial communities, respectively (Fig. 6). RDA analysis showed that environmental factors explained 75.33 % of the variation in the microbial community of the water body,



**Fig. 3.** Non-metric multidimensional scaling ordination based on the Bray-Curtis similarity showed bacterial community structure. (A) Bacterial community structure in water. (B) Bacterial community structure in rhizosphere. Control: control group, WS: water spinach group, WS-ECB: water spinach - exogenous compound bacteria group.

with RDA1 and RDA2 explaining 52.08 % and 23.25 %, respectively. All seven environmental factors significantly influenced the variation in the bacterial community of the water body. Environmental factors explained 63.56 % of the variation in the composition of the inter-root microbial community, with RDA1 and RDA2 explaining 48.65 % and 14.91 % of the variation, respectively, and  $\text{COD}_{\text{Mn}}$  and  $\text{NH}_4^+\text{-N}$  were the most important drivers of the rhizosphere microbial community. In addition, there was a significant correlation between Bray-Curtis distance and physicochemical factor matrix in water (Mantel:  $r = 0.3914$ ,  $p = 0.001$ ) and root bacterial community structure (Mantel:  $r = 0.3842$ ,  $p = 0.002$ ). Mantel test further verified the relative effects of key environmental factors on microbial community  $\beta$ -diversity (Table 3).

Both stochastic and deterministic factors are important in forming bacterial community assemblages. The Sloan neutral model was used to fit the relationship between the frequency of occurrence of OTUs and their relative abundance in the bacterial community, and determine the contribution of neutral processes to the construction of the bacterial community. The results showed that the occurrence frequency of OTUs in bacterial communities in Control group, WS group and WS-ECB group in water samples (Control:  $R^2 = 0.636$ ; WS:  $R^2 = 0.688$ ; WS-ECB:  $R^2 = 0.702$ ; Fig. 7A, B, C) and the WS group and WS-ECB group in root samples (WS:  $R^2 = 0.579$ ; WS-ECB:  $R^2 = 0.539$ ; Fig. 7D, E) were consistent with the neutral model.

## 4. Discussion

### 4.1. Nutrient removal performance in aquaculture wastewater

Recently, the integrated bioremediation system composed of different units (precipitation, aeration, biofilm, large animal and artificial wet) with different purification function has become a new aquaculture wastewater treatment technology [38]. Phytoremediation has been effectively applied to the removal of pollutants in water environment [39,40]. In this study, the wastewater before treatment had high chemical oxygen demand and nitrogen and phosphorus content, whereas, after treatment,  $\text{COD}_{\text{Mn}}$ , TN and TP were removed to a certain extent in WS and WS-ECB (Table 1). The reduction in the concentration of  $\text{COD}_{\text{Mn}}$  involves microorganisms that are responsible in breakdown of organic compounds during phytoremediation [41]. Typically, aeration treatment played a dominant role in  $\text{COD}_{\text{Mn}}$  removal throughout the purification process during the experimental time [42]. Increased dissolved oxygen (DO) in the water column promotes microbial colonization and accelerates the metabolism of organic pollutants. On the other

hand, a mature biofilm system provides an aerobic layer for microorganisms where organic matter is degraded [43]. Since the suspended particles in aquaculture wastewater contain a large amount of N and P, subsequently during the process TN and TP are also effectively removed in the precipitation and filtration stage [44].

In fact, the inorganic compounds produced during aquaculture largely meet the nutritional requirements of plants [45]. Consistent with the results of previous studies, the treatment effect of  $\text{PO}_4^{3-}$  in the two treatment groups was comparable and much higher than that in Control group in this study (Table 1). Studies reported that the growth of plants needs to absorb phosphorus from water, and the existence of rhizosphere biofilm can promote phosphorus metabolism [15,43].

The introduction of exogenous bacterial consortia into traditional floating bed remediation systems has been applied to the removal of heavy metals and organic matter from polluted waters, and some studies have shown that the addition of ECBs has potential for purifying polluted aquaculture waters [29,46,47]. Microorganisms can form root biofilms by using roots as biological substrates in ecological floating bed systems. However, the direct addition of ECB leads to the free existence of bacteria in the water body. This addition method cannot avoid the loss of microorganisms and the shortcomings of uneven growth cycle and affect their attachment [48]. The inoculation of ECB in the root of plant in floating-bed can further enhance their effectiveness in degrading pollutants [49]. Moreover, the biofilm carrier with ECB could be suspended below the planted floating-bed to improve the stability of ECB [32,47]. In wetland systems,  $\text{NH}_4^+$  is the most easily removed form of nitrogen, with a removal rate of  $>28\%$  to  $90\%$  [13]. In this study, the removal rate of ammonia nitrogen was higher in the WS-ECB group. In many reports, bacteria prefer to use  $\text{NH}_4^+$  as one of the nitrogen sources, and the ECB added in this study (including photosynthetic bacteria and Bacillus) have the ability to remove ammonia nitrogen [50–52]. In addition, the roots of aquatic plants may have higher DO concentration, forming aerobic micro-zones that can promote  $\text{NH}_4^+$  nitrification, and the ECB can continuously carry out nitrification in the micro-zones to reduce the nitrogen content in the culture wastewater [13,30,53].

In the present study, the reduction in nutrient levels in the control group may be due to sedimentation and the role played by indigenous microorganisms in the water body, and micro-aeration was added to all the experimental setups in this experiment, which also facilitated microbial nitrification and denitrification processes [54]. In general, compared with the control group, the restoration of water spinach and water spinach combined with ECB had a significant effect on nutrient removal of aquaculture wastewater. Typically, phytoremediation is the

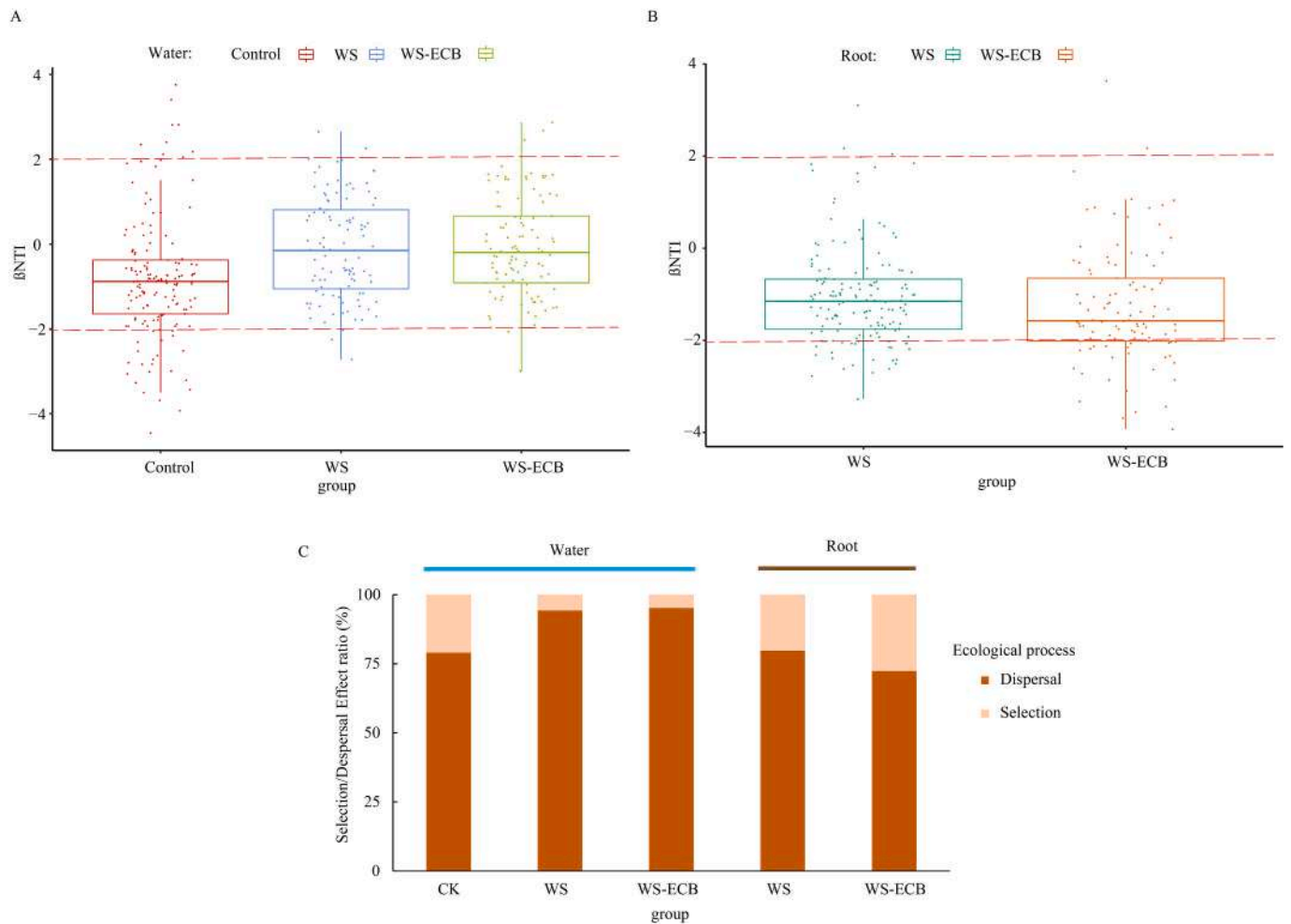


Fig. 4. The LefSe cladogram of abundant biomarkers. (A) The LefSe cladogram of abundant biomarkers in water; (B) The LefSe cladogram of abundant biomarkers in rhizosphere. Control: control group, WS: water spinach group, WS-ECB: water spinach - exogenous compound bacteria group.

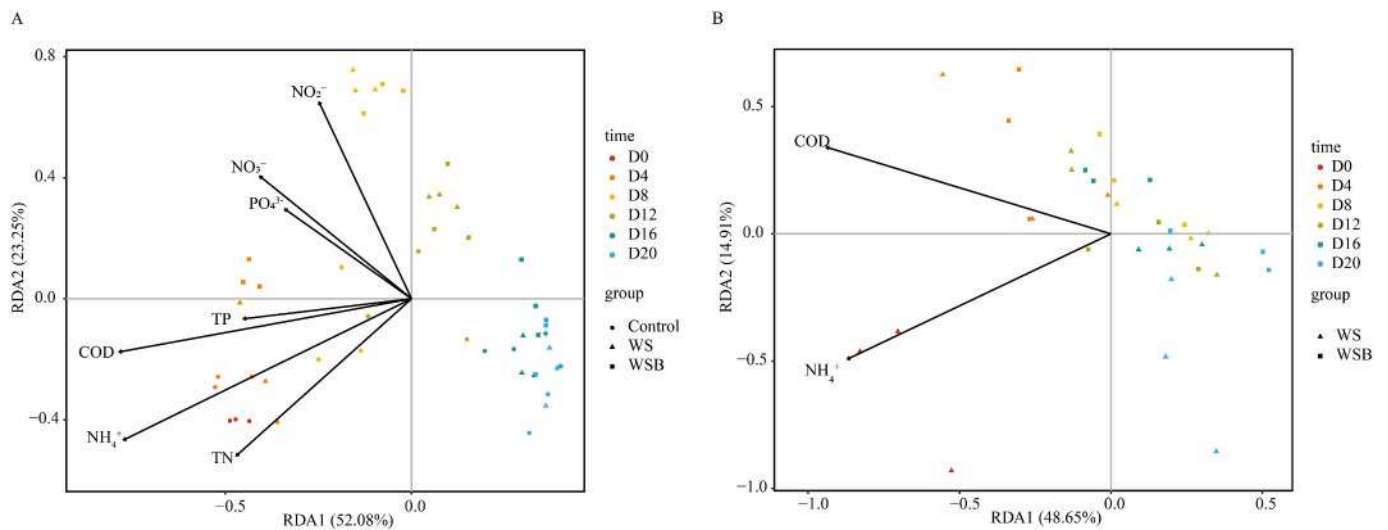
final treatment step for residual nutrients in an integrated bioremediation system. When it is properly allocated with other components such as biofilms and aquatic animals, the removal efficiency of pollutants can be improved through more kinds of treatment mechanisms, which has been shown in many studies [16,38,55].

4.2. Composition and succession process of bacterial communities in different treatments

In the process of bioremediation, microorganisms play an important role in the biogeochemical cycle of elements, the transformation of nitrogen and phosphorus, and the decomposition of organic matter. The



**Fig. 5.** Ecological processes of microbial communities in water and rhizosphere. (A) Distribution of  $\beta$ -NTI value in water and rhizosphere. The dashed horizontal lines represent the importance thresholds above (+2) and below (-2). (B) Selection and dispersal ratio of water and rhizosphere microbial communities.



**Fig. 6.** Redundancy analysis (RDA) showed the changes of microbial communities. (A) RDA analysis of water samples; (B) RDA analysis of rhizosphere samples. Control: control group, WS: water spinach group, WS-ECB: water spinach - exogenous compound bacteria group.

microbial community in water and rhizosphere had a strong influence on the removal of pollutants, and the microbial communities also changed in response to environmental conditions [10,56].

Microbial community diversity and composition determine the stability and effectiveness of aquaculture wastewater treatment process. The addition of ECB significantly increased the Chao1 index and

**Table 3**

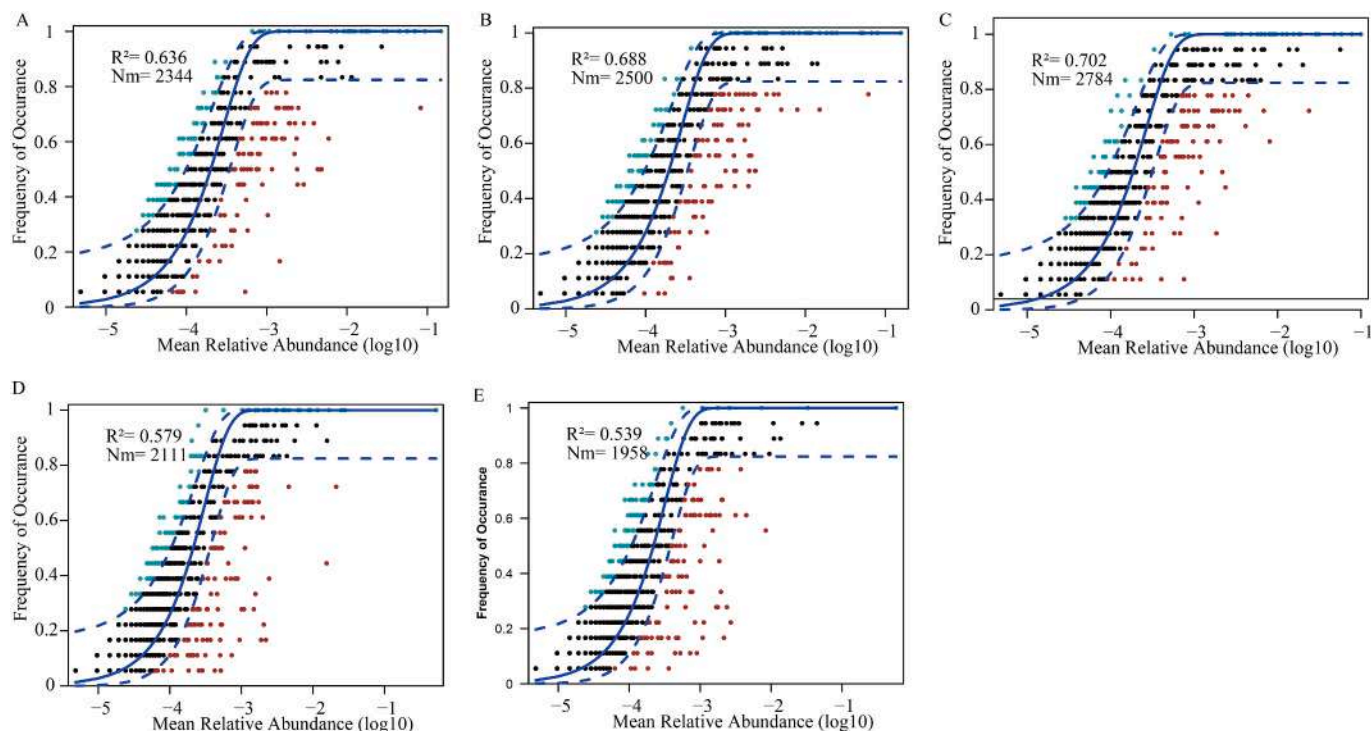
Mantel tests for the correlations between water variables (Based on Euclidean distance) and bacterial communities (Based on Bray Curtis similarity) with 999 permutations.

Samples	Environmental variables	r	p
Water	COD	0.3408	0.001
	NH <sub>4</sub> <sup>+</sup>	0.3616	0.001
	TN	0.2571	0.001
	TP	0.191	0.001
	NO <sub>2</sub> <sup>-</sup>	0.2443	0.001
	NO <sub>3</sub> <sup>-</sup>	0.2549	0.001
	PO <sub>4</sub> <sup>3-</sup>	0.0681	0.093
Root	COD	0.347	0.01
	NH <sub>4</sub> <sup>+</sup>	0.2789	0.021

Shannon index of water bacterial community in part of the WS-ECB group (Fig. 1A, B), indicating that adding ECB to the floating bed system of water spinach can improve the richness and population diversity of water bacterial community and make it more stable. The Proteobacteria, Actinomycetes and Bacteroidetes, which are the dominant bacterial groups in wastewater treatment systems, were found to be the dominant groups in this study (Fig. 2A). They have a variety of metabolic functions and play an important role in the biodegradation process [57]. Alphaproteobacteria was the group with the highest relative abundance in the water bacterial community in this study. According to previous studies, Alphaproteobacteria can carry out a variety of metabolic reactions including photosynthesis, nitrogen fixation, ammonia oxidation and methylation, and is related to biological nitrogen and phosphorus removal [57–59]. The addition of water spinach and exogenous compound bacteria may lead to the increased abundance of some bacterial with ability to regulate water quality. Compared with the control group, the relative abundance of Gammaproteobacteria in WS was higher at the end of the treatment, and its reported that

Gammaproteobacteria contained a large number of functional bacteria of nitrogen and phosphorus [47]. The relative abundance of Actinomycetes in WS-ECB was higher, and can contribute to the removal rate of COD<sub>Mn</sub> [60]. The water bacterial community structure was affected by both time and treatment methods (Table 2). With the progress of the experiment, it was observed that the bacterial community in water of WS and WS-ECB had similar evolution patterns, but was different from the control group (Fig. 3A). This indicated that plants and ECB changed the succession process of bacterial community in water, and the exudates secreted by plant roots during the growth process stimulated microbial activities, thus promoting the production and aggregation of microorganisms [61,62]. After the addition of ECB, it was inhibited by the competition of indigenous microorganisms in the water environment at the initial stage, so the community structure was not different from the WS group. The succession of rhizosphere bacterial community was only divided into two stages, indicating that it is more stable than water bacterial community.

LefSe analysis based on LDA can be used to identify the taxa most likely to explain the differences in treatment. A total of 25 dominant taxa (Fig. 4A) were observed in the water microbial communities of WS, many of which have physiological characteristics and functions that are crucial to the purification process of aquaculture wastewater. *Lewinella* belongs to the Bacteroidetes and its members are often found in activated sludge [63]. *Sphingobium* is a key bacterium in denitrification, and most species of this genus play a role in bioreactor and biodegradation in soil and wastewater [64,65]. *Halomonas* is easily adapted to the environment, and some species are denitrifiers [66]. *Hydrogenophaga* is a marker which was noted to exist in the WS. It is an important denitrifying bacterium with better nitrogen removal function and is commonly used for wastewater treatment [67,68]. *Caulobacter*, which is closely related to the removal of phosphate from wastewater, were discovered in WS [69]. Rhizobium belongs to denitrifying bacteria and can participate in the process of biological denitrification [70]. At the



**Fig. 7.** Fit of the neutral community model (NCM) of community assembly. (A) Neutral modeling of the Control group in water; (B) Neutral modeling of the WSB group in water; (C) Neutral modeling of the WSB group in rhizosphere; (D) Neutral modeling of the WS group in rhizosphere; (E) Neutral modeling of the WSB group in rhizosphere. Blue and red dots indicate OTU occurring more or less frequently than given by the model. The solid blue line best fits the neutral community model (NCM) and the dashed blue line indicates the 95 % confidence interval around the NCM prediction. Nm indicates the metacommunity size times immigration, R2 indicates the fit to this model.

same time, *Rhizobium* can convert insoluble phosphorus into a form that can be absorbed and utilized by plants by secreting organic acids and phosphatases [43]. These results suggest that the floating beds of water spinach promoted the enrichment of functional microorganisms in water and pollutant removal performance. Among the biomarkers of aquatic bacterial communities at WS-ECB, *Reyranella* has been reported to be associated with the degradation of COD<sub>Mn</sub> [71]. The rhizosphere bacterial community also changed under the influence of treatment and time during the experiment, but it was relatively slow compared with the water body. In the rhizosphere bacterial community, the biomarker level of WS was higher than that of WS-ECB, which may indicate that the rhizosphere of water spinach has a more diverse bacterial community (Fig. 4B). The genus *Sphingobium* with denitrification appeared in the rhizosphere of WS. Due to the resolution of the 16S rRNA gene sequence, some biomarkers are not classified at the generic level, so future studies are needed to better determine their ecological role.

#### 4.3. Microbial community assembly driven by deterministic and stochastic processes

Microbial community composition in different environments is usually controlled by different ecological processes. After years of research, several processes influence the assembly of bacterial communities, including deterministic factors (e.g., environmental variables) as well as stochastic processes (e.g., diffusion) [59,72]. Stochastic processes in intensive tidal flat aquaculture account for only 3 % of bacterial community assembly in, while environmental and other non-stochastic factors drive bacterial community assembly [73]. However, Ofițeru [74] suggest that the neutral community model should be used as the basis for the description of open biological systems. The inclusion of environmental factors allowed us to explain more of the variation, but the relative abundance of microbial communities was still driven by neutral processes.

In order to elucidate the ecological processes of bacterial community changes in the operation of floating bed restoration system, we emphasize the role of selection and diffusion processes (Fig. 5). The neutral model is able to quantify the spread and ecological drift of microbial communities, and many researchers have applied it to explain microbial community aggregation in a wide range of ecological phenomena [35]. In this study, the neutral model successfully estimated the relationship between the frequency of most OTUs and their relative abundance changes (Fig. 7), indicating that random processes play a dominant role in the assembly of water and root microbial communities. But neutral models cannot explain 100 % of species occurrence, and deterministic processes (e.g., environmental selection) may alter the relationships that obey neutral distributions generated by neutral processes [75]. In general, water nutrients are closely related to the bacterial community in aquaculture wastewater, and the concentration of nitrogen, phosphorus, and other nutrients can change the diversity and composition of the microbial community [76]. In this study, the concentrations of COD<sub>Mn</sub>, NH<sub>4</sub><sup>+</sup> and TN had the highest correlation with the composition of water bacterial community. In the process of aquaculture, the input of organic matter and nutrients makes the level of TN and COD in the water body higher, and then changes the composition of the microbial community, which is also consistent with the results of previous studies [77,78]. The rhizosphere bacterial community also had the highest correlation with the concentrations of COD<sub>Mn</sub> and NH<sub>4</sub><sup>+</sup> (Fig. 6B). Aquatic rhizosphere microorganisms participate in many activities, including COD degradation and nitrogen removal, and the roots provide a favorable environment for aerobic microorganisms to degrade nutrients and thus be utilized by plants [12]. The deterministic contribution of rhizosphere microbial community in WS-ECB was greater than that in WS. The addition of ECB may make the assembly of root microbial community shift to deterministic process.

In conclusion, both environmental factors and neutral processes are important for the formation of both water and rhizosphere bacterial

communities during bioremediation. Both factors should be taken into account when analyzing the community assembly process. In this study, neutral processes seem to have a greater impact on community structure.

## 5. Conclusion

In this study, 16S rRNA technology was used to evaluate the performance of water spinach and exogenous compound bacteria in the ecological floating bed system for the treatment of aquaculture wastewater. The results showed that water spinach and water spinach combined with exogenous compound bacteria changed the bacterial community structure of water and rhizosphere thus enhancing water purification. We also found that the introduction of water spinach and exogenous compound bacteria increased some biomarkers related to nitrogen and phosphorus degradation. Bacterial communities are mainly formed by random processes, but water environment factors are also important regulators of bacterial community structure. This study provides a new understanding of the dynamic changes of microbial communities during the removal of nutrients from ecological floating beds. However, this study is a preliminary investigation on the effects of phytoremediation and phyto-microbial remediation on water quality and bacterial community. Further research on the changes in bacterial community function during this process will be more helpful to guide the remediation of aquaculture wastewater.

#### CRedit authorship contribution statement

**Jiaojiao Xu:** Writing – original draft. **Regan Nicholaus:** Writing – review & editing. **Wen Yang:** Writing – review & editing, Supervision. **Yangcai Wang:** Funding acquisition, Project administration. **Jinyong Zhu:** Writing – review & editing. **Zhongming Zheng:** Writing – review & editing, Supervision, Funding acquisition.

#### Declaration of competing interest

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

#### Data availability

Data will be made available on request.

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