



The compound *Bacillus* and sea purslane (*Sesuvium portulacastrum*) enhanced aquaculture wastewater treatment efficiency: Insights from a study on microbial community distributions

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ABSTRACT

As the aquaculture industry continues to grow rapidly, the environmental consequences of wastewater discharge from aquaculture have become a major concern. The biological treatment method has proven to be an effective and beneficial approach for wastewater treatment. In this experiment, aquaculture wastewater was treated using two different ratios of *Bacillus subtilis* and *Bacillus licheniformis* composites in combination with sea purslane (*Sesuvium portulacastrum*) to investigate the wastewater treatment efficiency and microbial community. Sea purslane effectively removed nutrients from aquaculture wastewater. The addition of compound *Bacillus* significantly increased the removal rates of COD_{Mn}, TP, and PO₄³⁻-P, which were 54.9–56.4 %, 78.2–79.9 %, and 85.0–89.6 %, respectively. Moreover, the compound *Bacillus* notably influenced the microbial community composition in the water and root systems, reducing species abundance while increasing community diversity, with Treatment 2 exhibiting the highest community stability. The addition of the compound *Bacillus* significantly increased the relative abundance of Alphaproteobacteria. Most of the important operational taxonomic units (OTUs) that significantly impacted the groups belonged to Alphaproteobacteria. Different biomarkers in the water and root system were primarily from Alphaproteobacteria, Gammaproteobacteria, and Bacteroidia, including *Idiomarina* and *Alteromonas*, *Ruegeria*, *Vibrio*, *Algoriphagus*, and *Maribacter*, among others. These microorganisms play a significant role in promoting the removal rate of nitrogen and phosphorus. Furthermore, the compound *Bacillus* notably affected the abundance of nitrogen cycle-related sequences and increased the relative abundance of microorganisms associated with the nitrogen cycle. These findings provide valuable insights for optimizing aquaculture wastewater treatment processes.

1. Introduction

As shrimp aquaculture intensifies, feed waste and shrimp excretions led to the accumulation of nitrogen and phosphorus pollutants in water, including ammonia nitrogen, nitrite nitrogen, and reactive phosphate (Kokkuar and Srisapome, 2021; Xu et al., 2024). The discharge of wastewater from shrimp farming contributes significantly to environmental pollution, including water eutrophication, soil salinization, and the accumulation of residues such as antibiotics, disinfectants, and other chemicals. Currently, an integrated wastewater treatment system is considered one of the most effective methods for treating shrimp-

aquaculture wastewater (Lukwambe et al., 2024). This system typically include sedimentation, microalgae (Yang et al., 2024), biofilms (Wei et al., 2024a, 2024b, Wei et al., 2022), shellfish (Lukwambe et al., 2020, Lukwambe et al., 2018; Nicholas et al., 2023), and macrophytes/microalgae (Xu et al., 2024). The elevated salinity of mariculture wastewater complicates its treatment, making salt tolerance a key consideration when choosing organisms for wastewater treatment, such as shellfish and macrophytes. Artificial wetlands (floating islands) are a low cost and environmentally friendly technology for wastewater treatment (Vásquez et al., 2022). Aquatic plants are an integral component of these artificial floating islands, providing good conditions

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for filtration, phytoremediation, and a large surface area for the growth of attached microorganisms (Carrasco et al., 2017; Stefanakis, 2018). Biological nitrification-denitrification and plant uptake are often the two most important mechanisms for nitrogen removal in artificial wetlands (Vásquez et al., 2022). Microorganisms attach to the root system, forming biofilms that are more stable than the microbial communities in water (Yuan et al., 2024). In the aerobic root zone microorganisms stabilize organic matter and nitrify ammonium nitrogen to nitrate nitrogen. In the anoxic zone, microbial-mediated denitrification converts nitrate to nitrogen gas, which is then released into the atmosphere (Vázquez-Burney et al., 2015). Salinity tolerance is crucial when selecting plants for mariculture wastewater treatment, while many macrophytes are effective in treating low salinity wastewater, such as *Ipomoea aquatica* Forsskal (Xu et al., 2024), *Iris pseudacorus* L. (Wu et al., 2017a, 2017b), *Canna indica* L. (Zuo et al., 2020). Fewer species can tolerate hypersaline conditions, one of such plants is *Sesuvium portulacastrum*, commonly known as sea purslane. The sea purslane has high edible, medicinal, and economic values (Magwa et al., 2006) and can absorb nitrogen and phosphorus to improve the marine environment (Boxman et al., 2017). The use of sea purslane for the treatment of high-salinity wastewater demonstrated a COD_{Mn} removal rate of 45–48 %, which significantly influenced the microbial community structure (Yuan et al., 2024). As a floating bed plant, sea purslane can be harvested regularly for use as vegetable or animal feed, providing both environmental and economic benefits. It effectively removes pollutants such as nitrogen and phosphorus from wastewater.

Bacillus is a genus of spore-forming bacteria known for its resistant ability to harsh physical and chemical conditions, which enables it to survive in diverse environments, including freshwater and marine sediments (Soltani et al., 2019). The role of *Bacillus* in aquaculture is extensive. For instance, the addition of *Bacillus subtilis* to aquaculture water has been shown to improve the growth performance, immune response, and resistance to *Vibrio harveyi* infection in juvenile shrimp (*Litopenaeus vannamei*) (Zokaeifar et al., 2014). Furthermore, *Bacillus* is highly effective in aquaculture wastewater treatment and a key component of many microecological formulations. It improves water quality by reducing ammonia and nitrite concentrations (Kuebutornye et al., 2019). By introducing organic carbon, *Bacillus* can enhance ammonia removal (Pinho et al., 2017). Among the various biotechnologies available for nitrogen reduction in aquaculture wastewater, biological denitrification stands out as one of the most effective, environmentally friendly, and cost-efficient methods (Barman et al., 2017).

The concentration of harmful nitrogen, such as ammonia, nitrite, and nitrate in aquaculture water can be effectively controlled by adding appropriate microorganisms (Kuebutornye et al., 2019; Soltani et al., 2019). Xu et al. (2024) demonstrated that the combined use of compound bacteria (*Rhodospseudomonas palustris* and *Bacillus subtilis*) along with water spinach (*Ipomoea aquatica* Forsskal) effectively treated low-salinity *Penaeus vannamei* aquaculture wastewater, achieving an ammonia removal rate of 93.79 %. Plant root systems influence microorganisms in two main ways: by releasing dissolved oxygen, which promotes the activity and growth of nitrifying bacteria and accelerates the nitrification process (Han et al., 2020), and by secreting carbon-rich compounds that intensify the denitrification process (Wu et al., 2017a, 2017b). Dominant heterotrophic microorganisms such as *Bacillus* have a wide optimal pH range (5–10), which aligns well with the optimal pH range for plants (Martins et al., 2019). Furthermore, the plant root system provides an ideal habitat for microorganisms to form biofilms. Thus, the simultaneous treatment of aquaculture effluent by plants and microorganisms warrants a promising application for wastewater management.

Bacillus subtilis, one of the commonly used microbial ecological agents (Shao et al., 2020), is known for its high stability, resistance, and bacteriostatic activity (Subtil et al., 2019). The removal of NH₄⁺-N, NO₃⁻-N, NO₂⁻-N, and TN by *Bacillus subtilis* reached 81 %, 87 %, 52 %, and 91

%, respectively, demonstrating a significant purifying effect on water quality (Shao et al., 2021). Nitrite, ammonia, and nitrate concentrations in shrimp aquaculture water were significantly reduced to tolerable levels by adding of *Bacillus subtilis* at a 10⁸ CFU/mL to the aquaculture water (Zokaeifar et al., 2014). *Bacillus licheniformis* is also widely used as an ecological agent in aquaculture to remove NH₄⁺-N from water (Geng et al., 2022). In this experiment, we used sea purslane to treat the shrimp wastewater and introduced the compound *Bacillus* (*Bacillus subtilis* and *Bacillus licheniformis*) into the system. We investigated the treatment effect of sea purslane and compound *Bacillus* on wastewater and its influence on the microbial community structure/dynamics using 16S rRNA high-throughput sequencing technology. The main objectives of this experiment were: (1) to study the treatment effect of compound *Bacillus* on wastewater; (2) to assess changes in the microbial community structure in the water and the root system of sea purslane; and (3) to predict the roles and functions of key microorganisms in nitrogen and phosphorus removal. The results of this experiment will provide valuable insights for optimizing aquaculture tailwater treatment systems.

2. Materials and methods

2.1. Experimental design

The experiments were conducted at Xianxiang farm in the Yinzhou district of Ningbo, where there is a *Penaeus vannamei* farming facility. The wastewater was sourced from a shrimp farming pond with a stocking density of 70,000 shrimp per 667 m². The initial concentrations of chemical oxygen demand (COD_{Mn}) and nutrients in the wastewater are presented in the Table 1. The shrimp culture wastewater treatment system consisted of four biofilter compartments (sedimentation, microalgae, shellfish, and sea purslane), and the compound *Bacillus* was injected into the sea purslane area. Compound *Bacillus* 1 consist of *Bacillus subtilis* and *Bacillus licheniformis* in a 1:5 ratio, while compound *Bacillus* 2 has a *Bacillus subtilis* and *Bacillus licheniformis* ratio of 2:5. Compound *Bacillus* 1 and Compound *Bacillus* 2 were determined by preliminary experiments. The experiment was carried outdoors using white barrels, each containing 100 L of wastewater. It was divided into three groups: the control group (water: WC, root: RC) with 20 indiv sea purslane; Treatment 1 (water: WT1, root: RT1) with 20 indiv of sea purslane and an injection of compound *Bacillus* 1 every eight days; and Treatment 2 (water: WT2, root: RT2) with 20 indiv of sea purslane and an injection of compound *Bacillus* 2 every eight days. Each group had three replicates. Throughout the experiment, 30 % the volume of water was replaced every two days to replicate the wastewater treatment process. The injection dose was 2 ‰ of the total water volume, resulting in *Bacillus* concentration of 10⁸ cfu/L. The experiment lasted for 16 days. Temperature, pH, salinity, and dissolved oxygen levels were measured daily, while COD_{Mn}, total nitrogen, total phosphorus, nitrite, nitrate, ammoniacal nitrogen, and active phosphate levels were examined every four days (D4, D8, D12, and D16) before changing the water. Simultaneously, the samples of planktonic microorganisms from the water and root system of the sea purslane were collected.

Water samples were taken using polythene bottles and filtered through 0.2 μm filter membrane using a filter pump. The membrane was

Table 1
The initial concentrations of the pollutants in the wastewater (Mean ± SD).

Pollutants	Concentration (mg/L)
COD _{Mn}	16.50 ± 1.96
phosphate (PO ₄ ³⁻ -P)	1.17 ± 0.17
nitrite (NO ₂ ⁻ -N)	3.83 ± 0.48
nitrate (NO ₃ ⁻ -N)	12.16 ± 1.22
ammonia (NH ₄ ⁺ -N)	5.38 ± 1.03
total nitrogen (TN)	19.82 ± 2.23
total phosphorus (TP)	3.17 ± 0.61

placed into a 5 mL EP tube and stored at -80°C for further analysis. The roots (5 g) were cut with sterile scissors and placed into 10 mL EP tubes for storage. Each tube contained a composite sample of roots from five plants. All samples were transported in an ice box to the laboratory within 2 h and stored at -80°C for further analysis.

2.2. Environmental parameters measurement

The water pH, temperature, and dissolved oxygen levels were measured using a multifunctional water quality detector (Multi 3620 IDS, Germany). Salinity (S) was measured with a refractive index salinometer. After collecting water samples, the COD_{Mn} was determined through oxidation with alkaline potassium permanganate. The total phosphorus (TP) and total nitrogen (TN) were determined via oxidation with potassium persulfate. The collected water samples were filtered using a glass fiber filter membrane with a pore size of $0.45\ \mu\text{m}$. Active phosphate ($\text{PO}_4^{3-}\text{-P}$) was measured using the ascorbic acid-reduced phosphomolybdenum blue, nitrate ($\text{NO}_3^{-}\text{-N}$) was determined by zinc-cadmium reduction, the nitrite ($\text{NO}_2^{-}\text{-N}$) by naphthalene ethylenediamine hydrochloride, and the ammonia ($\text{NH}_4^{+}\text{-N}$) by hypobromite oxidation.

2.3. DNA extraction and sequencing

Bacterial DNA was extracted using a DNA extraction kit (minkgene Water DNA kit, Guangzhou Findrop Biotechnology Co., Ltd., Guangzhou, China) and DNA concentration and purity were determined using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, MA, USA). To amplify bacterial colonies universal primers 338F (5'-ACTCCTACGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGGTWTCTAAT-3') were used targeting V4 region of the 16S rRNA gene. A 250-bp double-end-paired sequence was generated using Illumina's HiSeq 2500 platform (Guangdong Megagenics Biotechnology Co., Ltd., Guangzhou, China). High-throughput sequencing was performed on the Illumina MiSeq sequencing platform at Guangzhou Megger Genetics with the resulting sequencing data processed using USEARCH (V11.0.667.18). To enhance data quality, the combined sequences were denoised using the UNOISE3 algorithm (unoise_alpha = 2, midsize = 8, according to the default settings). This denoising step corrected errors and removed chimeras, (tesselations), resulting in the generation of zero-radius operation taxon units (ZOTUs), ZOTUs were quantified and compared with the SILVA database for taxonomic information.

2.4. Statistical analysis

One-way ANOVA was used to compare differences in nutrient removal rates, Chao1 and Shannon indices among treatment groups, with Duncon's Multiple Comparisons applied for post hoc analysis. When doing ANOVA for nutrient removal rates, raw data were arcsin transformed. All results were visualized using R 4.4.1 (R Core Team, 2024) and RStudio (2023.03.0 + 386). Alpha diversity was plotted using the geom_boxplot() function in the ggplot package. Cluster analysis was performed using the hclust() function in the package "vegan" to characterize the similarity of bacterial composition between groups. Principal coordinate analysis (PCoA) was performed using the cmdscale() function in the "ape" package and the ddpoly() function in the "plyr" package to visualize the overall structure of the bacterial community. Random forest regression was performed using the "randomForest" package, with 30 OTUs showing significant effects selected for further analysis five ten-fold cross-validations, were performed based on the calculated values of the importance of each OUT (e.g., "IncNodePurity"). After ranking the OTUs from highest to lowest, the top 10 OTUs were finally selected. Random forest regression and variable significance analysis were executed using the "rfPermute" package, while species abundance maps of these 10 OTUs over time were generated using

the "reshape" package and "circlize" for visualization. Linear discriminant analysis effect size (LEfSe) (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=PICRUST_normalize), was utilized to identify statistically significant taxa between the different treatments categorized by the microbial community. Functional gene prediction was performed using FAPROTAX to screen for genes related to nitrogen metabolism, these results were analyzed and visualized using the "ComplexHeatmap" package.

3. Results

3.1. Nutrient removal

The COD_{Mn} removal rates for Treatment 1 and Treatment 2 were 56.4 % and 54.9 %, respectively, both of which were significantly higher ($P < 0.05$) than that of the control group (5.4 %). However, no statistically significant difference was observed between the two treatment groups (Fig. 1). The TN removal rates for control, Treatment 1 and Treatment 2 were 45.0 %, 59.1 % and 54.8 %, respectively. No significant differences were observed among the three groups (Fig. 1). The TP removal rates for Treatment 1 and Treatment 2 were 79.9 % and 78.2 %, respectively, both significantly higher ($P < 0.05$) than the control group (66.9 %) (Fig. 1). The $\text{PO}_4^{3-}\text{-P}$ removal rates for Treatment 1 and Treatment 2 were 85.0 % and 89.6 %, respectively, both significantly higher than the control group (63.3 %) ($P < 0.05$) (Fig. 1). Additionally, the removal of $\text{NO}_2^{-}\text{-N}$, $\text{NO}_3^{-}\text{-N}$ and $\text{NH}_4^{+}\text{-N}$ was above 98 % in all three groups, with no significant difference observed. Overall, Treatment 1 showed the highest removal of COD_{Mn} , TN, and TP, while Treatment 2 achieved the highest removal of $\text{PO}_4^{3-}\text{-P}$ (Fig. 1).

3.2. Correlation between environmental factors and microbial community

Spearman's correlation analysis was performed on the top 10 classes, environmental factors, and *Bacillus*. In the water, the relative abundance of Alphaproteobacteria was higher in Treatment 1 compared to the other two groups (Fig. 2A). It was significantly negatively correlated with $\text{NH}_4^{+}\text{-N}$ and positively correlated with TP and TN (Fig. 2B). The relative abundance of Cyanobacteria in the control group was higher than that in the treatment groups (Fig. 2A), and it was strongly negatively correlated with TP. Acidimicrobiia was more abundant in treatment group 2 than in the other two groups (Fig. 2A), and it was remarkably negatively correlated with $\text{PO}_4^{3-}\text{-P}$, TP, and TN (Fig. 2B). Gammaproteobacteria was more abundant in Treatment 1 compared to the control group (Fig. 2A), and it was positively correlated with TN and TP (Fig. 2B). Parcubacteria showed a strong negative correlation with COD_{Mn} , while Saccharimonadia was prominently negatively correlated with $\text{PO}_4^{3-}\text{-P}$ (Fig. 2B). *Bacillus* was significantly negatively correlated with TP and TN (Fig. 2B). The main environmental factors affecting the composition of

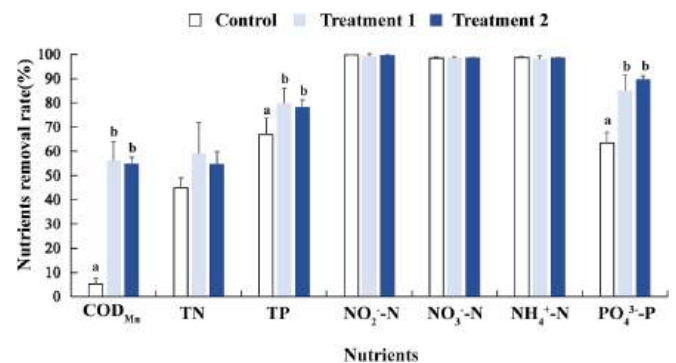


Fig. 1. Nutrient removal performance. Different letters indicate a significant difference between different treatments of the same nutrients ($P < 0.05$). The error bar stands for standard deviation.

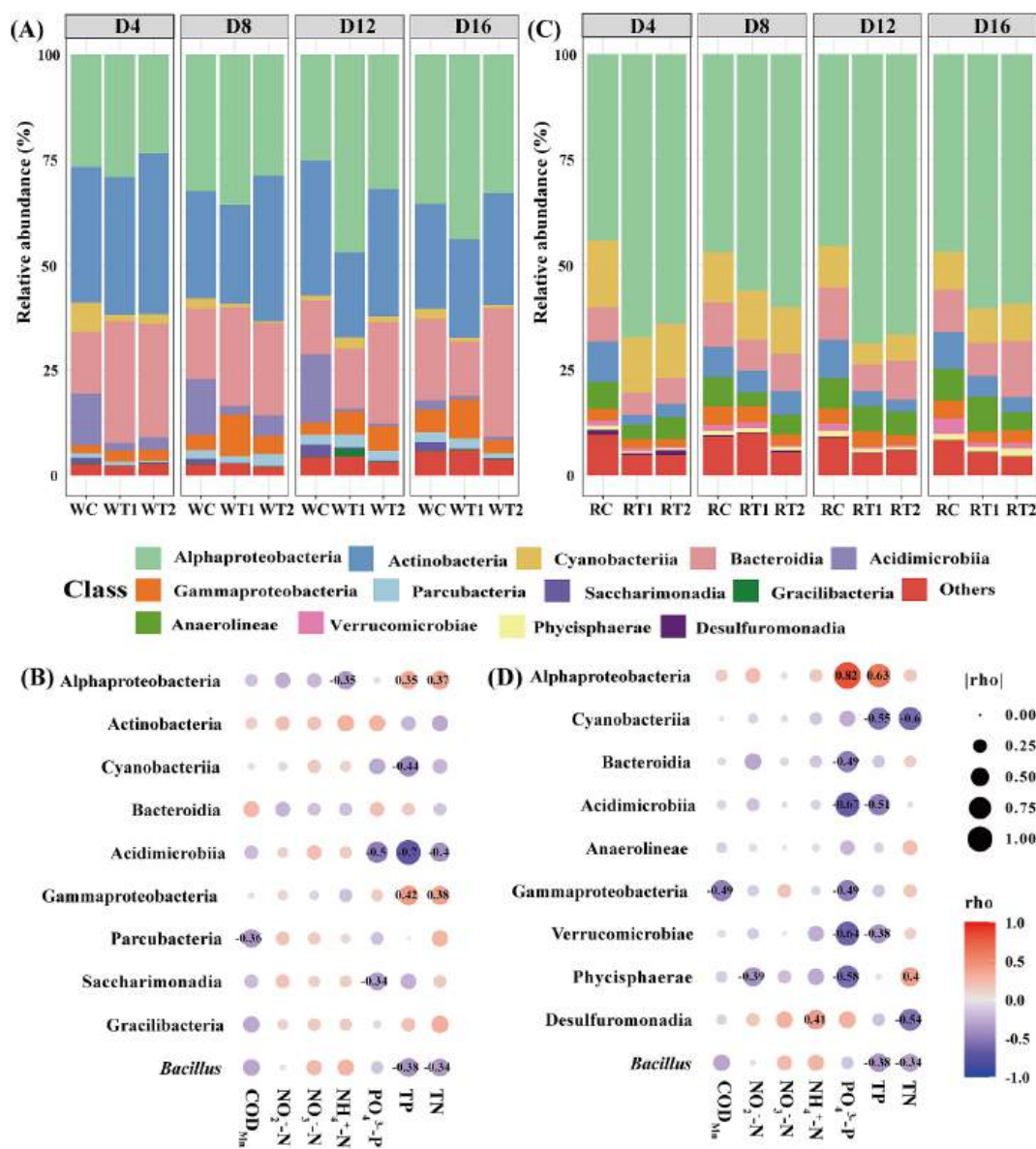


Fig. 2. Relative abundance of top 10 class in the water column (A) and root system (B) and Spearman's correlation analysis of Top 10 class and *Bacillus* in (C) water column and (D) root system with environmental factors. WC: the water of the control group, WT1: the water of Treatment 1, WT2: the water of Treatment 2. RC: the root system of a control group, RT1: the root system of Treatment 1, RT2: the root system of Treatment 2.

microbial communities were PO₄³⁻-P, TN, TP, and *Bacillus* (Fig. 2B).

In the root system, the relative abundance of Alphaproteobacteria was higher in treatment groups compared to the control group (Fig. 2C). It showed a significant positive correlation with PO₄³⁻-P and TP. Cyanobacteria exhibited a significant negative correlation with TN and TP. Bacteroidia showed a significant negative correlation with PO₄³⁻-P. The Acidimicrobiia and Verrucomicrobiae correlate dramatically negatively with PO₄³⁻-P and TP (Fig. 2D). Gammaproteobacteria were significantly negatively correlated with COD_{Mn} and PO₄³⁻-P. Phycisphaerae showed a significant negative correlation with NO₂⁻-N and PO₄³⁻-P and a significant positive correlation with TN (Fig. 2D). Desulfuromonadia showed a significant positive correlation with NH₄⁺-N and a significant negative correlation with TN (Fig. 2D). *Bacillus* showed a significant negative correlation with TP and TN (Fig. 2D). The main environmental factors affecting the composition of microbial communities were COD_{Mn}, PO₄³⁻-P, TN, and TP (Fig. 2D).

3.3. Microbial community diversity

In the water, no significant differences were observed in the Richness and Simpson indices on day 4. However, by day 8, the richness index was significantly higher in the control group compared to Treatment 2, while the Simpson index was notably lower ($P < 0.05$) in both the control and Treatment 1 relative to Treatment 2. On days 12 and 16, the richness index remained significantly higher in control than in Treatment 2 (Fig. 3A). In the root system, on day 4, the Richness index was significantly higher ($P < 0.05$) and Simpson was notably lower ($P < 0.05$) in the control group compared Treatment 2. No significant difference was found between the two treatment groups (Fig. 3B).

The stability of microbial communities was evaluated using the average variation degree (AVD) index, where lower AVD values represent higher community stability. Based on the AVD index, the order of community stability in the water (Fig. 3C) and the root systems (Fig. 3D) was as follows: control, Treatment 1, and Treatment 2, with Treatment 2 exhibiting the highest community stability.

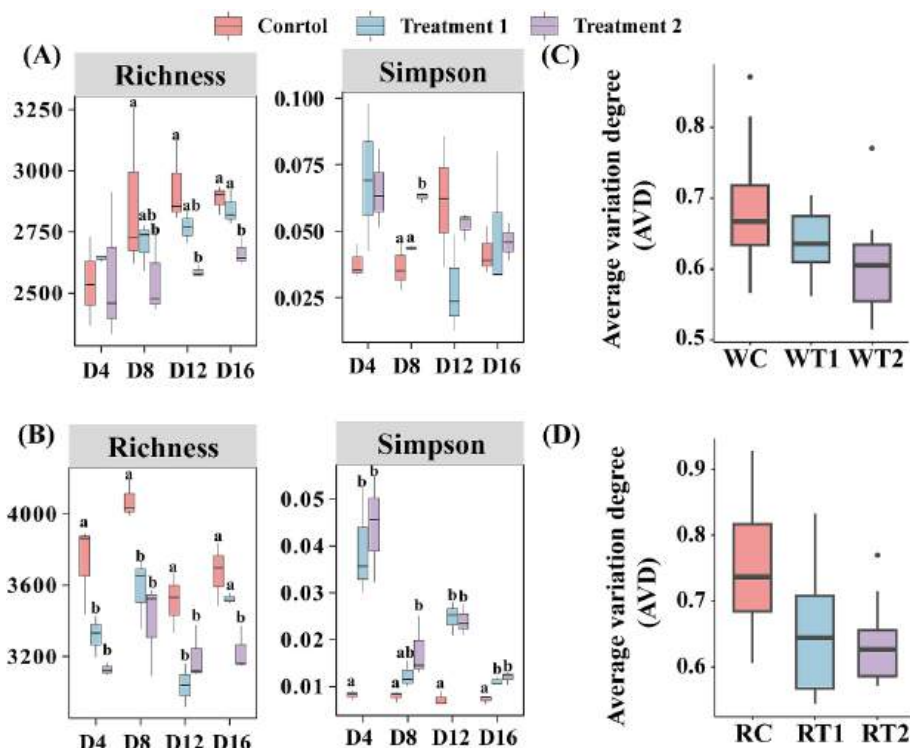


Fig. 3. α -diversity of bacterial communities in the water (A) and on the root system (B) and the relationship between bacterial OTU richness and average variation degree (AVD) of re-assembled bacterial communities in the water (C) and on the root system (D). WC: the water of the control group, WT1: the water of Treatment 1, WT2: the water of Treatment 2. RC: the root system of the control group, RT1: the root system of Treatment 1, RT2: the root system of Treatment 2. Different letters indicate a significant difference between different treatments simultaneously ($P < 0.05$). The error bar stands for standard deviation.

To better understand the dynamic processes within the bacterial community, PCoA was performed on the bacterial community in both the water and root system. Significant differences in bacterial communities between control and treatment groups were observed in both water and root systems. The microbial communities in each group fluctuate over time, with significant aggregation at the same time. While significant differences were observed between the control and treatment groups at the same time, there was no significant differences between the two treatment groups (Fig. 4).

3.4. Potential biomarkers to regulate bacterial communities

To identify the potential main drivers between different groups, we used random forest (RF) analysis to determine the key microbial

predictors. The top 10 OTUs (Fig. 6) that contributed most to the differences between groups were identified and their abundance at different times in each group was analyzed. The significance of these predictors was estimated using the percentage increase in MSE (Mean Square Error) with higher MSE% values implying more important predictors. Potential biomarkers of difference between groups were analyzed using LDA.

Actinobacteriota (OTU_255), Bacteroidota (OTU_233 and 475), Cyanobacteria (OTU_109), Dependientia (OTU_111), Proteobacteria (OTU_220, 341, 146 and 110), and Verrucomicrobiota (OTU_626) were the top 10 important OTU in the water. Of these, *Marivians*, *Litorimicrobium* and Flavobacteriaceae were significant (Fig. 5A). On day 8, *Litorimicrobium* and *Marivians* were more abundant in control and Treatment 1 and were higher in Treatment 1 than in control (Fig. 5C).

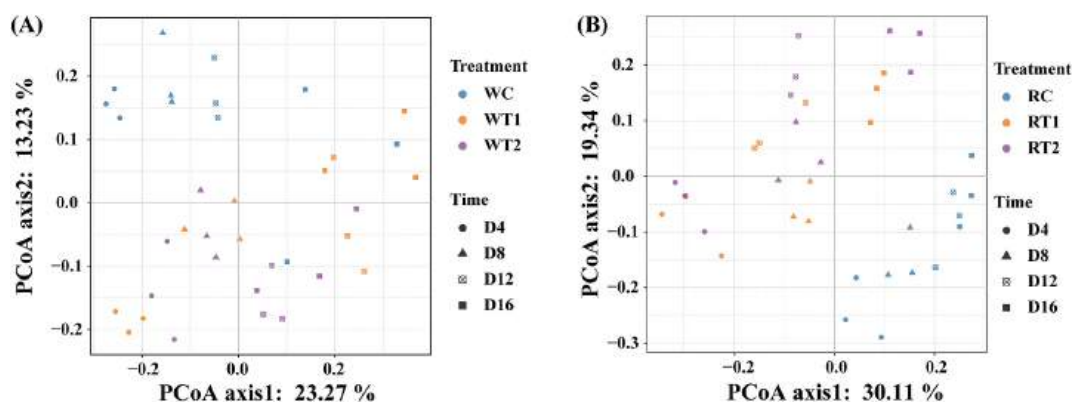


Fig. 4. The principal co-ordinates analysis (PCoA) of bacterial communities in the water (A) and on the root system (B). WC: the water of the control group, WT1: the water of Treatment 1, WT2: the water of Treatment 2. RC: the root system of the control group, RT1: the root system of Treatment 1, RT2: the root system of Treatment 2.

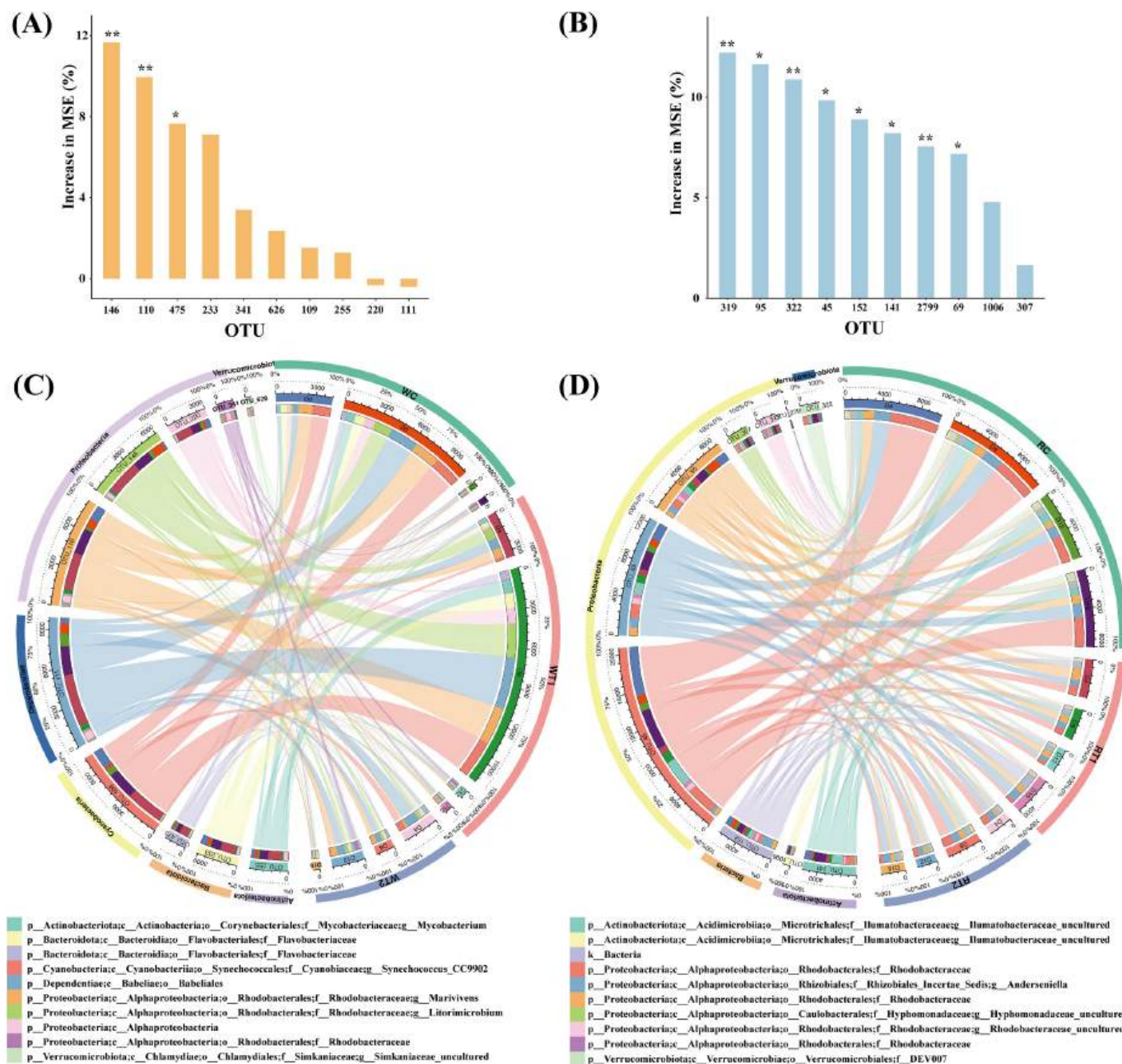


Fig. 5. Random Forest Regression of bacterial communities in the water (A) and in the root system (B) and sample-species abundance correlation in the (C) water and (D) root system. WC: the water of the control group, WT1: the water of Treatment 1, WT2: the water of Treatment 2. RC: the root system of the control group, RT1: the root system of Treatment 1, RT2: the root system of Treatment 2. $P < 0.05$ is indicated by *, and $P < 0.01$ by **.

Flavobacteriaceae were more abundant in Treatment 1 than in the other groups. These with significantly impacts OTUs had the highest abundance in Treatment 1 and the lowest in Treatment 2 (Fig. 5C). The potential biomarkers in Treatment 1 were all from the Proteobacteria, with four belonging to Gammaproteobacteria, including *Idiomarina* and *Alteromonas*, and one (*Ruegeria*) from the Alphaproteobacteria (Fig. 6A). Potential biomarkers in Treatment 2 were all from the Proteobacteria and Bacteroidota, mainly concluding *Roseibacterium*, *Vibrio*, *Algoriphagus* and *Maribacter* (Fig. 6A).

Among the top 10 OTUs in the root system, Actinobacteriota (OTU_141 and 1006), Proteobacteria (OTU_45, 69, 95, 307, 319, and 2799), and Verrucomicrobiota (OTU_322), are the top 10 important OTU in the root system. Among them, OTU_319, 322 and 2799 are highly significant, while OTU_95, 45, 152, 141, and 69 is significant with others (Fig. 5B). The abundance of these OTUs with significant

effects was highest in the control group and lowest in Treatment 2 (Fig. 5D). The potential biomarkers in Treatment 1 were all from the Proteobacteria, with *Photobacterium* from the Gammaproteobacteria and *Ruegeria* from the Alphaproteobacteria (Fig. 6B). The only potential differential marker in Treatment 2, *Maribacter*, was from the Bacteroidia (Fig. 6B).

3.5. FAPROTAX function prediction

Nine classes of functional genes related to nitrogen and phosphorus cycling were identified using Functional annotated database of prokaryotes (FAPROTAX) functional prediction. In the water (Fig. 7A), there was a consistent trend in the percentage of sequences related to the various functional processes, except for nitrogen fixation and nitrate reduction. On day 4, the number of sequences related to the remaining

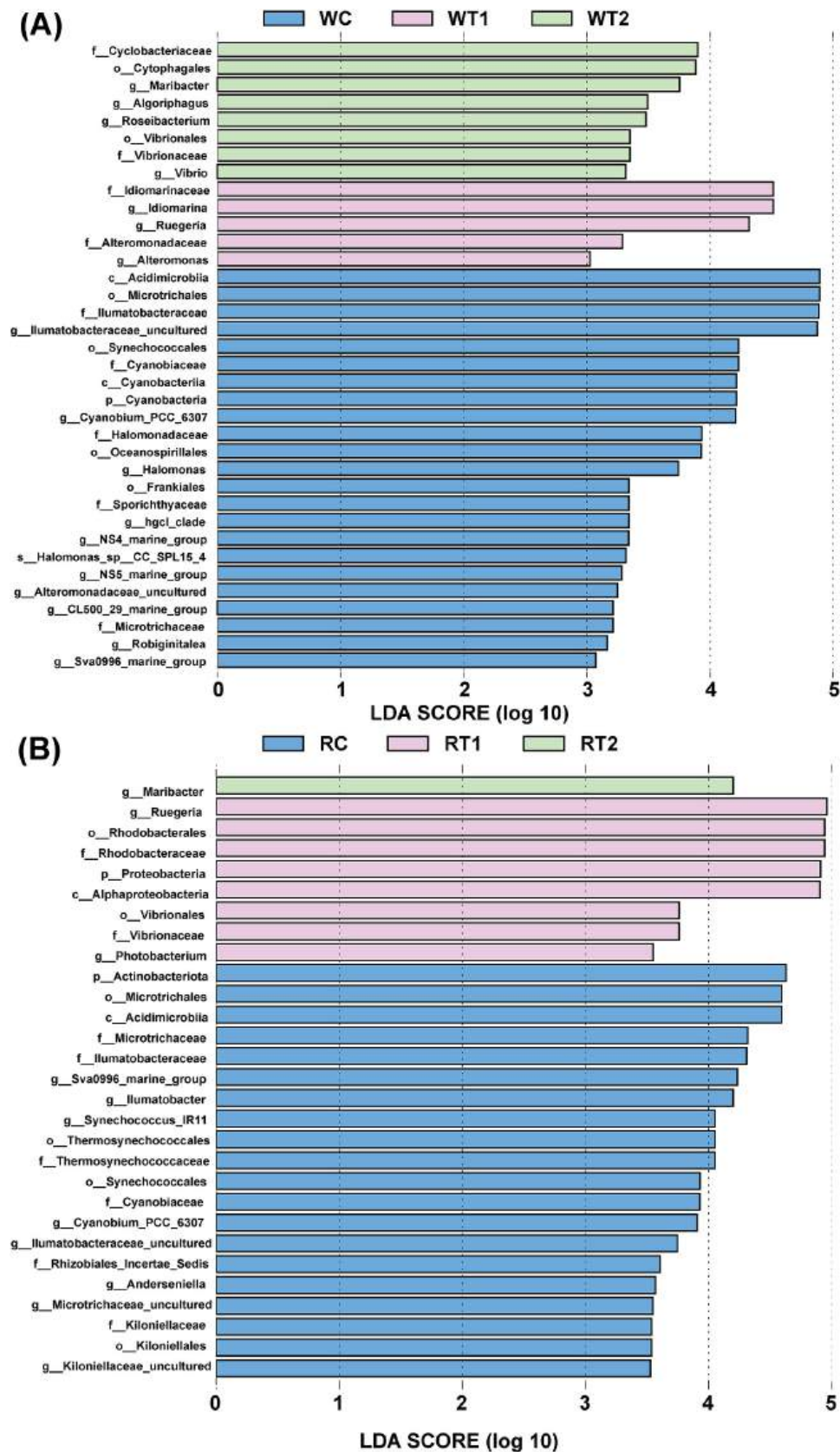


Fig. 6. LEfSe analysis of the bacterial composition of the water (A) and root system (B). WC: the water of the control group, WT1: the water of Treatment 1, WT2: the water of Treatment 2. RC: the root system of the control group, RT1: the root system of Treatment 1, RT2: the root system of Treatment 2.

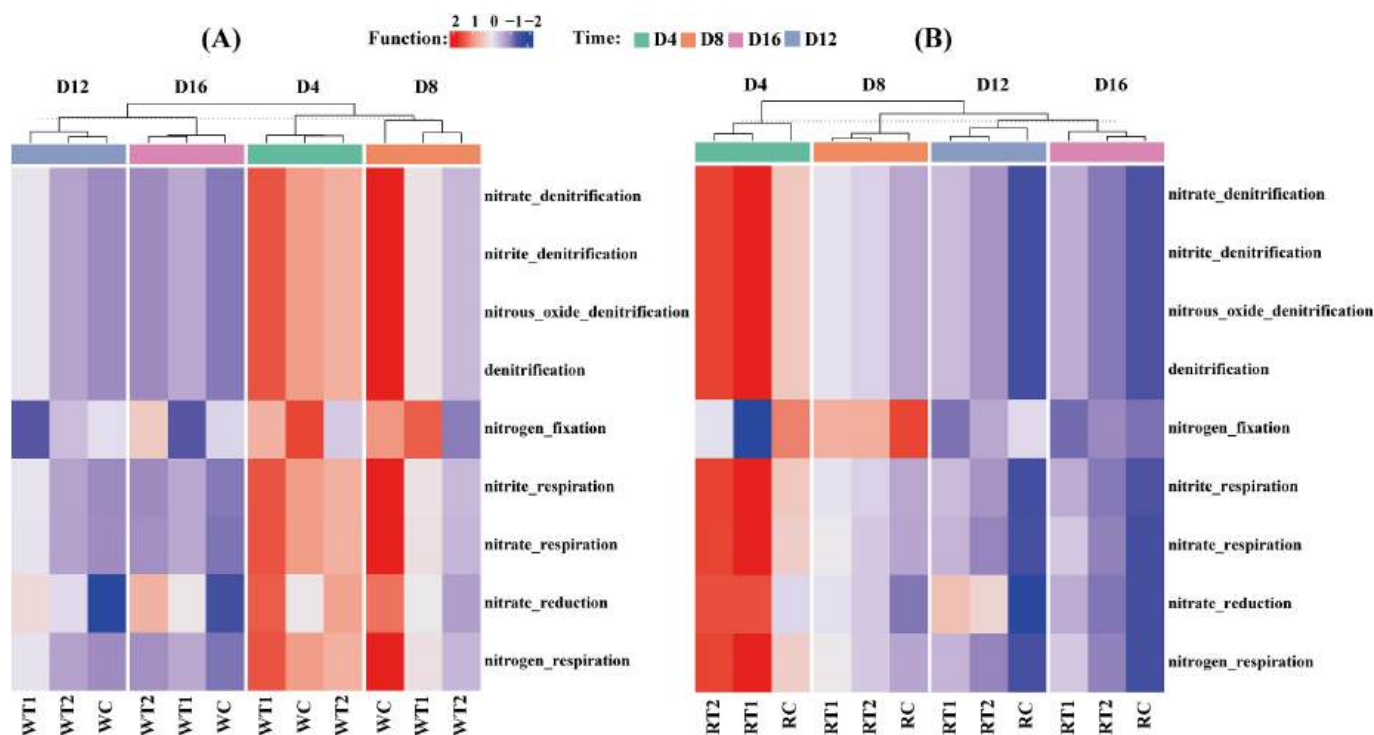


Fig. 7. The function prediction of bacterial communities in the water (A) and on the root system (B). WC: the water of the control group, WT1: the water of Treatment 1, WT2: the water of Treatment 2. RC: the root system of the control group, RT1: the root system of Treatment 1, RT2: the root system of Treatment 2.

functions was highest in Treatment 1, followed by the control group and Treatment 2. On day 8, the control group showed an increase in sequences related to these functions. With the control group having the highest number followed by Treatment 1, which was higher than Treatment 2. A decreasing trend was observed on days 12 and 16, where the control group had the lowest number of sequences and Treatment 1 had the highest. Nitrogen fixation was highest in Treatment 1 and lowest in Treatment 2 on day 4 and 8. But it was highest in control and lowest in Treatment 1 on day 12 and 16. The nitrate reduction was highest in Treatment 1 and lowest in control on day 4, highest in control and lowest in Treatment 2 on day 8, and lowest in control on day 12 and 16.

In the root system (Fig. 7B), a similar consistent trend was observed in the percentage of sequences related to the remaining functional processes, except for nitrate reduction. The number of sequences related to the remaining functional processes declined over the course of the experiment, with Treatment 2 consistently having the highest number followed by Treatment 1, and finally the control group. Nitrate reduction showed an increasing trend from day 4 to day 8 followed by a decline from day 8 to day 16. It was consistently higher in the control group than in the treatment groups, and higher in Treatment 2 than in Treatment 1.

4. Discussion

An integrated wastewater treatment system is a novel aquaculture treatment technology (Lukwambe et al., 2024). It is characterized by environmental friendliness, low cost, and low operation and maintenance requirements. The macrophyte serves as one of the key treatment filters in the system. Many reports on macrophyte treatment of aquaculture wastewater have shown promising results (Xu et al., 2024; Zuo et al., 2020). However, most of these studies focused on freshwater or low-salinity aquaculture wastewater. There is a significant correlation between salinity and the nutrient removal efficiency of macrophytes (Lymbery et al., 2013). Elevated salinity tends to reduce the nutrient removal rate by plants. The salinity in this study was relatively high (25 ‰). The wastewater treatment using only sea purslane showed effective

removal of all nutrients. However, adding compound *Bacillus* significantly increased the removal of COD_{Mn} , TP, and $\text{PO}_4^{3-}\text{-P}$ (Fig. 1). Compound *Bacillus* significantly promotes the conversion or uptake of phosphorus by sea purslane. Phosphorus plays a vital role in plant growth, serving as a fertilizer source that stimulates plant growth (Nájera de Ferrari et al., 2015). Plant absorbs phosphorus from water, and the presence of inter-root biofilms promotes phosphorus metabolism (Liu et al., 2023). The reduction of COD_{Mn} may be largely related to the activities of aquatic plants, which host microorganisms capable of decomposing organic matter during phytoremediation (Adabembe et al., 2022). The addition of the compound *Bacillus* increased COD_{Mn} removal rate (Fig. 1), likely due to the enhanced oxidation of organic matter in the water, which boosts microbial metabolism (Akinbile et al., 2016). Fahid et al. (2020) found that adding exogenous bacteria to the plant treatments significantly increased COD removal compared to plant treatments alone. The abundance of *Bacillus* in both the water and root system was significantly and negatively correlated with the concentration of TP and TN (Fig. 2), promoting the removal of these nutrients. Adding *Bacillus subtilis* and *Bacillus licheniformis* to the aquaculture process can reduce nutrient concentration in aquaculture water (Geng et al., 2022). Inoculating exogenous compound bacteria on the roots of floating bed plants can further enhance their pollutant degradation (Fahid et al., 2020). In this study, the compound *Bacillus* bacteria effectively enhance aquaculture wastewater treatment efficiency by sea purslane. Previous studies have indicated that increased plant inter-root bacteria stimulate the phytoremediation system restoration potential (Hussain et al., 2019; Saleem et al., 2018; Nicholaus et al., 2024). In this study, two different ratios of the compound *Bacillus* were used, but there were no significant different in nutrient removal between them (Fig. 1).

Microorganisms play an important role in the transformation of nitrogen and phosphorus as well as in the decomposition of organic matter. Microbial communities in both the water and root system significantly influence pollutant removal, and these microbial communities are also shaped by environmental factors (Xu et al., 2024). Microbial stability can be characterized by two key aspects: community resilience, which refers to the process by which a community recovers

and returns to an alternative steady state following a disturbance, and community resistance, which denotes the ability of a community to remain unchanged in the face of such disturbances (Allison and Martiny, 2008). The addition of compound *Bacillus* reduced species richness in both the water and root system while it increased the diversity of the common species. This led to an overall increase in community diversity. The Simpson's index was higher in the treatment groups compared to the control group (Fig. 3 A, B). The AVD values for the groups treated with compound *Bacillus* were lower than those of the control group (Fig. 3C, D). Bacterial communities with higher diversity tend to have lower AVD values, reflecting a positive correlation between microbial stability and biodiversity (Xun et al., 2021), communities with high diversity are generally more stable and resistant to disturbances. Therefore, the addition of the compound *Bacillus* enhanced both the community diversity and stability. Treatment 2 was particularly favorable for promoting microbial community diversity and stability.

OTUs in the water column that significantly contributed to intergroup differences were mainly from Proteobacteria, Actinobacteriota, Bacteroidota, Cyanobacteria, and Verrucomicrobiota (Fig. 5A). In the root system OTUs with significant effects were primarily from the Actinobacteriota, Proteobacteria, and Verrucomicrobiota (Fig. 5B). Alphaproteobacteria and Gammaproteobacteria was found to significantly influence nutrient removal efficiency in both the water column and the root system. The relative abundance of Alphaproteobacteria was higher in the treatment groups than in the control group (Fig. 2A, B). Alphaproteobacteria was significantly negatively correlated with $\text{NH}_4^+\text{-N}$ (Fig. 2C), playing an important role in removing $\text{NH}_4^+\text{-N}$ due to its excellent denitrification ability (Gao et al., 2020). *Ruegeria* (Alphaproteobacteria), identified as a potential differential marker in the water and root system of Treatment 1 (Fig. 6), promotes denitrification and accelerates the reduction of inorganic nitrogen to gaseous nitrogen compounds in the water (Wei et al., 2024a). *Idiomarina*, *Alteromonas*, and *Photobacterium* (Gammaproteobacteria) were the potential differential markers in Treatment 1, while *Vibrio* (Gammaproteobacteria) was a potential differential marker in Treatment 2 (Fig. 6). *Idiomarina* is a desulfurating and thermophilic bacterium involved in the degradation of difficult-to-degrade compounds under saline conditions (De Graaff et al., 2011). *Photobacterium* isolated from a recirculating aquaculture system achieved a maximum removal rate of 4.5 mg/L/h for $\text{NO}_2^-\text{-N}$ (Liu et al., 2019). *Vibrio*, a group of gram-negative, curved, rod-shaped, autotrophic bacteria with lipopolysaccharide outer membranes are found in aquatic ecosystems, which can cause disease in humans and animals (Ibanga et al., 2023). *Algoriphagus* and *Maribacter* (Bacteroidia) were identified as the potential differential markers in treatment 2 (Fig. 6). Bacteroidota is a heterotrophic bacterium involved in the breakdown of complex carbon and protein-rich material (Gu et al., 2019). It helps converting complex organic matter into smaller molecules facilitating the decomposition of highly concentrated organic wastes (Zhou et al., 2020). Two important OTUs in the water column belonged to the Flavobacteriaceae family, which is responsible for phosphate removal and may positively affect nutrient removal (Zhang et al., 2022). In the root system, Verrucomicrobiae showed a significant negative correlation with TP and $\text{PO}_4^{3-}\text{-P}$ (Fig. 2D), suggesting its involvement in phosphorus removal. Some Verrucomicrobia isolated from seawater are aerobic chemically energetic heterotrophs that use mono- or disaccharides as a carbon source and energy source to convert nitrate nitrogen to nitrite nitrogen (Yoon et al., 2008). The addition of exogenous compound bacteria during the wastewater treatment process with water spinach increased the abundance of microorganisms responsible for regulating water quality and altered the microbial community succession (Xu et al., 2024). Microbial intervention accelerates nutrient cycling and facilitates plants' removal of nitrogen and phosphorus pollutants (Wu et al., 2013). In this study, the abundance of these important OTUs was higher in treatment 1 than in treatment 2 (Fig. 6).

The FAPROTAX was used to predict the metabolic or ecologically

relevant functions of prokaryotes (Louca et al., 2016). Nine functions related to the nitrogen cycle were identified in this study. The number of functionally related sequences was higher in the treatment groups compared to the control group, except for nitrogen fixation (Fig. 7). The addition of compound *Bacillus* decreased the number of sequences related to nitrogen fixation in the water while increasing the number of other sequences related to other nitrogen cyclin processes, including denitrification and nitrification. Most Aerobic denitrifying bacteria are gram-negative, and many hydrogenotrophic denitrifying bacteria belong to α -, β - and γ - proteobacteria (Isanta et al., 2015). The relative abundance of α -proteobacteria in the root system and water column of the treatment groups was higher than that of the control group (Fig. 2A, B), and the addition of compound *Bacillus* in the sea purslane treatment promoted the denitrification process in the water. The *Bacillus* mineralizes nitrogenous wastes through nitrification or denitrification, thereby reducing ammonia and nitrite levels and improving water quality (Xie et al., 2013). As the experiment progressed, the number of functional sequences related to nutrient cycling in both the water and root systems decreased. However, the number of sequences remained higher in the treatment groups than in the control group, with Treatment 1 showing a higher number of sequences than Treatment 2 (Fig. 7). This may be due to the gradual decrease in nutrient concentration over time leading to decline in microorganisms related to nitrogen cycling. Nevertheless, the addition of the compound *Bacillus* increased the number of sequences related to nitrogen cycling and influenced the relative abundance of microorganisms involved in these processes.

5. Conclusion

The present study investigated the efficacy of a combined approach utilizing *Bacillus subtilis* and *Bacillus licheniformis* in conjunction with sea purslane for the treatment of aquaculture wastewater, focusing on its impact on microbial community composition and dynamics. The Compound *Bacillus* significantly enhanced the removal rate of COD_{Mn} , TP, and $\text{PO}_4^{3-}\text{-P}$. The addition of the compound *Bacillus* had a substantial impact on the microbial community composition and function, significantly influencing both the microbial composition in the water and in the rhizosphere. It also increased the community stability, while Treatment 2 exhibiting higher stability than in Treatment 1. The *Bacillus* significantly increased the abundance of Alphaproteobacteria, and many of the key OTUs were derived from this class. These results highlight the potential of integrating plant-based and microbial-based treatments for sustainable aquaculture wastewater treatment, while also emphasizing the need for further research to optimize application strategies.

CRedit authorship contribution statement

Hongmei Yuan: Writing – original draft, Methodology, Investigation, Data curation. **Junxian Li:** Data curation. **Haihang Wang:** Data curation. **Regan Nicholas:** Writing – review & editing. **Muhammad Naeem Ramzan:** Writing – review & editing. **Wen Yang:** Writing – review & editing. **Zhongming Zheng:** Writing – review & editing, Project administration, Methodology. **Yangcai Wang:** Project administration, Methodology, Data curation.

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.

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Data availability

The raw sequences have been deposited in the NCBI Sequence Read Archive with the Bio-Project number PRJNA1110728.

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