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Exogenous compound bacteria enhance the nutrient removal efficiency of integrated bioremediation systems: Functional genes and microorganisms play key roles

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ABSTRACT

With the continuous development of intensive mariculture, the application of the integrated bioremediation system of aquaculture wastewater (IBSAW) is increasingly promoted. However, the process and nutrients removal performance of the IBSAW need to be further optimized due to its immature technologies. In this study, exogenous compound bacteria (ECB) were added to IBSAW to investigate its pollutants removal efficiency and the relevant mechanisms. High-throughput sequencing and Geochip gene array were used to analyze the correlation between nutrients and bacteria, and the abundance of N and P cycling genes were quantified. Multivariable statistics, dimensionality reduction analysis, and network analysis were applied to explore the mechanisms of IBSAW operation. The results showed that the nutrients decreased significantly after adding ECB, with the brush treatment group significantly outperforming the ceramsite in removing NO_3^- and PO_4^{3-}. Ceramsite has an advantage in removing NO_2^--N. The addition of ECB and different substrates significantly affected the composition of bacterial communities. The contents of nosZ and nirKS related to denitrification in the treatment groups were significantly higher than those in the control group, and the contents in the brush treatment group were significantly higher than that of ceramsite. The biomarkers *Psychroserpens* and *Ruegeria* on the biofilm of the brush treatment group were positively correlated with nirKS, while *Mycobacterium*, *Erythrobacter* and *Paracoccus*, *Pseudohaliea* in the ceramsite group were positively correlated with nirS and nirK, respectively. Therefore, it is speculated that the ECB significantly promoted the increase of denitrification bacteria by affecting the composition of bacterial communities, and the ECB combined with functional genera improved the efficiency of nutrients removal in the system. This study provided a reference for understanding the process and mechanism of nutrients removal, optimizing the wastewater purification technology of the IBSAW and improving the performance of the system.

1. Introduction

Mariculture is one of the fastest-growing food-producing industries in the world (Zhang et al., 2022). However, with the development of mariculture, a large amount of aquaculture wastewater is discharged, which promotes the eutrophication of coastal waters (Farmaki et al., 2014; Filgueira et al., 2017). Since Naylor et al. questioned the impact of mariculture on the environment, increasing attention has been paid to the harm of mariculture wastewater (Naylor et al. 1998, 2000).

Mariculture wastewater has the characteristics of a wide source and complex composition. Most importantly, it contains a large amount of salt, especially Cl^-, which limits the use of conventional wastewater treatment methods (Ni et al., 2022). The use of a single physical, chemical or other methods is obviously not enough to deal with this problem, so the integrated bioremediation systems of aquaculture wastewater (IBSAW) which integrates various methods have been

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widely used and reported (Lukwambe et al., 2019; Nicholaus et al., 2020). The IBSAW mainly includes units of sedimentation, biofilm, filter-feeding shellfish, and macroalgae (Lukwambe et al., 2020). The substrate on which the microbial activity occurs determines the performance of the bioreactor (Ahmad et al., 2022). In actual wastewater treatment, biofilms are mainly used for nitrate removal, but they also include many other unnecessary microbial processes. In order to improve the performance and the process of the system in purifying wastewater, it is necessary to study the element cycling and microbial composition in the biofilms (Aalto et al., 2022). The cycle of nutrient elements is closely related to microorganisms, among which the load and transformation of nitrogen (N) and phosphorous (P) elements are the main aspects of attention. Nitrite (NO_2^--N), nitrate (NO_3^--N), ammonium (NH_4^+-N) and N_2 are all existing forms in the process of N cycling. In wastewater treatment, ammonia oxidation, nitrification and denitrification are usually associated with microbial metabolism (Kleindienst et al., 2014). Similarly, the transformation of P is under close and delicate microbial control (Duhamel et al., 2021). Dissolved organic phosphorus (DOP) is poorly characterized at the molecular level, yet operationally includes organic and inorganic polymeric forms of P within three main bond classes: P-esters (including mono (P–O–C) and diesters (C–O–P–O–C)), P-anhydrides or polyphosphates (P–O–P) and phosphonates (P–C) (Duhamel et al., 2021). Microbial communities can utilize DOP extensively in each of the major bond classes through the activities of various P-hydrolases, including alkaline phosphatases (Duhamel et al., 2021).

In the IBSAW, the biofilm depend on synergistic interactions between substrate and microorganisms (Yang et al., 2018). Salinity is also conducive to the formation of biofilm in general (Rousseau et al., 2014). Brushes are widely used in aquaculture due to their low cost, easy availability, and low pollution. They can effectively intercept large particulate matter and provide a site for microbial attachment, which is conducive to efficient removal of pollutants (Li et al. 2020, 2022; Liu et al., 2023). Porous matrix (e.g. ceramsite) has been widely used in wastewater treatment systems due to superior absorbability and habitats for microbes (Tan et al., 2021). These advantages contribute to an increase in the abundance and activity of bacteria (e.g. nitrifying bacteria, heterotrophic bacteria and autotrophic denitrifying bacteria) and promote N transformation (Wang et al., 2020a). For example, heterotrophic nitrification and aerobic denitrification (HNAD) bacteria can simultaneously remove N and organic matter in a single reaction unit under aerobic conditions, which has attracted more and more researchers' attention (Huang et al., 2017; Li et al., 2019). Many HNAD include *Acinetobacter*, *Bacillus*, *Cupriavidus*, *Halomonas*, *Klebsiella*, *Marinobacter*, *Pseudomonas*, *Photobacterium*, and *Rhodococcus*. The majority of these HNAD have been extensively studied and isolated from a variety of environments (Huang et al., 2020). *Bacillus*, as a widely used probiotic, is often used to treat aquaculture wastewater. For example, the application of *Bacillus licheniformis* in wastewater purification (Ji et al., 2018) could promote the removal of phosphorus, and when studying the biological characteristics of *Bacillus megaterium*, it was also found that it can effectively reduce organic and inorganic phosphorus in aquaculture water (Hlordzi et al., 2020). The application of *Bacillus subtilis* in aquaculture water can stabilize pH and significantly remove TN and NO_3^--N (Wang et al., 2022). *Chlorella vulgaris*, *B.licheniformis* and *B. subtilis* were selected as the microalgae species for the treatment in fish wastewater due to their short breeding cycle and effectiveness at survival in different types of environments (Aranda-Vega et al., 2024). *B. subtilis* and *B.licheniformis* added to an aquaculture wastewater purification device changed the microbiota composition and significantly reduced the chemical oxygen demand (Li et al., 2023). In eutrophic environments, HNAD with different combinations of N removal pathways may be more efficient and stable than a single strain (Huang et al., 2020). For example, the interaction of *B.subtilis*, *Pseudomonas stutzeri* and *Rhodococcus* sp. can effectively achieve the removal of TN and organic C from nitrate-rich sewage (Zhang et al., 2019). Compared with single and pure strains, mixed strains have many advantages, especially in the removal of complex contaminants (Aparicio et al., 2018; Patel et al., 2018; Shang et al., 2018). From the perspective of microbial ecology, the coexistence and interaction of mixed strains in the culture system of mixed strains have novel biological functions, such as quorum sensing (Whiteley et al., 2017), deception effect (Leinweber et al., 2017), mutualism (Mujtaba and Lee, 2017). In addition, mixed strains are more effective in removing contamination because the multiple metabolic pathways and capabilities of the mixed strains are more powerful than those of a single pure isolate (Bruger and Waters, 2018). Our preliminary experiments also showed that 75% TN could be efficiently removed by adding the exogenous compound bacteria (ECB) to aquaculture wastewater (author's unpublished data). In this study, ECB were added to the IBSAW to investigate its pollutants removal efficiency and the relevant mechanisms. The objectives of the study were (1) to analyze the correlation between key bacteria and nutrients in the systems with ECB, (2) to assess the abundance of genes related to N and P cycling under different treatments and establish the correlation network between functional genera and related genes responsible for the process, and (3) to demonstrate the mechanisms how nutrients of were degradated and cycled in the IBSAW.

2. Materials and methods

2.1. Experimental system set-up

This study was conducted in the Pilot Test Base of Ningbo University at Meishan, where IBSAW with four treatment units (the sedimentation, biofilm, filter-feeding shellfish, and macroalgae) were built. Each unit is a white polyethylene (PE) barrel, which carries 70L of water. Wastewater was collected from a high-density greenhouse shrimp Farm (Haohai Aquaculture Farm, Yinzhou District, Ningbo City, Zhejiang Province) and sent to the Pilot Test Base. The biofilm carrier is polyethylene brush (extended diameter 0.2m, length 0.5m) and ceramsite (1 g/grain), which are placed separately. The density of the brush biofilm unit is 4 brushes per barrel, and the ceramsite unit is 3 bags per barrel. They were equipped with oxygen-enhancing equipment. To simulate the process of wastewater treatment, 20L of wastewater were flowed to the next treatment unit every 2 days in the order: sedimentation to biofilm to filter-feeding shellfish to macroalgae. The ECB used in the experiment is a bacterial suspension with a concentration of $OD_{600} = 0.5$, which is composed of five strains of *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Micrococcus luteus* (purchased from BeNa Culture Collection) with a ratio of 2: 2: 3: 1: 2. In the treatment group, 7×10^8 cfu/mL of ECB were added into biofilm units (including brush group and ceramsite group), while no ECB were added in the control group. The experiment lasted 25 days.

2.2. Sample collection and water quality index analysis methods

On the 25th day of the experiment, water samples for nutrients were taken and filtered with a 0.45 μm filter membrane. The filtered water samples were collected with a centrifuge tube and stored at −20 °C. Microbial water samples were taken in the biofilm unit and filtered with a 0.2 μm filter membrane and stored at −80 °C. The biofilm samples were taken from the surface of polyethylene brushes and ceramsite. Placed in sterile EP tubes, then stored in −80 °C.

NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P, TN, and TP were determined by the automatic discontinuous chemical analyzer SmartChem. Among them, the concentration of NH_4^+-N was determined by hypobromite oxidation method, TN and TP were determined by potassium persulfate oxidation method, NO_3^--N concentration was determined by cadmium column reduction method, NO_2^--N is determined by naphthalene ethylenediamine spectrophotometry, PO_4^{3-}-P concentration was determined by phosphoro-molybdenum blue spectrophotometry.

2.3. DNA extraction and sequencing

Bacterial DNA was extracted using a DNA extraction kit (Minkgene Water DNA Kit). A NanoDrop 2000 spectrophotometer (Thermo, USA) at OD 260/280 nm was used to quantify the integrality of the extracted DNA. The V4 unit of the 16S rRNA gene was amplified using the universal primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) for bacterial colonies (Caporaso et al., 2011). The 250bp paired-end sequences were generated using the Illumina HiSeq 2500 platform (Guangdong Magigene Biotechnology Co., LTD., Guangzhou, China). Sequence data were uploaded to the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov) with bioproject number PRJNA1010708 and accession number SRP457489. The bioinformatics processing of sequencing data was performed by USEARCH (V11.0.667_I8) (Edgar, 2010). Use the UNOIS3 algorithm to denoise the combined sequence (unoise_alpha = 2, minsize = 8), correct the error and remove the mosaic, generate the zero-radius operation Taxon unit (ZOTUs), quantify the ZOTU (Edgar, 2016) and compare it with the SILVA (v 138) database (Quast et al., 2012) to obtain the classification information. A total of 3897273 raw reads were produced. The quality of all samples was qualified, and the correct base recognition rate was 99.9%. The rarefaction curves indicated a good sequencing depth, and the results could reflect the real microbial community (Fig. S1).

2.4. Detection of carbon, nitrogen, phosphorus, and sulfur functional gene chip

After obtaining the original experimental sample, the total microbial DNA in the sample was extracted, and then the total amount and purity of the DNA sample were tested. After passing the test, DNA samples and reagents used for qPCR were added to the 384-well plate as Sample Sourceplate, and primers and reagents used for qPCR were added to the other 384-well plate as Assay Sourceplate. The sample plate and primer reagent were added to the nanopore of the high-throughput qPCR chip using a high-throughput automatic microsampling device. The qPCR reaction and fluorescence signal detection were performed in the SmartChip Real-Time PCR System, and the amplification curve and dissolution curve were automatically generated (Nolan et al., 2006). Canco software was used to obtain the detection status and Ct value (amplification cycle number) of each gene in the sample, and 16S rRNA was used as the internal reference to standardize the data to obtain the relative quantitative information of each gene in the sample. Absolute quantitative information of the 16S rRNA gene was obtained according to Roche instrument detection, and absolute quantitative information of other genes was obtained after conversion (Mardis and McCombie, 2017).

2.5. Statistical analysis

Data of nutrient content were all subjected to one-way ANOVA with Tukey's multiple comparisons. The difference in means of genes between treatment groups was compared in IMB SPSS statistics 21 software packages (IBM, USA) and plotted using GraphPad Prism 9 and R 4.0.4. The absolute quantification of functional genes was drawn in Excel, the star map was drawn by the package "ggplot2" in R 4.0.4, and the circulation path was created by Adobe Illustrator (2023). Primary co-ordinate analysis was performed using the cmdscale () function from the "ape" package and the ddply () function from the "plyr" package, and the community structure of bacteria in different media was visualized. The correlation between biomarkers and environmental factors was calculated using the corr.test () function in the "corrplot ()" package, and visualized by the Corrplot () function. Linear discriminant analysis (LDA) was done in the galaxy hutlab website to evaluate and identifies the biomarkers (Segata et al., 2011). The specific differences between groups were distinguished by the Kruskal-Wallis rank test, then the consistency of differences were estimated by Wilcoxon rank test, and finally the influence of biomarkers on significantly different groups based on LDA scores was evaluated (Chang et al., 2022). The correlation coefficient between the abundance of microbial and the abundance of functional genes was calculated by the package "Hmisc", then the network was obtained using package "igraph", and finally visualized in Gephi 0.10.1.

3. Results

3.1. The performance of nutrients removal

Compared with the untreated wastewater, the nutrient content in the wastewater treated with the biofilm unit of the IBSAW decreased significantly (Fig. 1, $P < 0.05$). Compared with the control group, the contents of NO_3^--N, NH_4^+-N, TN, PO_4^{3-}-P, TP in the biofilm of brush were significantly decreased (Fig. 1b-f, $P < 0.05$). The removal rate of NO_3^--N and TN in the brush treatment group was significantly higher than the ceramsite group, with rates of 88% and 61%, respectively (Fig. 1b and d, $P < 0.05$). And the removal rate of NH_4^+-N, PO_4^{3-}-P and TP is more than 80%. The ceramsite treatment group had a more significant effect on the degradation of NO_2^--N and P than the brush group (Fig. 1a and e, $P < 0.05$). The removal rate of NO_2^--N reached 70%, and the removal rate of PO_4^{3-}-P reached more than 80%. Overall, the brush unit optimized by ECB significantly improved the removal efficiency of NO_3^--N and TN, while the ceramsite unit enhanced the removal of NO_2^--N.

3.2. The quantification and proportion of genes, N and P metabolic pathways

N and P functional genes were directly related to nutrient content. The abundance of 31 genes related to N and P cycling was determined by Geochip. These included aerobic ammoxidation, organic N mineralization, nitrification, anaerobic ammonium oxidation, denitrification, N fixation, ammonification, organic P mineralization and other circulation paths. In the water collum, the ureC abundance of ammonification path reached 1.06×10^4 and 2.18×10^4 in brush and ceramsite treatment groups, respectively (Fig. 2a). The amoB of nitrification in the treatment group was significantly higher than that of the respective control group (Fig. 2e, $P < 0.05$). The phnK of organic P mineralization in brush group reached 2.97×10^4 (Fig. 2a). In the treatment group of ceramsite, the nirS2, nosZ of denitrification, phoD of organic P mineralization reached more than 2000 (Fig. 2a) and nosZ was significantly higher than other groups (Fig. 2e, $P < 0.05$). Overall, the gene abundance related to organic P mineralization and denitrification in the water treatment group was significantly higher than that in the control group, and the whole increase in the ceramsite group was greater than that in the brush group (Fig. 2c).

The gene abundance on biofilm was significantly higher than that in the water column (Fig. 2b and d). The abundance of nirS2, nosZ and napA of denitrification in brush treatment group reached 10^5 (Fig. 2b), nirKS and nosZ were significantly higher than the control group (Fig. 2f, $P < 0.05$). The ureC reached 2.38×10^5, phnK reached 1.16×10^4 (Fig. 2b) and both were significantly higher than the control group (Fig. 2f, $P < 0.05$). nirS1, nirS3, nirK, amoB, gdhA of organic N mineralization, phoX and ppx of organic P mineralization reached more than 10^4 in brush with ECB (Fig. 2b) and both were significantly higher than other groups (Fig. 2f, $P < 0.05$). Except for hzsB, the abundance of other genes in the ceramsite treatment group was higher than that in the control group (Fig. 2f, $P < 0.05$). On the biofilm of the treatment group, the distribution of functional genes was opposite to that of water collum, showing a greater increase in gene expression in the brush group than in the ceramsite (Fig. 2d).

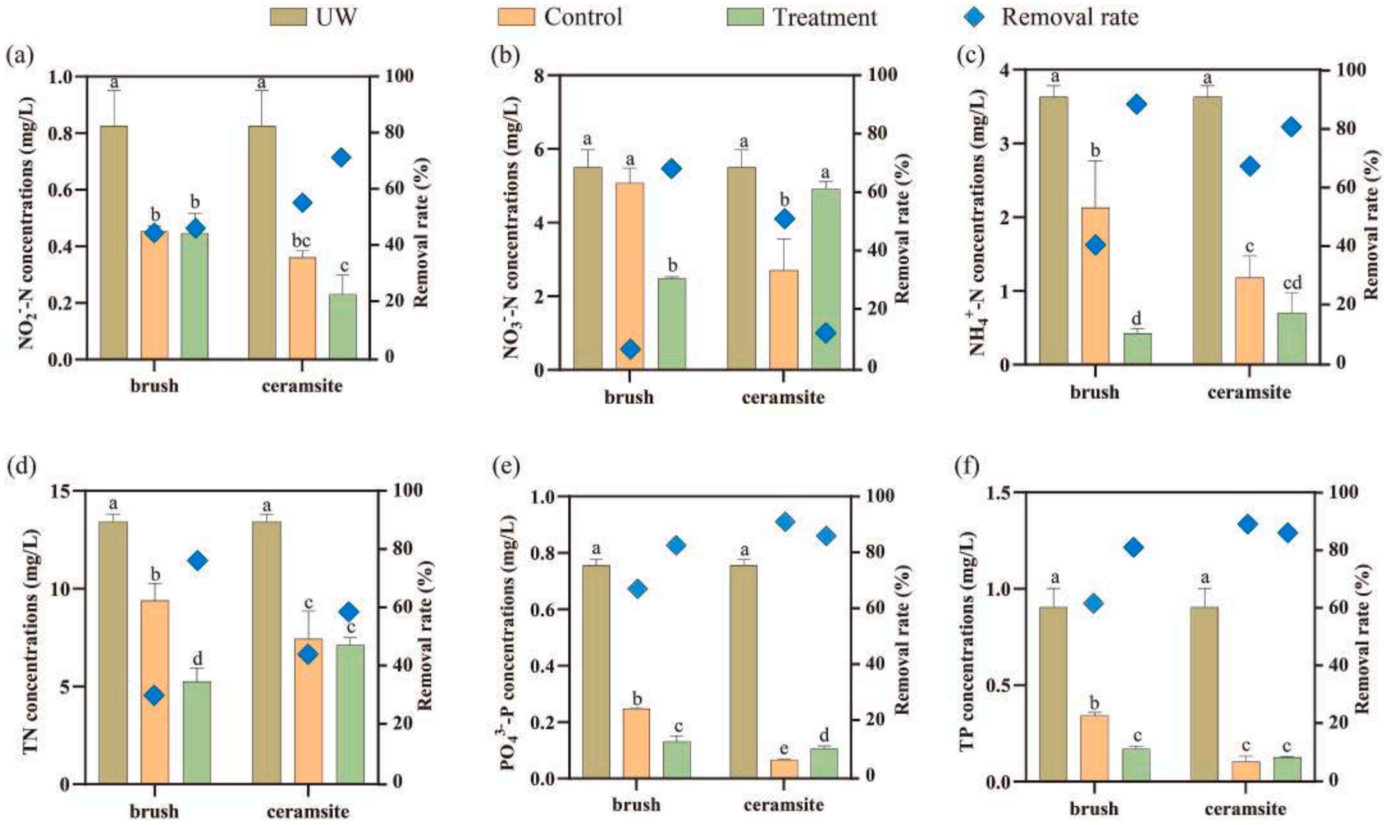


Fig. 1. Variations in levels of NO_2^--N (a), NO_3^--N (b), NH_4^+-N (c), TN (d), PO_4^{3-}-P (e) and TP (f) in IBSAW. UW: untreated wastewater; TN: total nitrogen; TP: total phosphorus. Different lower letters above the error bars symbolize the significant differences ($P < 0.05$, one-way ANOVA) among different groups.

3.3. The composition of bacterial communities in IBSAW

Principal co-ordinates analysis showed that the bacterial communities of the control group and the treatment group were separated on both sides of the PC1 axis (24.9% of the total variability) in the water column, while the brushes and ceramic particles were separated on the PC2 axis (19.7% of the total variability) (Fig. 3a). The distribution of bacterial communities on biofilms also showed similar patterns, with a contribution the PC1 axis was 36% and the PC2 axis was 23% (Fig. 3b). Further analysis using one-way PERMANOVA analysis based on Bray-Curtis illustrated that ECB significantly affected the composition of bacterial communities in the water column and in both brush and ceramsite biofilms (Table 1, $P < 0.05$).

At the phylum level, the proportion of Bacteroidota in the water and on the biofilm of the brush treatment group significantly increased (Fig. 3c and d), and the proportion of Firmicutes in the water of treatment groups also significantly increased (Fig. 3c and e). The proportion of Actinobacteriota in the water and on the biofilm of the ceramsite group also increased significantly (Fig. 3d and f). At the class level, the Bacteroidia of the brush treatment group and the Actinobacteria of the ceramsite treatment group both increased significantly (Fig. 3g-j).

3.4. Association of bacteria and nutrients in IBSAW

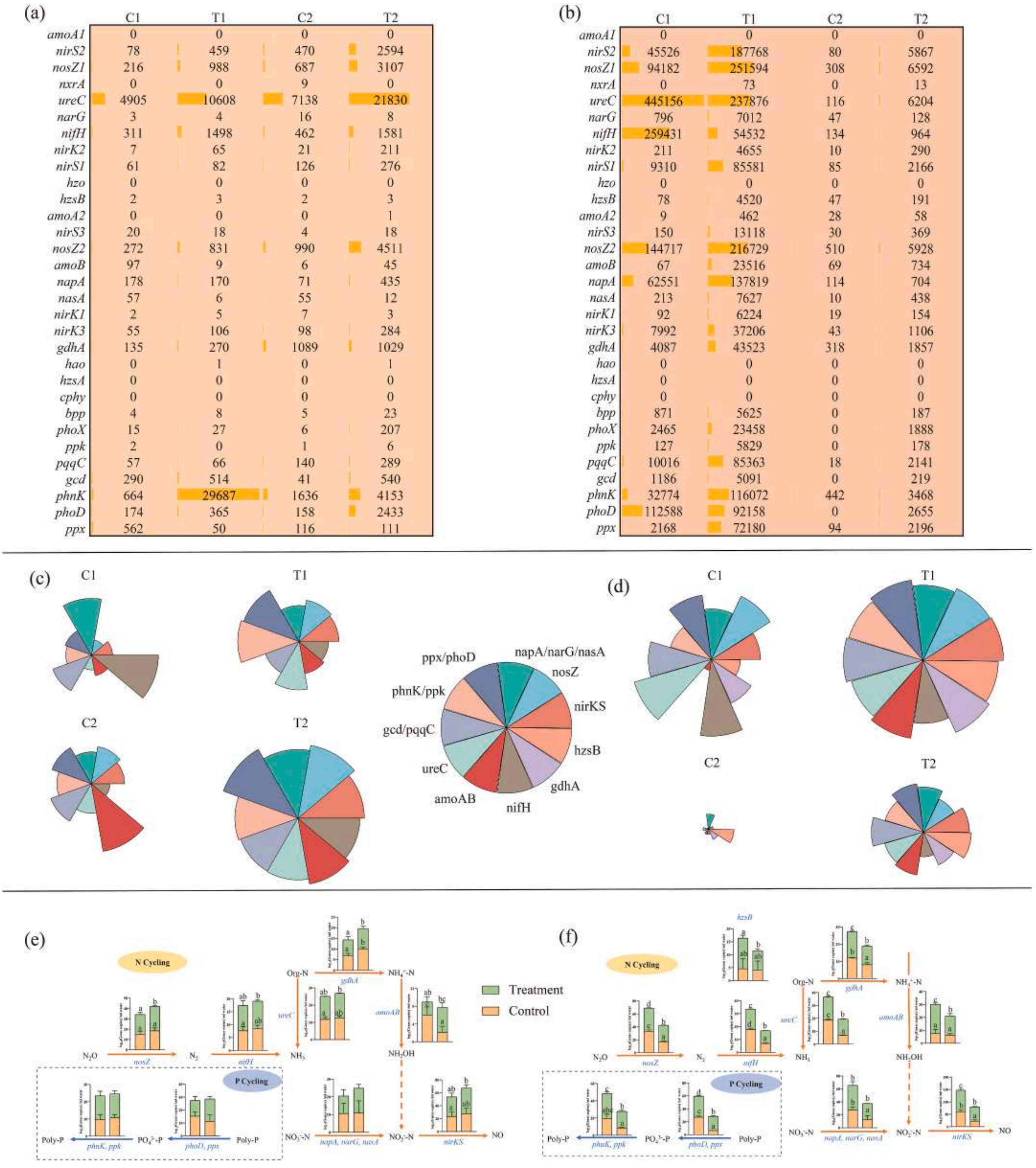
The application of Linear discriminant analysis Effect Size (LEfSe) can help find biomarkers, 91 bacterial genera with significant difference in quantity were screened, including 32 in the water column and 59 on the biofilm. *Bacillus* became a common biomarker in the treatment group (Fig. 4a–c).

To explore the relationship between bacteria and nutrients, the correlation heatmap was used to reflect the relationship between them. In the water collum, the biomarkers *Pseudonocardia* and *Denitromonas* belonging to the ceramsite treatment group had a strong negative correlation with NO_2^--N, *Marivivens* and *Edaphobaculum* had a strong positive correlation with NO_3^--N (Fig. 4b, $P < 0.01$), *Kocuria* and *Ruegeria* had a significant negative correlation with NH_4^+-N (Fig. 4b, $P < 0.05$). Biomarker *NS3a_marine_group* of brush treatment group was strongly positively correlated with TP (Fig. 4b, $P < 0.001$). *Celeribacter*, *Tenacibaculum* and *Polaribacter* were significantly negatively correlated with NH_4^+-N, and *Tenacibaculum* was also negatively correlated with TN (Fig. 4b, $P < 0.05$).

Compared with water, the biomarkers on the biofilm were more closely related to nutrients. *Jejudonia* in brush group was strongly negatively correlated with NH_4^+-N, *Ruegeria* was strongly negatively correlated with TN (Fig. 4d, $P < 0.001$), and *Rhodospirillaceae uncultured* was significantly negatively correlated with NO_3^--N (Fig. 4d, $P < 0.05$). *Bacillus* and *Kocuria* of ceramsite group were strongly negatively correlated with NH_4^+-N (Fig. 4d, $P < 0.01$), *Allorhizobium Neorhizobium Pararhizobium Rhizobium*, *Zhihengliuella*, *Erythrobacter* and *Photobacterium* were strongly negatively correlated with NO_2^--N, *Iamia* and *Vitellibacter* were strongly negatively correlated with PO_4^{3-}-P (Fig. 4d, $P < 0.01$), *Cyanobium PCC-6307*, *Zhihengliuella*, *Erythrobacter* and *Mycobacterium* were strongly negatively correlated with TP (Fig. 4d, $P < 0.001$).

3.5. Linkages among key genera and functional genes

A network was constructed to analyze the relationship between bacteria and genes in IBSAW. All the side Spearman correlation coefficients were greater than 0.8, and the q value was less than 0.05. The number of nodes and edge relationships involved in the composition of the biofilm network was greater than that of the water collum (Table 2), which indicated that the biofilm network was much more complex than that of the water. In the water collum, *Bacillus* in brush treatment group



(a)

	C1	T1	C2	T2
amoA1	0	0	0	0
nirS2	78	459	470	2594
nosZ1	216	988	687	3107
nxrA	0	0	9	0
ureC	4905	10608	7138	21830
narG	3	4	16	8
nifH	311	1498	462	1581
nirK2	7	65	21	211
nirS1	61	82	126	276
hzo	0	0	0	0
hzsB	2	3	2	3
amoA2	0	0	0	1
nirS3	20	18	4	18
nosZ2	272	831	990	4511
amoB	97	9	6	45
napA	178	170	71	435
nasA	57	6	55	12
nirK1	2	5	7	3
nirK3	55	106	98	284
gdhA	135	270	1089	1029
hao	0	1	0	1
hzsA	0	0	0	0
cphy	0	0	0	0
bpp	4	8	5	23
phoX	15	27	6	207
ppk	2	0	1	6
pqqC	57	66	140	289
gcd	290	514	41	540
phnK	664	29687	1636	4153
phoD	174	365	158	2433
ppx	562	50	116	111

(b)

	C1	T1	C2	T2
amoA1	0	0	0	0
nirS2	45526	187768	80	5867
nosZ1	94182	251594	308	6592
nxrA	0	73	0	13
ureC	445156	237876	116	6204
narG	796	7012	47	128
nifH	259431	54532	134	964
nirK2	211	4655	10	290
nirS1	9310	85581	85	2166
hzo	0	0	0	0
hzsB	78	4520	47	191
amoA2	9	462	28	58
nirS3	150	13118	30	369
nosZ2	144717	216729	510	5928
amoB	67	23516	69	734
napA	62551	137819	114	704
nasA	213	7627	10	438
nirK1	92	6224	19	154
nirK3	7992	37206	43	1106
gdhA	4087	43523	318	1857
hao	0	0	0	0
hzsA	0	0	0	0
cphy	0	0	0	0
bpp	871	5625	0	187
phoX	2465	23458	0	1888
ppk	127	5829	0	178
pqqC	10016	85363	18	2141
gcd	1186	5091	0	219
phnK	32774	116072	442	3468
phoD	112588	92158	0	2655
ppx	2168	72180	94	2196

Fig. 2. Absolute quantification of N and P cycle genes in the water column (a) and on the biofilm (b), the star map in the water column (c) and on the biofilm (d), and the circulation path in the water column (e) and on the biofilm (f). C1: Control group in brush unit; C2: Control group in ceramsite unit; T1: Treatment group in brush unit; T2: Treatment group in ceramsite unit. In the column diagram (c) and (d), the brush group is on the left column, and the ceramsite group is on the right column.

was highly positively correlated with nirS, nosZ and ureC, *Tenacibaculum* was positively correlated with gcd and phnK (Fig. 5a). *Bacillus* in ceramsite treatment group was positively correlated with nirS, nosZ and phnK, *Kocuria*, *Edaphobaculum*, *Anderseniella* and *Ruegeria* were positively correlated with gcd, *Muricauda* and *Sedimentitalea* were positively correlated with nosZ, nirS etc. which were related to denitrification (Fig. 5b). On the biofilm of the brushes with ECB, *Hanstruepera* and *Maribacter* were positively correlated withnarG and phoD, *Jejudonia* was positively correlated with nirS, ureC, amoB and napA, *Muricauda* was positively correlated with nifH and bpp, *Psychroserpens* and *Ruegeria*

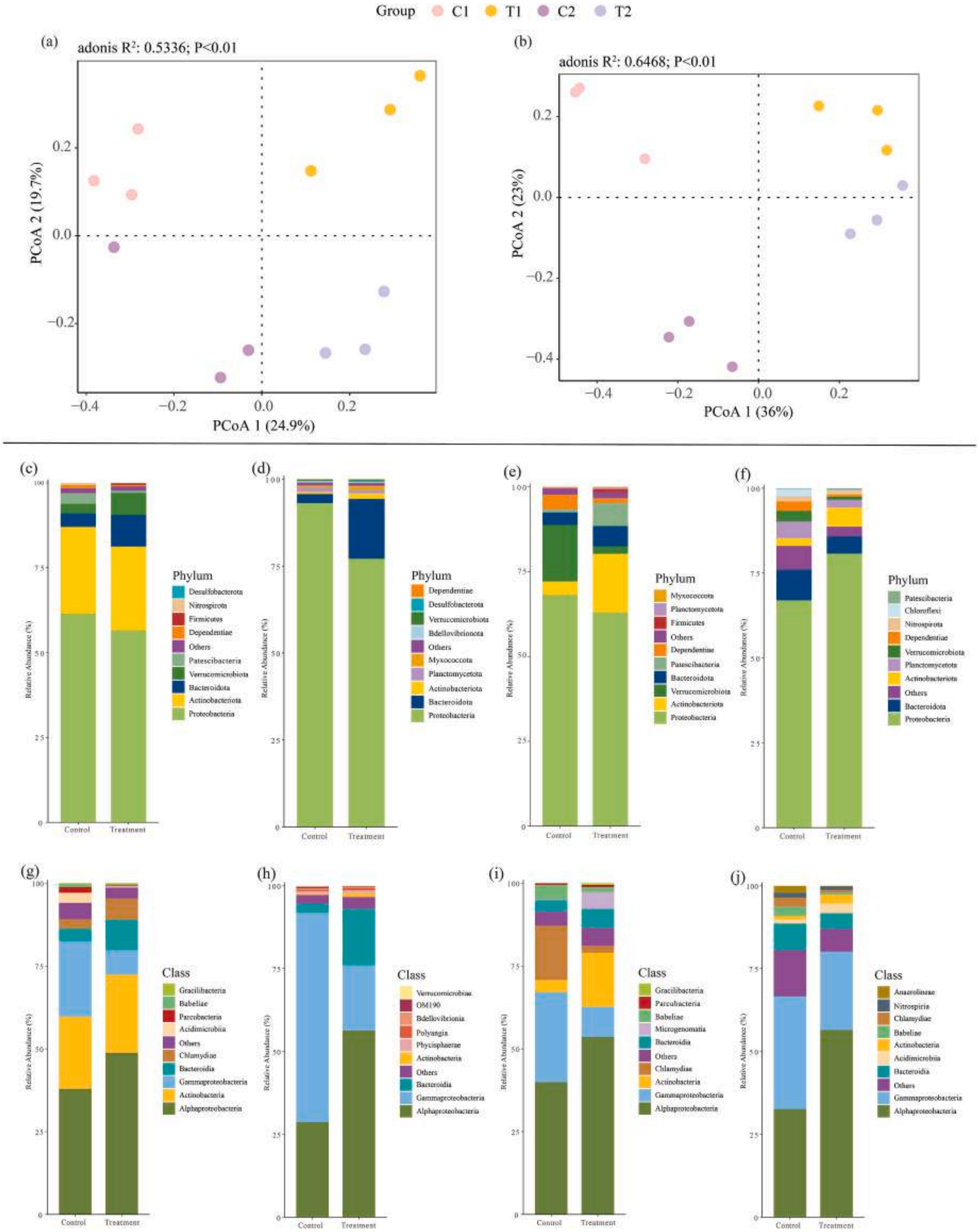


Fig. 3. Primary co-ordinate analysis of bacterial community in the water (a) and on the biofilm (b), dominant phylum of bacteria (top 10) in the water of the brush unit (c), on the biofilm of the brush unit (d), in the water of the ceramsite unit (e) and on the biofilm of the ceramsite unit (f), and dominant class of bacteria (top 10) in the water of the brush unit (g), on the biofilm of the brush unit (h), in the water of the ceramsite unit (i) and on the biofilm of the ceramsite unit (j).

were positively correlated with genes related to denitrification, anaerobic ammonium oxidation, assimilatory nitrate reduction, organic N mineralization and P transformation (Fig. 5c). *Bacillus*, *Kocuria* and *Photobacterium* in ceramsite treatment group was positively correlated with phoX and phnK, *Mycobacterium* and *Erythrobacter* were positively correlated with genes related to denitrification, anaerobic ammonium oxidation, assimilatory nitrate reduction and P transformation, *Algisphaera* and *Maritalea* were positively correlated with nirS, gdhA and phoD, *Paracoccus* and *Pseudohaliea* were positively correlated with nirK, bpp and ppk (Fig. 5d).

4. Discussion

4.1. Correlation between nutrients and biomarkers in IBSAW

The IBSAW has been widely used in the treatment of aquaculture

Table 1
Quantitative impact of adding ECB treatment on differences in bacterial community structure with PERMANOVA analysis based on Bray-Curtis.

Substrate	Medium	Treatment	
		R^2	P
Brush	Water	0.597	0.030
	Biofilm	0.597	0.028
Ceramsite	Water	0.644	0.025
	Biofilm	0.449	0.029

wastewater due to its low cost and environmental friendliness (Ahmad et al., 2022; Khanjani et al., 2022; Wei et al., 2022). However, the technologies of the IBSAW are not fully mature, the process and nutrients removal performance of the system could be further optimized (Aquilino et al., 2020; Tom et al., 2021). Microorganisms of the systems are closely related to the nitrogen-phosphorus cycle, and many microorganisms are conducive to the removal of nitrogen-containing compounds in wastewater (Deng et al., 2021). Bacteria can transform N through nitrification, denitrification, uptake and anaerobic oxidation of ammonia by nitrate and N fixation (Baneras et al., 2012). For example, *Enterobacter*, *Azospirillum*, *Pseudomonas*, *Klebsiella*, and *Vibrio* are common nitrogen-fixing bacteria (Lamers et al., 2012, López Guerrero et al., 2012). In this study, nutrients removal performance of the IBSAW were significantly promoted by the synergy of the ECB and functional genera (Fig. 4). In the water collum and biofilms, *Bacillus* belonging to ECB became the biomarker in the ceramsite treatment group and showed a significant negative correlation with NH_4^+-N, promoting the removal of NH_4^+-N (Fig. 4a, c and d). Biomarkers refer to organisms with significant differences in each group, which to reflect the situation of the environment. In the process of wastewater treatment, beneficial and functional microorganisms can achieve better treatment effects if they can dominate the community. In this study, compared with the control group without ECB, many functional genera in the water and biofilm of the treatment group became biomarkers, such as *Tenacibaculum*, *Polaribacter*, *Kocuria* and *Ruegeria*, etc., which promoted the removal of nutrients (Fig. 4). The results of the correlation between biomarkers and nutrients in the water column showed that *Tenacibaculum* in brush treatment groups was negatively correlated with NH_4^+-N, suggesting that *Tenacibaculum* inhibits the concentration of NH_4^+-N (Fig. 4b). In network analysis, *Tenacibaculum* was negatively correlated with nifH acting on the N fixation process (Fig. 5a), suggesting that *Tenacibaculum* inhibits N fixation and reduced the transition from N_2 to NH_4^+-N. *Tenacibaculum* supported efficient N removal through traditional aerobic nitrification and anoxic denitrification or simultaneous nitrification and denitrification (Liu et al., 2022). In addition, some studies have verified that *Tenacibaculum*'s cultivable HNAD strains participate in high salinity wastewater treatment through microbial community analysis (Cho et al., 2018; Lu et al., 2018). *Polaribacter* was significantly negatively correlated with NH_4^+-N (Fig. 4b). When probiotics with *Polaribacter* as the dominant population were added, the concentration of organic substances and ammonia in the aquaculture wastewater could be significantly reduced (Wang et al., 2020b). *Kocuria* and *Ruegeria* in ceramsite treatment group were negatively correlated with NH_4^+-N and ureC (Figs. 4b and 5b). The ureC is the gene that dominates the ammonification, so it can be inferred that *Kocuria* and *Ruegeria* inhibit ureC, leading to a decrease in NH_4^+-N. As a N cycling bacterium, *Ruegeria* plays an important role in the denitrification process and is often reported (Magalhães et al. 2011, 2012; Choi et al., 2016). In addition, *Pseudonocardia*, *Denitromonas* and NO_2^--N were negatively correlated (Fig. 4b). *Pseudonocardia* belongs to actinomycetes, and N degradation can occur through different ways of actinomycetes species (He et al., 2021). The study on the efficient removal of ammonia and nitrite in the brackish and freshwater aquaculture system by the microbial enrichment alliance showed that *Denitromonas* became the dominant group at the genus level in the alliance (Tuyet et al., 2022). All these have confirmed the advantages of ceramsite treatment group in removing NO_2^--N.

The microbial composition on biofilm was obviously different from that of water column, and the colonized microorganisms on different carriers were also significantly different. *Bacillus* was a biomarker in the water column and on the biofilm of the ceramsite treatment group, and its abundance was strongly negatively correlated with NH_4^+-N on the biofilm (Fig. 4d). Many studies have reported the effect of *Bacillus* in purifying aquaculture wastewater. In the study of purifying coastal aquaculture wastewater, *B.subtilis* and *B.amyloliquefaciens* have achieved a removal rate of more than 80% of NH_4^+-N (Shao et al., 2021a; Shao et al., 2021b). *Hanstruepera*, *Jejudonia*, *Maribacter*, *Psychroserpens* and *Ruegeria* in brush group were negatively correlated with TN (Fig. 4d), and they were all positively correlated with genes related to denitrification (Fig. 5c). So, it was probably that they promoted denitrification, and accelerated the process of reducing inorganic N in the water to gaseous N compounds. *Mycobacterium*, *Algisphaera* and *Erythrobacter* in ceramsite treatment group were negatively correlated with NO_2^--N and TP (Fig. 4d). They were positively correlated with genes related to denitrification and P transformation (Fig. 5d). Thus, they promoted the transformation of NO_2^--N and P. The report on the study of membrane bioreactor also shows that *Erythrobacter* contributes to the denitrification process (Choi et al., 2021). The maximum NO_2^--N removal rate of the isolated *Photobacterium* from the circulating water aquaculture system reaches 4.5 mg/L/h (Liu et al., 2019). *Rhodospirillaceae* in brush treatment group was significantly negatively correlated with NO_3^--N (Fig. 4d) and denitrifying strains belonging to *Rhodospirillaceae* could grow anaerobically in the dark and reduce NO_3^--N to N_2 (Sirisena et al., 2018). As a word, in the IBSAW improved by ECB, many microorganisms in the water and biofilm that were positively correlated with denitrification pathways have become biomarkers, leading the removal of nutrients in wastewater. And possibly due to the porous nature of ceramsite, more functional genera were attached to them, which were active in various pathways of denitrification.

4.2. N and P cycle and functional gene distribution

To understand the mechanisms of nutrients removal efficiency of the systems more intuitively and deeply, this study measured the absolute quantitative data of genes related to N and P cycle using the Geochip gene array. In the water environment, the abundance of ureC was relatively higher in treatment groups (Fig. 2a). In the biological denitrification process, ammonification needs to be completed first, and then the next step of nitrification (Forbis Stokes et al., 2020). Nitrification is an effective method to remove ammonia and organic pollutants (Jin et al., 2017). The abundance of amoAB was also significantly higher than that of the control group (Fig. 2a). The high salinity of seawater will lead to an increase in viscosity, which to some extent affects the diffusion of oxygen (Zhang et al., 2017). Under the restriction of oxygen, ammonia is oxidized to nitrite by ammonia-oxidizing bacteria (Li et al., 2017). The remaining ammonia and nitrite are removed by anammox process, and then nitrite and nitrate are reduced to N_2 by denitrification (Lu et al., 2020). The nosZ in ceramsite treatment group was significantly higher than that in other groups (Fig. 2a).

The core of the biofilm unit is the biofilm structure formed by bacterial adhesion on the biological carrier (Zhang et al., 2017). Therefore, biological carriers play a crucial role in influencing biological nitrification performance (Deng et al., 2016), which directly control the growth and reproduction of bacteria during biofilm formation (Wang et al., 2018). Porous matrix (such as ceramsite) is widely used in wastewater treatment, it is not only due to superior absorbability and habitats for microbes, but also well reoxygenation in porous substrates by “tidal operation” which is beneficial to simultaneous nitrification and denitrification process (Tan et al., 2021). As a very durable biological carrier, PE material is also often used in sewage treatment processes (Zhao et al., 2019). The use of different types of biological carriers can

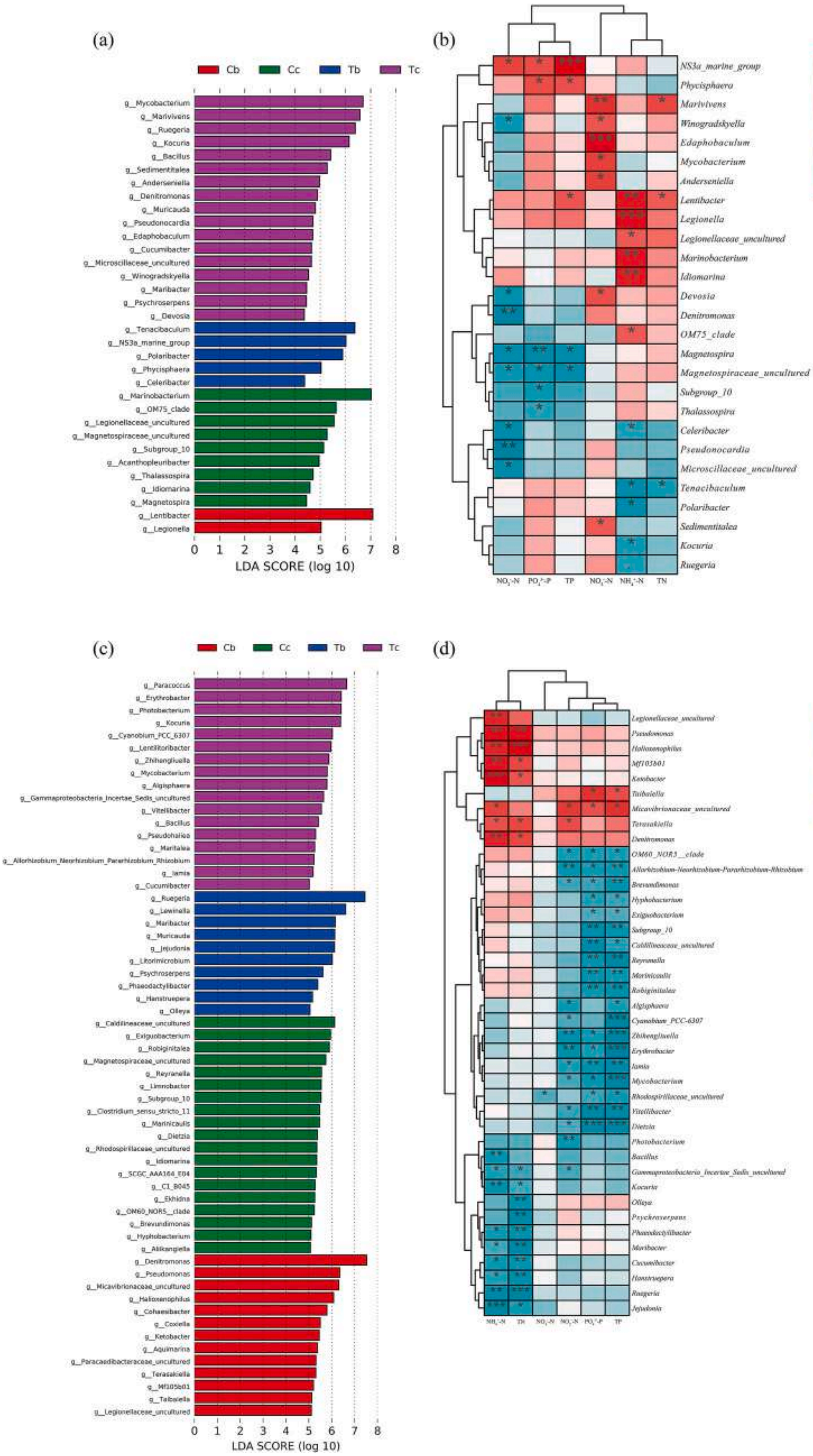


Fig. 4. LDA score histograms at a threshold of 4 in the water column (a) and heatmap of bacteria-nutrient correlations in the water column (b), LDA score histograms at a threshold of 5 on the biofilm (c) and heatmap of bacteria-nutrient correlations on the biofilm (d). C1: Control group in brush unit; C2: Control group in ceramsite unit; T1: Treatment group in brush unit; T2: Treatment group in ceramsite unit.

Table 2
Key topological parameters statistics of the network.

Groups		Nodes	Edges	Average degree	Modularity	ACC	Density	Diameter	APL
Water	Tb	35	123	7.029	0.455	0	0.207	3	1.6
	Tc	62	192	6.194	0.624	0	0.102	3	1.701
Biofilm	Tb	74	429	11.595	0.478	0	0.159	3	1.558
	Tc	78	450	11.538	0.607	0	0.15	3	1.573

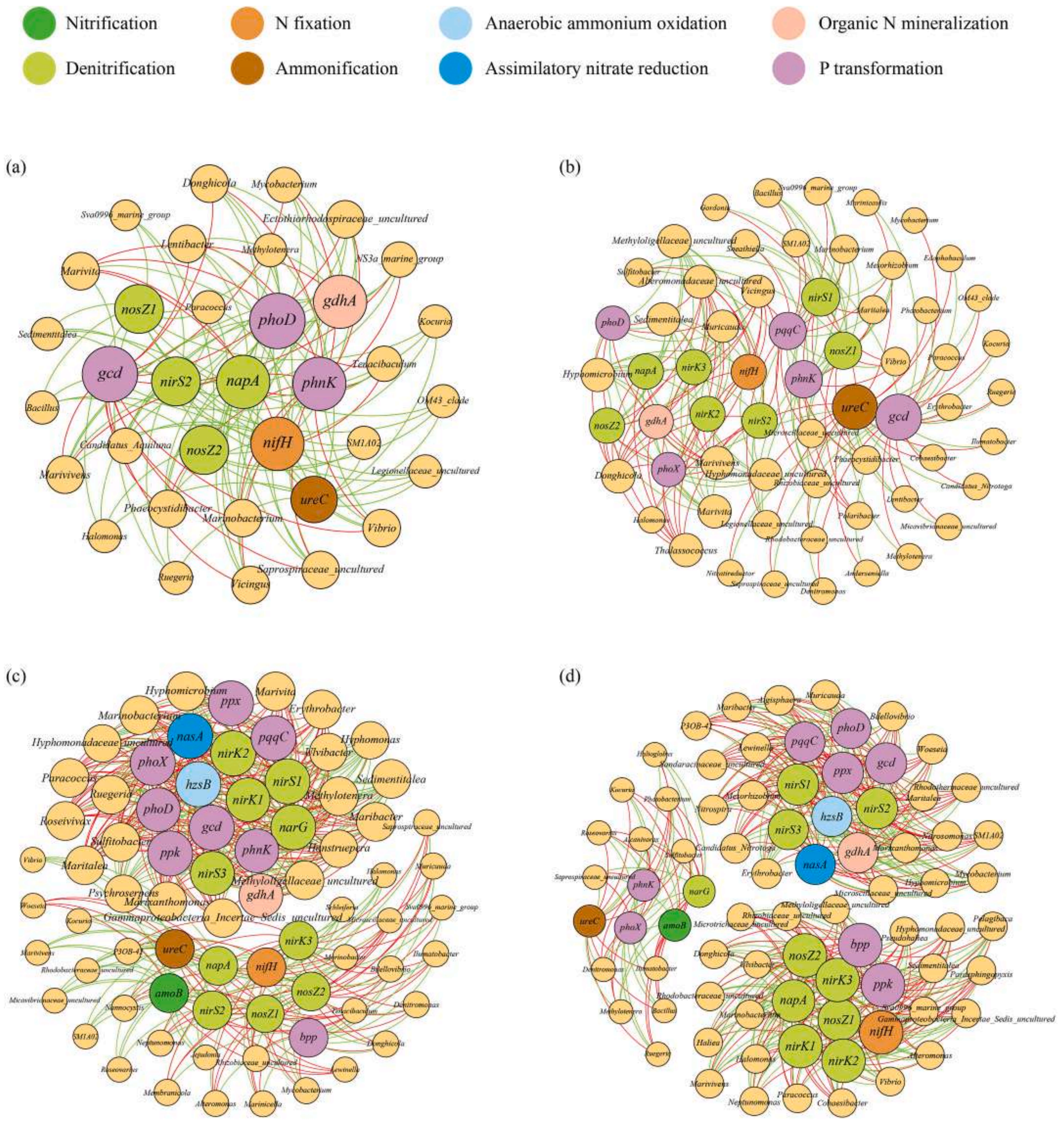


Fig. 5. Network analysis of relationships between genus and genes in the water column of brush treatment group (a) and ceramsite treatment group (b), on the biofilm of brush treatment group (c) and ceramsite treatment group (d). Line color represents positive and negative correlation, green is positive correlation, red is negative correlation.

change the abundance of N metabolism function genes during wastewater treatment (Zhu et al., 2022). The gene abundance of the brush group was significantly higher than that of the ceramsite group (Fig. 2). On the biofilm, the ureC abundance of the brush group was high, which indicated that the ammonification in the brush unit is active. The amoB abundance in the brush group with ECB has reached more than 10^4, the abundance of nirS2, nosZ and napA has reached 10^5 (Fig. 2b), indicating that the nitrification and denitrification in the subsequent N removal steps also follow up normally. But the abundance of related genes in the control group was far lower than that in the treatment group (Fig. 2f). The nitrification and denitrification efficiency of the control group was not as high as that of the treatment group, which was also reflected in the N content. The content of NO_3^--N, NH_4^+-N and TN of the treatment group was significantly lower than that of the control group (Fig. 1).

In terms of P cycle, in the brush treatment groups, the phnK in the water and on the biofilm reached 10^4 (Fig. 2). In bacteria, phnK is one of the most critical genes encoding the carbon-phosphorus lyase complex, which is necessary for the metabolism of unactivated phosphonates to phosphate (Yang et al., 2016). The phnK improves the absorption and utilization of phosphorus-containing compounds through transmembrane transport (Tian et al., 2021). The ppk and ppx catalyze the accumulation and release of P in aerobic and anaerobic processes respectively (Yang et al., 2019), and phoD gene is involved in the transport of organic phosphorus (Willis et al., 2019). The abundance of the phosphorus absorption genes ppk and phnK in the brush and ceramsite treatment groups were higher than those of the phosphorus release genes ppx and phoD. The abundance of the phosphorus absorption genes in the treatment group was also higher than that in the control group (Fig. 2), which indicated that the PO_4^{3-}-P removal effect of the system has been improved (Fig. 1).

4.3. The link between bacteria and genes

The *Bacillus* species have a large number of gene banks of nitrate reduction and denitrification, including dissimilatory nitrate/nitrite reduction to ammonium (DNRA), membrane-bound denitrification, and a periplasmic nitrate reductase pathway (Sun et al., 2016).

Bacillus showed a high positive correlation with nirS and nosZ in the brush and ceramsite treatment groups (Fig. 5). In the wastewater sequencing batch reactor strengthened by *Bacillus*, it was observed that the important functional genes nirS and nosZ involved in denitrification showed a continuous increase (Yang et al., 2017). The gene abundance on the biofilm was significantly higher than that of the water column. On the biofilm, the abundance of nirS and nosZ genes in the treatment groups were significantly higher than that of the control group (Fig. 2f). The higher abundance of nirS genes plays a key role in nitrite reduction (Yang et al., 2017). As a marker of complete denitrification, nosZ gene is used to encode nitrous oxide reductase responsible for N_2O to N_2 reduction (Stres et al., 2004). Its increase promotes the last step of denitrification, which not only leads to complete denitrification, but also may reduce N_2O emissions (Yang et al., 2017). *Bacillus* was positively correlated with nasA of assimilatory nitrate reduction in ceramsite treatment group (Fig. 5d). In the study of bioaugmentation using *Bacillus subtilis*, it is shown that the overexpression of nitrogen assimilation gene of *Bacillus subtilis* leads to the increase of denitrification (Rahimi et al., 2020). The increase of these important functional genes closely related to denitrification proves that *Bacillus* promotes denitrification. And denitrification process played a leading role in the nitrogen cycle (Zhang et al., 2021). *Psychroserpens* was positively correlated with hzsB in brush treatment group (Fig. 5c), and its absolute abundance was positively correlated with anammox activity (Zhang et al., 2020). Anammox bacteria used ammonia as electron donor, nitrite as electron acceptor, and produced nitrogen and nitrate (Kuenen, 2008). In the study of using artificial tidal wetland ecosystem to treat saline water, it was found that hzsB participates in controlling the conversion process of NH_4^+-N and TN (Zhang et al., 2023). In this study, the content of TN significantly decreased, especially in the brush treatment group, with a removal rate of over 50% (Fig. 1d). As a biomarker in the brush treatment group, *Psychroserpens* showed a highly significant negative correlation with TN (Fig. 4d, $P < 0.01$). Therefore, it can be inferred that the ECB significantly changed the bacterial composition of the original wastewater (Table 1, $P < 0.05$), causing the growth of functional bacteria like *Psychroserpens*, thereby controlling the TN content in the wastewater. In general, the abundance of ureC gene is proportional to the ammonia concentration (Sun et al., 2020), but the results in our study showed that the treatment group has a high removal rate of NH_4^+-N (Fig. 1), which may be due to the combination of ammonia and nitrite into nitrate and nitrogen, and the active anammox reaction in the ceramsite treatment group, leading to the lower nitrate degradation efficiency than the control group. The main manifestation in brush group was a significant decrease in the content of NH_4^+-N and TN (Fig. 1c and d), which was also due to the presence of multiple biomarkers highly positively correlated with denitrification genes, such as *Hanstruepera*, *Jejudonia*, *Maribacter*, *Muricauda*, *Psychroserpens* and *Ruegeria* (Fig. 4a and b). These functional genera all promoted the transformation of inorganic N. In ceramsite treatment groups, the NO_2^--N content decreased significantly (Fig. 1a), and denitrification also played a significant role. *Muricauda*, *Sedimentitalea*, *Mycobacterium*, *Algisphaera*, *Maritalea*, *Paracoccus*, *Erythrobacter* and *Pseudohaliea*, which were positively correlated with denitrification also became the biomarkers in ceramsite with ECB (Fig. 4a and c). The colonization of functional microorganisms in IBSAW was of great significance for nitrogen and phosphorus cycling. The beneficial microorganisms added from external sources can promote the formation of more efficient microbial communities to replace indigenous bacteria as the dominant species. This directly affected the abundance of functional genes, making like denitrification more active, thereby promoting the removal of nutrients and achieving the goal of purifying water.

5. Conclusion

In this study, the national standard method was used to determine the nutrient content, 16S rRNA high-throughput sequencing and Geochip gene array were used to analyze the correlation between nutrients and bacteria, and the abundance of N and P cycle genes was quantified. The results showed that the removal rate of NO_3^--N and TN in the brush treatment group was more than 50%, the removal rate of NH_4^+-N, PO_4^{3-}-P and TP was more than 80%. The removal rate of NO_2^--N and PO_4^{3-}-P in ceramsite treatment group reached 70% and 80% respectively. The brush unit optimized by ECB significantly improved the removal efficiency of NO_3^--N and TN, while the ceramsite unit enhanced the removal of NO_2^--N. In the water column, the amoAB of brush and ceramsite treatment groups was significantly higher than that of their control groups. *Bacillus* was positively correlated with nirS and nosZ in brush treatment groups, and nosZ in ceramsite treatment groups was significantly higher than that in other groups. The nirKS and nosZ on the biofilm in the brush treatment group were significantly higher than those in the control group. Several bacterial genera such as *Ruegeria* and *Sedimentitalea* were positively correlated with multiple genes. *Bacillus* as the biomarker on the biofilm of ceramsite treatment group, was strongly negatively correlated with NH_4^+-N, and positively correlated with nirS, nosZ, hzsB, nasA, pggC, gcd and phnK. Conclusively, the ECB significantly affected the bacterial composition in the original wastewater, promoted the community succession process of bacteria, and promoted multiple functional genera highly positively correlated with denitrification genes to become biomarkers, thereby promoting denitrification and improving the efficiency of N conversion.

CRediT authorship contribution statement

Yingzhen Wei: Writing – original draft, Visualization, Data curation. **Ding Shen:** Data curation. **Regan Nicholaus:** Supervision. **Yangcai**

Wang: Project administration. **Betina Lukwambe:** Writing – review & editing. **Jinyong Zhu:** Writing – review & editing. **Wen Yang:** Writing – review & editing. **Zhongming Zheng:** Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Zheng Zhongming reports financial support was provided by National Key R & D Program of China.

Data availability

I have shared the link to my data at the Materials and methods

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Appendix A. Supplementary data

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