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The Responses of Sediment Bacterial Communities in Chinese Mitten Crab (*Eriocheir sinensis*) Culture Ponds to Changes in Physicochemical Properties Caused by Sediment Improvement

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Abstract: The interaction between nutrients, heavy metals, and sediment bacterial communities play a key role in the health of crabs and the biogeochemical cycles of aquaculture systems. However, the effects of sediment improvement activities in crab culture on nutrients and heavy metals and the response of bacterial communities to the relevant changes are unclear. In this study, 24 water and sediment samples were collected from two aquaculture sites (total of 12 ponds, 6 at each site). High-throughput sequencing was used to determine the structure of the bacterial community and the diversity in water and sediment samples. The relationship between nutrients, heavy metals, and bacterial communities and the changes of the three before and after the improvement of the sediment were analyzed. The results showed that Proteobacteria, Bacteroidetes, Acidobacteria, Chloroflexi, and Firmicutes were predominant at the phylum level of sediment. Sediment improvement has an effect on NH_4^+-N, sulfide, total organic carbon (TOC), and heavy metals in sediments to varying degrees. In addition, redundancy analysis found that NH_4^+-N, NO_3^--N, TP, and heavy metals were key drivers in crab culture pond sediments. The results of functional prediction showed that carbon, nitrogen, and sulfur metabolism were the dominant processes in the two crab farming areas. Overall, changes in nutrients and heavy metals caused by sediment improvement further affected the structure and function of bacterial communities and may affect biogeochemical cycles. Our study has deepened the understanding of the effects of sediment improvement on nutrients, heavy metals, and bacterial communities in crab culture ponds.

Keywords: sediment improvement; bacterial community; nutrients; heavy metals; crab cultured area

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1. Introduction

Owing to the rising global population and the increasing demand for animal protein consumption, the aquaculture industry has developed rapidly in recent decades. And now with the restriction on capture fisheries, aquaculture has developed into a major global agricultural industry [1]. The growing aquaculture industry, with the corresponding increasing use of commercial feed, has brought huge economic benefits while causing a large amount of nutrient loading and sediment accumulation in the aquaculture system [2]. As a result of the accumulation of nitrogenous wastes from animal excreta and the degradation of uneaten feed, the environment of the aquaculture system tends to deteriorate, which affects the immunity of farmed animals and enhances their susceptibility to disease, resulting in a significant reduction in the survival rate of farmed animals [3].

In addition to nutrients, heavy metals adversely affect aquatic habitats, which is becoming a big issue since they can have severe toxic effects on numerous organisms [4].

Sediments serve as repositories of heavy metals in aquaculture systems, and some aquaculture practices (bioturbation/bioirrigation) will further release heavy metals into water bodies, posing a huge threat to farmed animals [5]. Most of the heavy metals stored in sediments (Co, Cr, Pb, Zn, Mn, and Ni) are persistently toxic and bioaccumulative in ecosystems, which has aroused widespread concern, and these properties directly or indirectly affect microorganisms in the aquatic environment [6]. The stimulation caused by the interaction of the sediment microbiome with heavy metals through physicochemical interactions affects the abundance of the sediment microbial communities [7]. For example, Yu et al. (2016) showed in a study of the structure and function of sediment microbial communities in the Lanzhou section of the Yellow River that the long-term accumulation of Cr significantly enriched microbes associated with Cr reduction [8]. Cyanobacteria and Gemmatimonadetes were significantly associated with Cu and As, respectively. The phylum commonly found in marine sediments such as Planctomycetes, Verrucomicrobia, and Gemmatimonadetes showed a significant positive correlation with Cr [9]. Since different species of microorganisms exhibit different sensitivities to heavy metals and are closely related to aquaculture system functions (carbon fixation, nitrogen fixation, sulfur metabolism, etc.), heavy metals can indirectly affect the function of aquaculture systems by influencing the function of microbial communities [10]. Therefore, by understanding the impact of heavy metals on microbial diversity, community structure, and function, the harm of heavy metals to farmed organisms can be further reduced, and the aquaculture environment can be restored [11].

Sediment microorganisms, as key participants in the degradation and recycling of essential elements, such as C, N, and P in sediments, are affected by changes in the concentration of related elements [12]. Some studies have shown that microbial communities in aquaculture environments (water and sediments) are susceptible to specific site conditions [1,13,14]. Undeniably, aquaculture ecosystems can be influenced by human activities like aquaculture practices. The input of nutrients and heavy metals from aquaculture practice activities may lead to changes in the nature of sediments and disturbances in the ecological function of the aquatic environment, and ultimately affect microbial communities that are closely related to the function of the aquatic environment and sediments. Studies have emerged on the effects of nutrient levels and heavy metals on the aquatic environment and microbial communities in sediments, but few on the sources of nutrient and heavy metal inputs. Some aquaculture practices (e.g., water exchange, sediment improvement, and exposure) are aimed at addressing eutrophication and heavy metal pollution in cultured waters, but how microbial communities in aquaculture ponds respond to these measures remains little known. Chinese mitten crab (*Eriocheir sinensis*) is one of the most popular cultured species in Jiangsu Province, which tends to live in benthic areas and is susceptible to changes in nutrient and heavy metal levels in sediments, so it is important to improve sediment during its culture process and in the preparation process before breeding [15]. The input of nutrients and heavy metals may affect sediment microbes, but it is unclear how sediment microbial communities respond to sediment improvement during pre-culture preparation of Chinese mitten crab. Sediment improvement is a common aquaculture activity to improve the breeding environment in the process of crab culture, which mainly occurs after the harvest period, including dredging, pond drying, and other activities.

Therefore, in this study, water and surface sediments of culture ponds were collected from two crab farming areas of the Gaochun District and the Pukou District of Nanjing City, which have a long history of crab farming. The effects of aquaculture practices on nutrient and heavy metal accumulation were explored by measuring nutrient and heavy metal levels in water bodies and sediments. 16S rRNA sequencing was used to determine the diversity and structural composition of microbial communities in water and sediments. This study aims to explore the following three objectives, (1) to investigate the changes in nutrients and heavy metals in the water and sediments of river crab culture ponds before and after sediment improvement; (2) to explore the response of microbial communities to

nutrients and heavy metals in river crab culture ponds; (3) to predict microbial function under the influence of nutrients and heavy metals in crab culture ponds.

2. Materials and Methods

2.1. Study Site and Sampling

Surface sediment (1–5 cm) samples and water samples were collected from two aquaculture farms in December 2021 (before sediment improvement) and April 2022 (after sediment improvement) from the Gaochun District (31.27° N, 118.88° E) and the Pukou District (32.08° N, 118.41° E) (Figure 1). Samples collected for the first time (i.e., December 2021) are labeled as GC (Gaochun District) and PK (Pukou District)), while the samples collected for the second time (i.e., April 2020) are labeled as TGC (Gaochun District) and TPK (Pukou District). These aquaculture areas have been operated for 13 years, and the area of these ponds ranges from 15,000 m^2 to 27,000 m^2, mainly for the cultivation of Chinese mitten crab (*Eriocheir sinensis*). A total of 12 sediment samples and the 12 water samples (6 water samples and 6 sediment samples each time) were collected in duplicate from each aquaculture farm using a water sampler and grabber, respectively. Samples were divided into eight groups (PKW, PKS, GCW, GCS, TPKW, TPKS, TGCW, TGCW, TGCS) based on sample type, sampling location, and time (Table 1). The number of sediment samples and water samples collected was sufficient for DNA extraction and analysis of physicochemical parameters. The sediment samples and the water samples were packed in polyethylene bags and sample bottles, respectively, and stored at −20 °C after sampling.

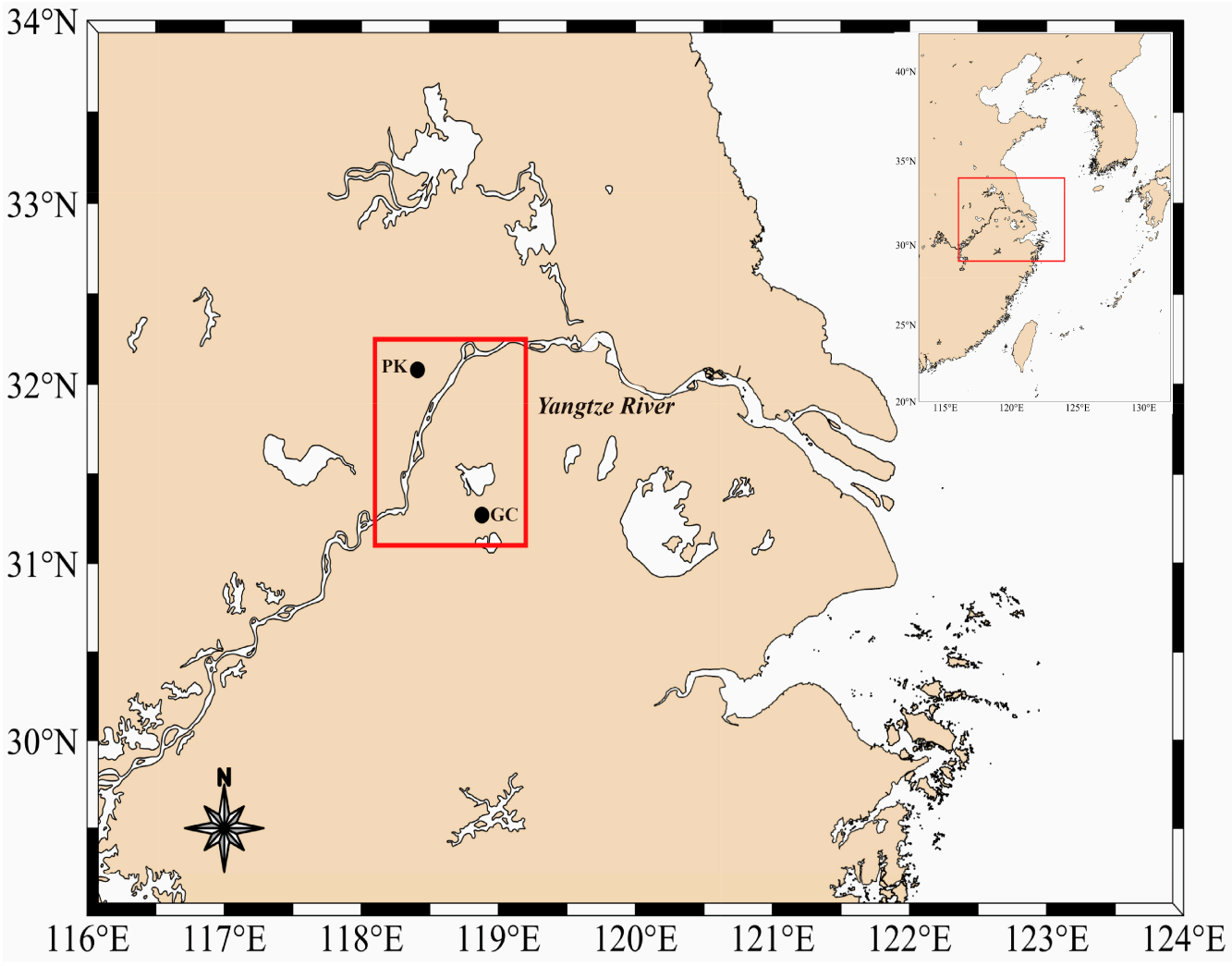

Figure 1. Location of the study area.

Table 1. Group information.

Sample Type	**Sediment**				**Water**			
Sampling period	Before sediment improvement		After sediment improvement		Before sediment improvement		After sediment improvement	
Sampling location	Gaochun District	Pukou District	Gaochun District	Pukou District	Gaochun District	Pukou District	Gaochun District	Pukou District
Groups	GCS	PKS	TGCS	TPKS	GCW	PKW	TGCW	TPKW

2.2. Determination of Physicochemical Properties and Metal Elements

The sediment samples were thoroughly mixed in the laboratory and divided into two parts, one part was subpackaged into three sterile polyethylene bags, sealed, and stored in a −80 °C cold storage for further 16srRNA analysis. The other part was dried in a vacuum freeze dryer after removing plant residues and debris, and then screened by a 100 mesh sieve and ground for the determination of metal elements and other physical and chemical indicators. Determination of the concentration of chemical parameters (NH_4^+-N, NO_3^--N, NO_2^--N, TN, TP, TOC, sulfide) and metal elements (Ca, Mg, Zn, Cr, Ni, Co, Cd, Pb, As, Hg) in sediment samples was conducted according to standard methods. The error of all data was expressed as mean value ± standard deviation.

300 mL of water was taken from each water sample, filtered with 0.22 μm PES membrane, and then the membrane was stored in sterile tubes at −80 °C until DNA was extracted, and the remaining samples were stored at 4 °C for chemical characterization, including NH_4^+-N, NO_3^--N and NO_2^--N, all of which were determined, with standard methods adopted (China Environmental Protection Administration, 2002).

2.3. DNA Extraction, PCR Amplification, and Sequencing Analysis

Total genomic DNA from water membranes and 500 mg frozen sediment were extracted using OMEGA kit E.Z.N. A™ Mag-Bind Soil DNA Kit according to manufacturer's instructions. The purity of genomic DNA was detected by 0.8% agarose gel electrophoresis, and its concentration was determined with Qubit® 3.0 fluorometer (Invitrogen, Waltham, MA, USA). The V3 and V4 regions of bacterial 16SrRNA genes amplified with universal primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′). PCR reactions were conducted in triplicate of each sample. The PCR mixture (total volume 30 μL) contained 20 ng template DNA, 15 μL 2×Hieff® Robust PCR Master Mix, 9~12 μL H_2O, and 1 μL of each primer. The PCR amplification conditions were as follows: initial denaturation at 95 °C for 3 min, followed by five cycles (denaturation at 94 °C for 30 s, annealing at 45 °C for 20 s, and extension at 65 °C for 30 s) and 20 cycles (20 s at 94 °C, 20 s at 55 °C, and 30 s at 72 °C), and final elongation at 72 °C for 5 min. The PCR products were measured by 2% agarose gel electrophoresis, and then a sequencing library was generated, and the library concentration was determined in Qubit® 3.0 fluorometer. The library was ultimately performed using Prep Kit (Illumina, San Diego, CA, USA) according to manufacturer's instructions and paired-end sequencing (2 × 300 bp) on the Illumina MiSeq platform at Sangon Biotech Co., Ltd. (Shanghai, China).

2.4. Bioinformatic Analysis

Raw sequence data was processed in USEARCH (Edgar et al., 2010) to remove low-quality and short sequences and obtain high-quality clean data [16]. The paired-end sequences were merged and relabeled by "-fastq_mergepairs"; and then "-fastq_filter" and "-derep_fulllength" filter were used, respectively, to filter and remove redundant sequences. After denoising by unoise3 to obtain ASVs (amplification sequence variant), chimeras are removed using VSEARCH. Then, the operational taxonomy units (OTUs) table was generated using "-otutab". Based on the Ribosome Database Project (RDP), Bayesian algorithm was used to obtain taxonomic affiliations of OTU sequences with a threshold of 0.6 and the species annotations were divided into five levels: phylum, class, order, family,

and genus [17]. All archaea, plasmids, and other sequences that cannot be assigned to bacteria were removed by species annotation (including five levels of phylum, class, order, family, and genus), resulting in an OTU feature table. To further analyze the complexity of microbial diversity within the sample, alpha diversity (i.e., Chao1, Richness, Shannon, Simpson) was calculated by USEARCH (v10.0.0). In order to evaluate the differences of microbial complexity among samples, the beta diversity based on both Euclidean and Bray-Curtis matrices was calculated by USEARCH (v10.0.0).

2.5. Statistical Analyses

The top 20 species with the greatest abundance in each sample were selected for the species annotation results at the phylum level and genus level to generate histograms of relative abundance of species. Based on Bray-Curtis distances, non-metric multidimensional scaling (NMDS) analysis was adopted to compare the differences between different types of samples, locations, and dates. The relationship between environmental factors and bacterial communities was examined using random forest models and redundancy analysis (RDA). The Pearson correlation coefficient calculated based on the Pearson correlation analysis was used to assess the statistical correlation between different variables, and the similarity matrix was obtained after the highly correlated variables ($r_p > 0.70$ and $r_p < -0.70$) were removed. FAPROTAX and PICRUSt2 (v.2.3.0) were used to predict the function of bacterial 16S amplicon sequencing data [18,19]. STAMP (v2.1.3) tested the distribution of dominant bacterial phyla and the statistically significant difference between pre- and post-improvement sample prediction function groups with Welch's *t*-test and multiple tests by Benjamini-Hochberg False Discovery Rate (FDR). Then, heat map clustering was obtained by R software (v 3.6.1) for analyzing the prediction functions of different groups. For the predicted functional groups, the relationships between the abundance of functional groups and environmental variables were examined by Spearman's rank correlations and plotted using origin6.0. One-way ANOVA using orgin6.0 was used to evaluate differences in physicochemical factors before and after sediment improvement.

3. Results

3.1. Physicochemical Characteristics of Water and Sediment Samples

To understand the changes in nutrient levels before and after aquaculture farm improvement, the physicochemical characteristics of surface water of the ponds, including NH_4^+-N, NO_3^-N, and NO_2^--N, were monitored in the laboratory. The results showed that the NH_4^+-N range of water samples in the GC group and PK group was 0.158–3.055 mg/L, and they reached a peak at the PK2 site. Compared with the samples before the sediment improvement (GC group and PK group), in the samples after the sediment improvement (TGC group and TPK group), the NH_4^+-N concentration of surface water samples in the Pukou District decreased (except TPK1), whereas the NH_4^+-N concentration of samples in the Gaochun District showed different levels of increase (Figure 2). The NO_3^--N concentration of the water samples in the Gaochun District decreased after the sediment improvement (i.e., TPK<PK), and the NO_3^--N concentrations of the two sample points peaked at site 3 (i.e., GC3 and PK3), and the NO_2^--N content of the samples in the Gaochun District was higher than that in the Pukou District.

The nutrient concentrations in sediments after sediment improvement (TGC and TPK groups) varied greatly from those before sediment improvement (GC and PK groups). NH_4^+-N concentrations in the TPK group and the sulfide content in the TGC group were reduced compared to those in the GC and PK groups (Figure 3). The total nitrogen (TN) and total phosphorus (TP) concentrations of the improved sediment samples in the Pukou area were higher than those of the pre- improved sediment samples (i.e., TPK > PK), while the change in total nitrogen concentrations in the Gaochun District (except GC1 and TGC1) was the opposite (Figure S1). The total organic carbon (TOC) content of both the TGC and TPK groups (except TPK1 and TPK3) was lower than that in the GC and PK groups (Figure 3). The concentration of heavy metals after sediment improvement (TGC and TPK groups) was

significantly different from that of pre-improved (GC and PK groups). The concentrations of Ca and Mg in the improved sediment samples increased overall compared to those before the sediment improvement, while the change in cadmium concentrations was the opposite (Figure 4). Despite the uneven distribution and great difference of the remaining seven heavy metal elements, none of them exceeded the standard value range specified by the state (Table 2).

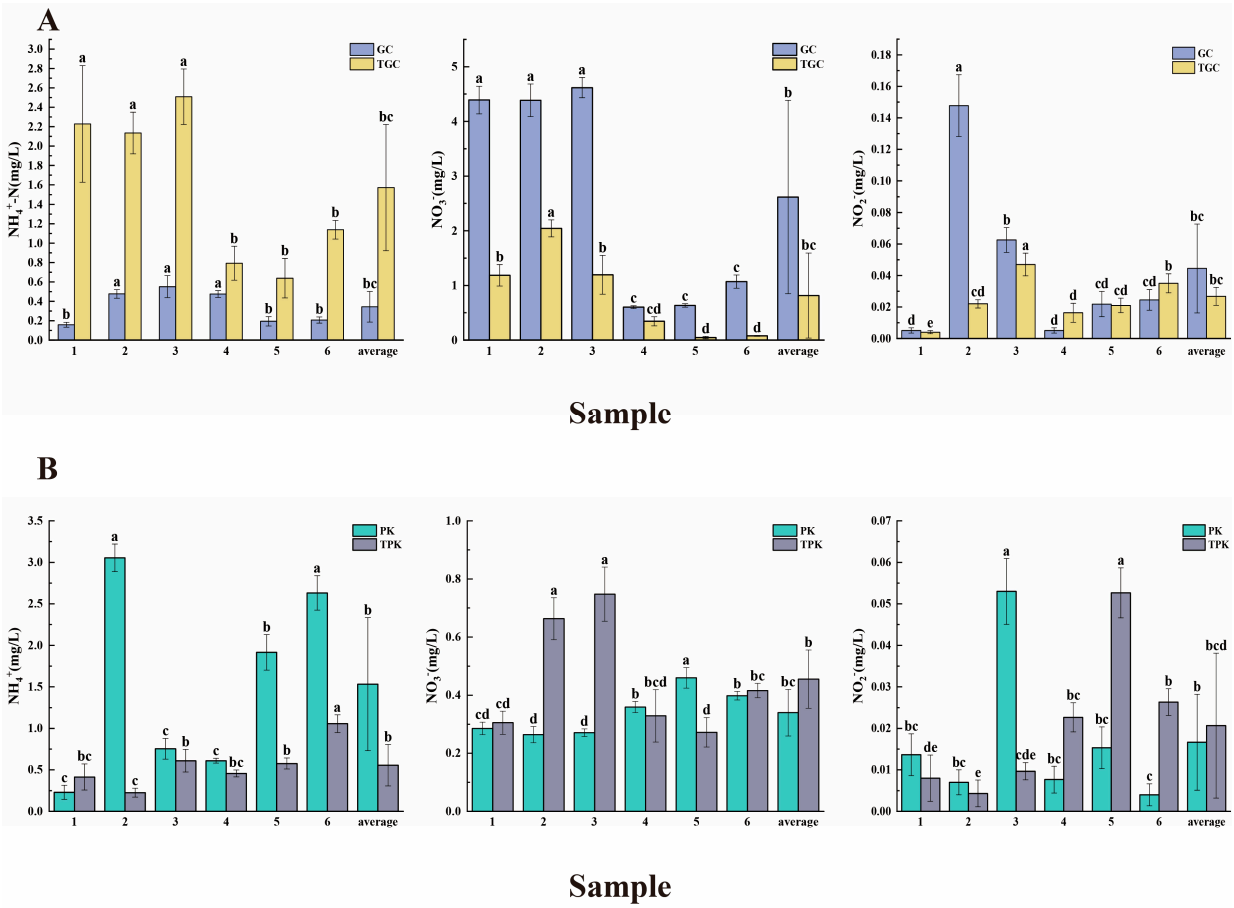

Figure 2. Physicochemical characteristics of water samples. (**A**) NH_4^+-N, NO_3^--N and NO_2^--N concentration of GC and TGC: GC: samples of the Gaochun District before sediment improvement; TGC: samples of the Gaochun District after sediment improvement; (**B**) NH_4^+-N, NO_3^-N, and NO_2^--N concentration of PK and TPK: PK: samples of the Pukou District before sediment improvement; TPK: samples of the Pukou District after sediment improvement; average: the average of GC, TGC PK, and TPK. Values were given as mean ± SD, and different letters in the upper (a, b, c, d, e) indicate a significant difference ($p < 0.05$) before and after sediment improvement based on the analysis of variance.

Table 2. Heavy metals of the sediment (mean value ± standard deviation).

Sample	Zn (mg/kg)	Cr (mg/kg)	Ni (mg/kg)	Cu (mg/kg)	As (mg/kg)	Pb (mg/kg)	Hg (mg/kg)
PK1	92.33 ± 1.53	81.33 ± 2.52	42.33 ± 3.06	51.33 ± 3.51	11.80 ± 1.13	21.33 ± 2.06	0.20 ± 0.02
PK2	97.67 ± 3.06	86.33 ± 2.89	38.67 ± 3.79	53.67 ± 4.16	16.60 ± 1.48	21.40 ± 3.20	0.16 ± 0.01
PK3	93.67 ± 3.06	90.67 ± 0.58	40.67 ± 0.58	52.33 ± 3.21	26.80 ± 1.40	20.70 ± 2.45	0.15 ± 0.01
PK4	84.67 ± 1.53	79.00 ± 4.36	37.00 ± 2.65	54.00 ± 2.65	11.57 ± 1.46	21.37 ± 1.78	0.15 ± 0.01
PK5	90.33 ± 2.31	82.00 ± 4.58	41.67 ± 4.16	50.67 ± 0.58	12.40 ± 0.92	23.63 ± 1.31	0.15 ± 0.03
PK6	90.67 ± 2.08	90.00 ± 3.00	41.67 ± 3.79	53.33 ± 3.06	9.18 ± 0.34	24.80 ± 1.93	0.18 ± 0.02
GC1	88.33 ± 2.52	63.33 ± 4.93	38.33 ± 2.52	43.00 ± 4.58	8.97 ± 0.48	30.53 ± 1.67	0.10 ± 0.01
GC2	72.00 ± 3.61	38.67 ± 4.51	22.67 ± 3.21	27.67 ± 3.79	5.53 ± 0.40	20.83 ± 2.49	0.09 ± 0.02

Table 2. *Cont.*

Sample	Zn (mg/kg)	Cr (mg/kg)	Ni (mg/kg)	Cu (mg/kg)	As (mg/kg)	Pb (mg/kg)	Hg (mg/kg)
GC3	80.33 ± 4.04	46.67 ± 3.21	28.33 ± 4.73	30.33 ± 2.08	5.89 ± 0.31	23.93 ± 2.10	0.09 ± 0.01
GC4	77.00 ± 2.00	49.33 ± 3.06	32.67 ± 2.52	45.00 ± 1.73	8.97 ± 0.21	25.23 ± 0.95	0.09 ± 0.01
GC5	85.00 ± 4.00	42.67 ± 1.53	35.33 ± 4.51	53.00 ± 1.73	11.67 ± 1.82	31.67 ± 1.16	0.12 ± 0.02
GC6	91.33 ± 1.53	50.33 ± 2.52	33.67 ± 4.73	46.33 ± 2.08	8.22 ± 0.31	31.13 ± 0.67	0.11 ± 0.00
TPK1	92.67 ± 3.06	72.00 ± 8.19	45.00 ± 3.46	45.00 ± 1.00	12.43 ± 1.50	23.27 ± 4.80	0.09 ± 0.01
TPK2	94.67 ± 2.08	71.33 ± 6.43	51.00 ± 1.73	45.00 ± 1.00	12.90 ± 1.75	22.07 ± 3.81	0.10 ± 0.01
TPK3	94.00 ± 7.55	66.67 ± 10.69	48.33 ± 6.66	48.33 ± 1.53	14.50 ± 0.96	23.63 ± 5.08	0.12 ± 0.02
TPK4	98.33 ± 1.15	91.00 ± 6.08	52.00 ± 3.46	45.33 ± 2.31	18.87 ± 3.25	26.07 ± 5.22	0.16 ± 0.03
TPK5	88.67 ± 3.21	50.00 ± 3.00	34.33 ± 2.08	38.33 ± 1.53	30.63 ± 5.16	29.90 ± 3.12	0.19 ± 0.01
TPK6	82.00 ± 1.00	63.67 ± 5.86	37.67 ± 5.51	41.33 ± 1.15	21.43 ± 3.42	28.20 ± 2.16	0.15 ± 0.01
TGC1	78.67 ± 2.08	51.33 ± 5.03	37.67 ± 4.51	29.33 ± 1.53	9.89 ± 1.16	22.00 ± 4.57	0.09 ± 0.01
TGC2	90.33 ± 0.58	45.00 ± 5.00	44.00 ± 4.00	36.33 ± 0.58	8.68 ± 1.22	26.40 ± 5.15	0.08 ± 0.01
TGC3	79.67 ± 0.58	51.00 ± 2.00	37.67 ± 2.89	34.67 ± 2.31	12.60 ± 0.92	22.87 ± 3.86	0.12 ± 0.01
TGC4	88.67 ± 3.06	49.67 ± 3.51	37.33 ± 2.52	34.00 ± 1.00	11.27 ± 1.36	25.00 ± 1.23	0.12 ± 0.01
TGC5	91.00 ± 3.46	44.67 ± 4.93	38.67 ± 4.16	37.00 ± 0.00	11.97 ± 1.01	20.90 ± 1.84	0.14 ± 0.02
TGC6	95.00 ± 1.00	65.67 ± 5.03	35.00 ± 4.36	37.33 ± 1.15	13.90 ± 2.40	24.97 ± 5.72	0.19 ± 0.04

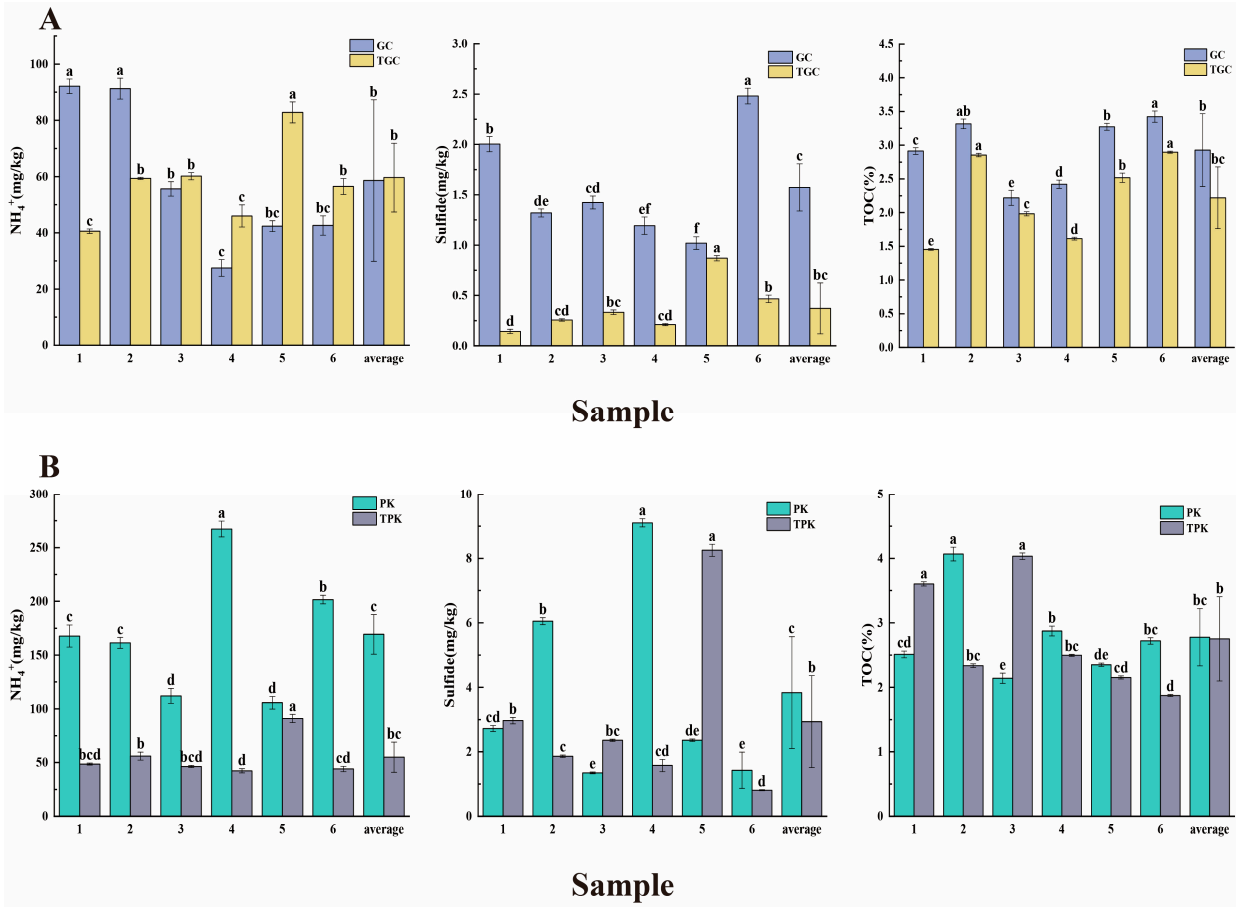

Figure 3. Concentrations of NH_4^+-N, sulfide and TOC in the sediments. (**A**) NH_4^+-N, sulfide and TOC concentration of GC and TGC: GC: samples of the Gaochun District before sediment improvement; TGC: samples of the Gaochun District after sediment improvement; (**B**) NH_4^+-N, sulfide and TOC concentration of PK and TPK: PK: samples of the Pukou District before sediment improvement; TPK: samples of the Pukou District after sediment improvement; average: the average of GC, TGC PK, and TPK. Values were given as mean ± SD, and different letters in the upper (a, b, c, d, e) indicate a significant difference ($p < 0.05$) before and after sediment improvement based on the analysis of variance.

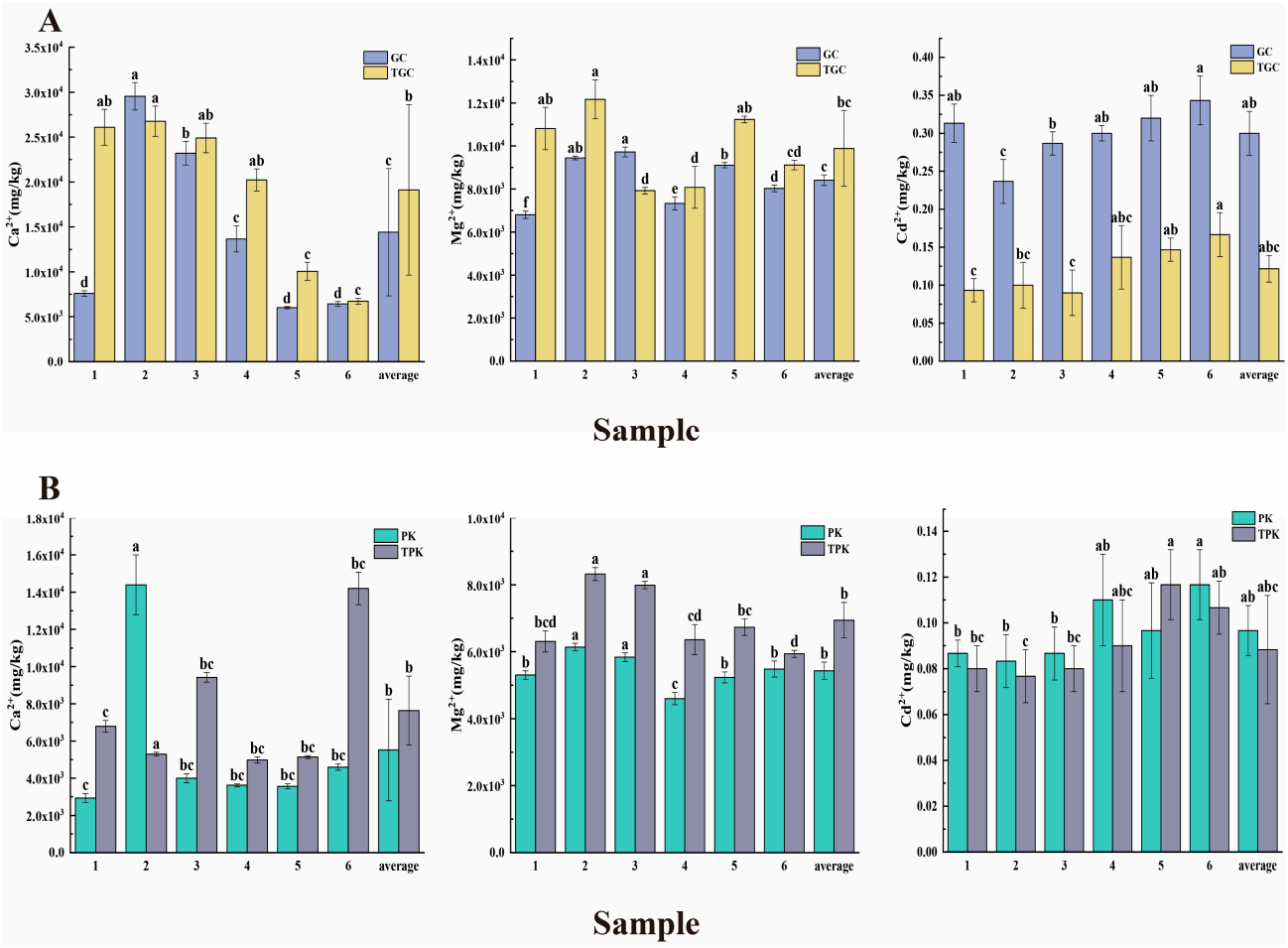

Figure 4. Concentrations of Ca, Mg, and Cd in the sediments. (**A**) Ca, Mg, and Cd concentration of GC and TGC: GC: samples of the Gaochun District before sediment improvement; TGC: samples of the Gaochun District after sediment improvement; (**B**) Ca, Mg, and Cd concentration of PK and TPK: PK: samples of the Pukou District before sediment improvement; TPK: samples of the Pukou District after sediment improvement; average: the average of GC, TGC PK, and TPK. Values were given as mean ± SD, and different letters in the upper (a, b, c, d, e) indicate a significant difference ($p < 0.05$) before and after sediment improvement based on the analysis of variance.

3.2. Diversity, Richness, and Structure of Microbial Communities

Samples were divided into eight groups (PKW, PKS, GCW, GCS, TPKW, TPKS, TGCW, TGCW, TGCS) based on sample type, sampling location, and time (Table 1). By sequencing the bacterial communities in water and sediment samples, a total of 5844 OTUs (operational taxonomic units) was defined with a similarity threshold of 97%. Alpha diversity (richness and Shannon index) of water and sediments varied widely from site to site. The α diversity of sediment samples at each site was significantly greater than that of water samples. In the sediment samples, the richness and Shannon index of TGCS were significantly higher than those of GCS, whereas the richness and Shannon index of TPKS were significantly lower than those of PKS, and there was no significant difference in the α-diversity of water samples (Figure 5A,B). Overall, the α diversity of the improved sediment samples (TGCS and TPKS groups) changed significantly compared to that of the sediment samples prior to sediment modification (GCS and PKS groups). Among the retrieved OTUs, a total of 28 bacterial phyla and 285 bacterial genera through species annotation were obtained. In all sediment samples, the dominant phyla were Proteobacteria (41.9%), Bacteroidetes (14.9%), Chloroflexi (11.7%), Acidobacteria (5.9%), and Firmicutes (4.6%). However, in the water samples, the relatively abundant phyla were Proteobacteria (46.1%), Actinobacteria

(22.0%), Bacteroidetes (16.1%), Flavobacteria (6.2%), and Verrucomicrobia (4.9%). The relative abundances of Flavobacterium, GP6 and Luteolibacter at the genus level (except for the unassigned genus) varied greatly among the various groups (Figure 5C,D). In addition, the distribution of dominant bacterial phyla in different groups was determined by the phylum-level Turkey–Kramer test. The abundance of Bacteroidetes in the TPKS group was significantly higher than that in the PKS and TPKW groups. The abundance of Actinobacteria in the four water sample groups (GCW, PKW, TPKW, TGCW) was significantly higher than that in the sediment groups (GCS, PKS, TGCS, TPKS), whereas the abundance of Chloroflexi displayed the opposite result (Figure S2). OTU-based non-metric multidimensional analysis (NMDS) showed that water sample groups and sediment groups were separated at the same sampling site. It was found that the GCW group and the TGCW group, the PKW group and the TPKW group, and the GCS group and the TGCS group were all separated (Figure 6A).

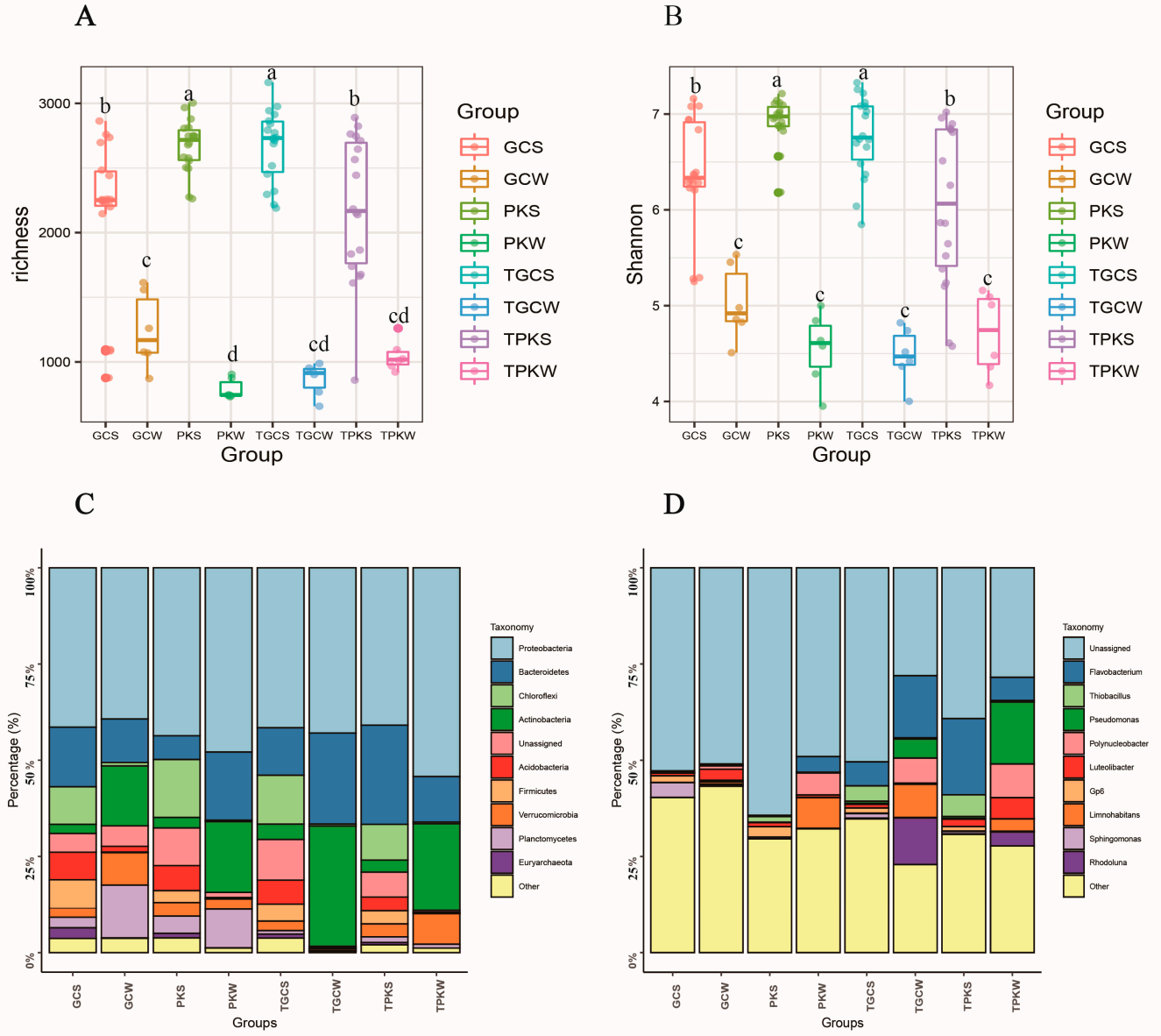

Figure 5. Alpha diversity (richness and Shannon index) of water and sediments and stacking of bacterial abundances at the phyla level and genus level by group. (**A**) richness of GCS, GCW, PKS, PKW, TGCS, TGCW, TPKS, and TPKW; (**B**) Shannon index of GCS, GCW, PKS, PKW, TGCS, TGCW, TPKS, and TPKW, (**C**) phyla level, (**D**) genus level. Different letters in the upper (a, b, c, d) indicate a significant difference ($p < 0.05$) between different groups based on the analysis of variance.

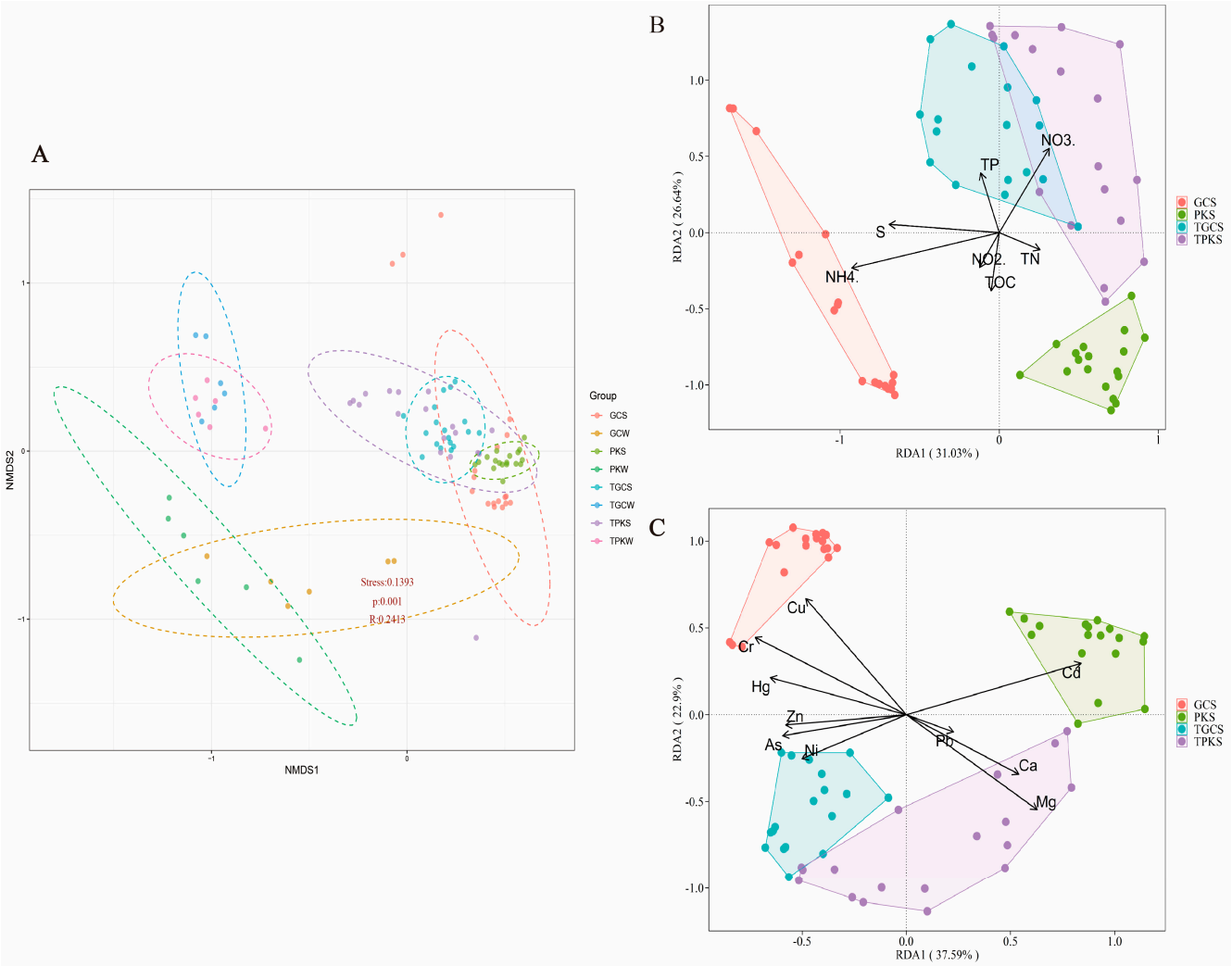

Figure 6. Non-metric multidimensional analysis (NMDS) of all groups and redundancy analysis (RDA) of bacterial communities and physicochemical characteristics in all groups. (**A**) NMDS; (**B**) RDA of bacterial communities and nutrients; (**C**) RDA of bacterial communities and heavy metals; NH4.: NH_4^+-N; NO3.: NO_3^--N; NO2.: NO_2^--N.

3.3. Relationships between Bacterial Community and Environmental Factors

By combining environmental variables such as sediment nutrients as well as heavy metals with bacterial communities, the correlation of sediment environmental factors with their community composition was determined and visualized by RDA. There was a close correlation between trimorphic nitrogen (NH_4^+-N, NO_2^--N and NO_3^--N), TN, TP, TOC, sulfide, heavy metals (e.g., Ca, Mg, Cd, etc.), and bacterial communities of GCS and PKS groups (Figure 6B,C). RDA1 and RDA2 accounted for 31.03% and 26.64%, 37.59%, and 22.9% of the total variance, respectively. Spearman correlation analysis was used to analyze the relationship between bacterial communities and environmental factors in aquaculture pond sediments. At the phylum level, there was no obvious correlation between the Proteobacteria in sediment samples prior to sediment improvement (GCS group and PKS group) with all the tested physicochemical factors. Bacteroidetes and Firmicutes were positively associated with Ca, Mg, and Cd ($p < 0.01$), whereas Zn, Cu, Ni, Hg, As, and Cr were negatively correlated ($p < 0.01$) (Figure 7A,B). All physical and chemical indicators measured for sediment and heavy metal concentrations played an important role in influencing improved bacterial communities (TGCS and TPKS groups) (Figure 7C,D). NO_3^--N had a significant positive effect on Nitrospirae, Latescibacteria, and Crenarchaeota

($p < 0.001$). Pb, Hg, and As had significant negative effects on Proteobacteria. Chloroflexi, Acidobacteria, Nitrospirae, Gemmatimonadetes, and Tenericutes were positively affected by Mg concentrations, with Cr showing the opposite pattern. Overall, sediment bacterial communities in both aquaculture areas were closely related to environmental factors.

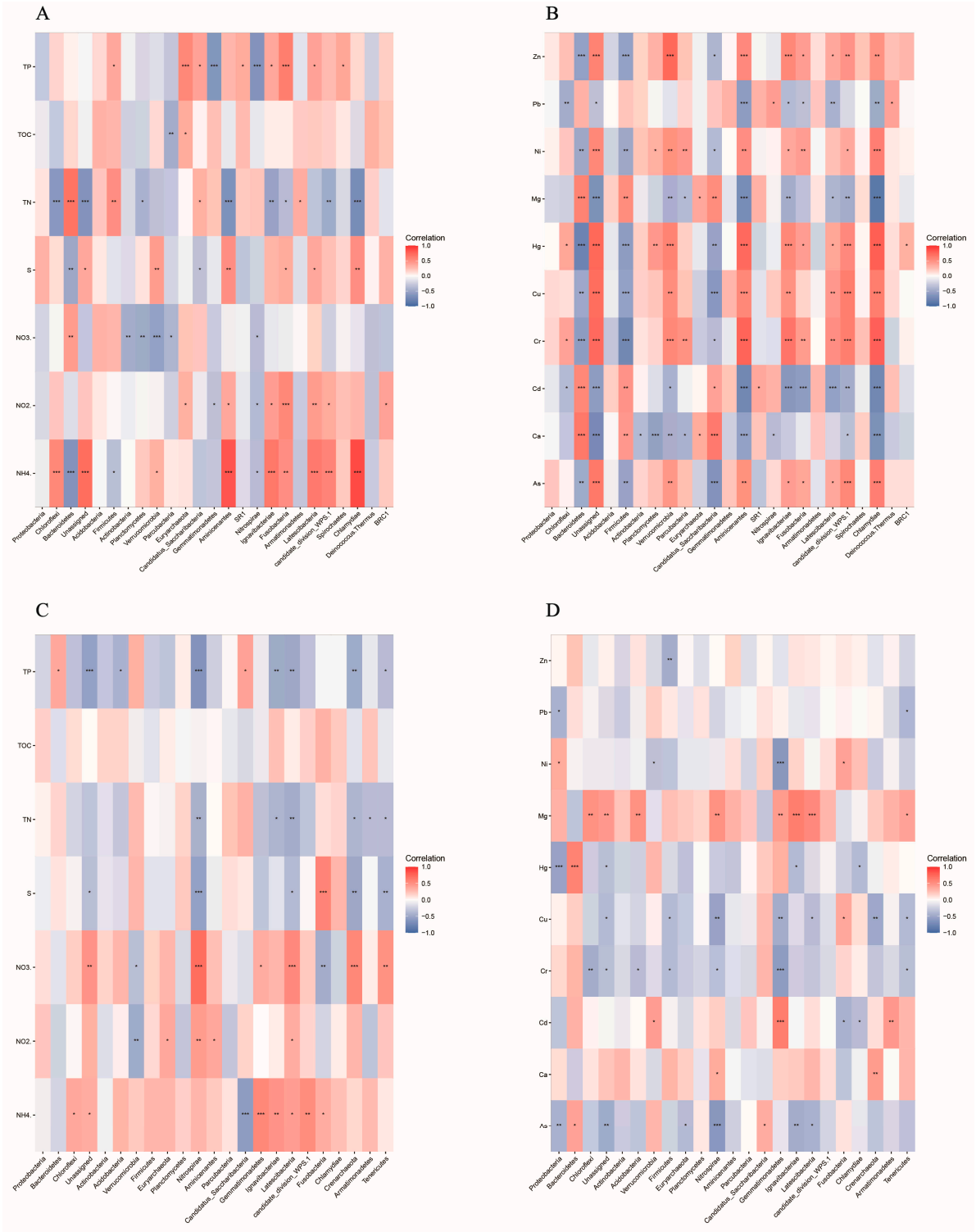

Figure 7. Correlations between significant environmental constraints of bacterial community variation and the relative abundance of bacterial community for the sediments. (**A**) nutrients of GCS and PKS group; (**B**) heavy metals of GCS and PKS group; (**C**) nutrients of TGCS and TPKS group; (**D**) heavy metals of TGCS and TPKS group; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

3.4. Predicted Function of Bacterial Community

Out of a total of 92 functional groups from FAPROTAX (v1.1) encompassing 5844 unique bacterial OTUs, we have detected 49 functional groups involved in carbon, nitrogen, and sulfur cycles in our samples. The abundances of functional groups involved in carbon, nitrogen, and sulfur cycles were different in different groups (Figure S3). In the sediment samples, the dark sulfide oxidation and dark oxidation of sulfur compounds abundance after the substrate modification were significantly greater than those of the pre-improved samples (i.e., TGCS > GCS, TPKS > PKS). However, the abundance of functions such as nitrite respiration, nitrate respiration, nitrous oxide denitrification, and nitrogen respiration involved in N cycling and methanogenesis involved in C cycling were conspicuously more abundant in the GCS and PKS groups than those in the TGCS and TPKS groups. In contrast, functional groups with significant differences in the water sample group were more similar.

Bacterial communities in aquaculture pond sediments had different sensitivities to environmental changes, while some key environmental drivers also displayed different effects on different functional groups of bacterial communities (Figure 8). The correlation between physicochemical factors and the predicted functional groups showed that the abundance of 10 functional groups involved in the N cycle, including nitrification, was positively correlated with nutrients (NH_4^+-N, NO_2^--N, TOC, sulfide). NH_4^+-N showed significant correlation with 14 functional groups involved in nitrogen cycling, except nitrogen fixation and nitrate reduction ($p < 0.05$). TN had a significant positive correlation with dark sulfide oxidation, dark thiosulfate oxidation, and dark oxidation of sulfur compounds ($p < 0.05$), while sulfides were the opposite.

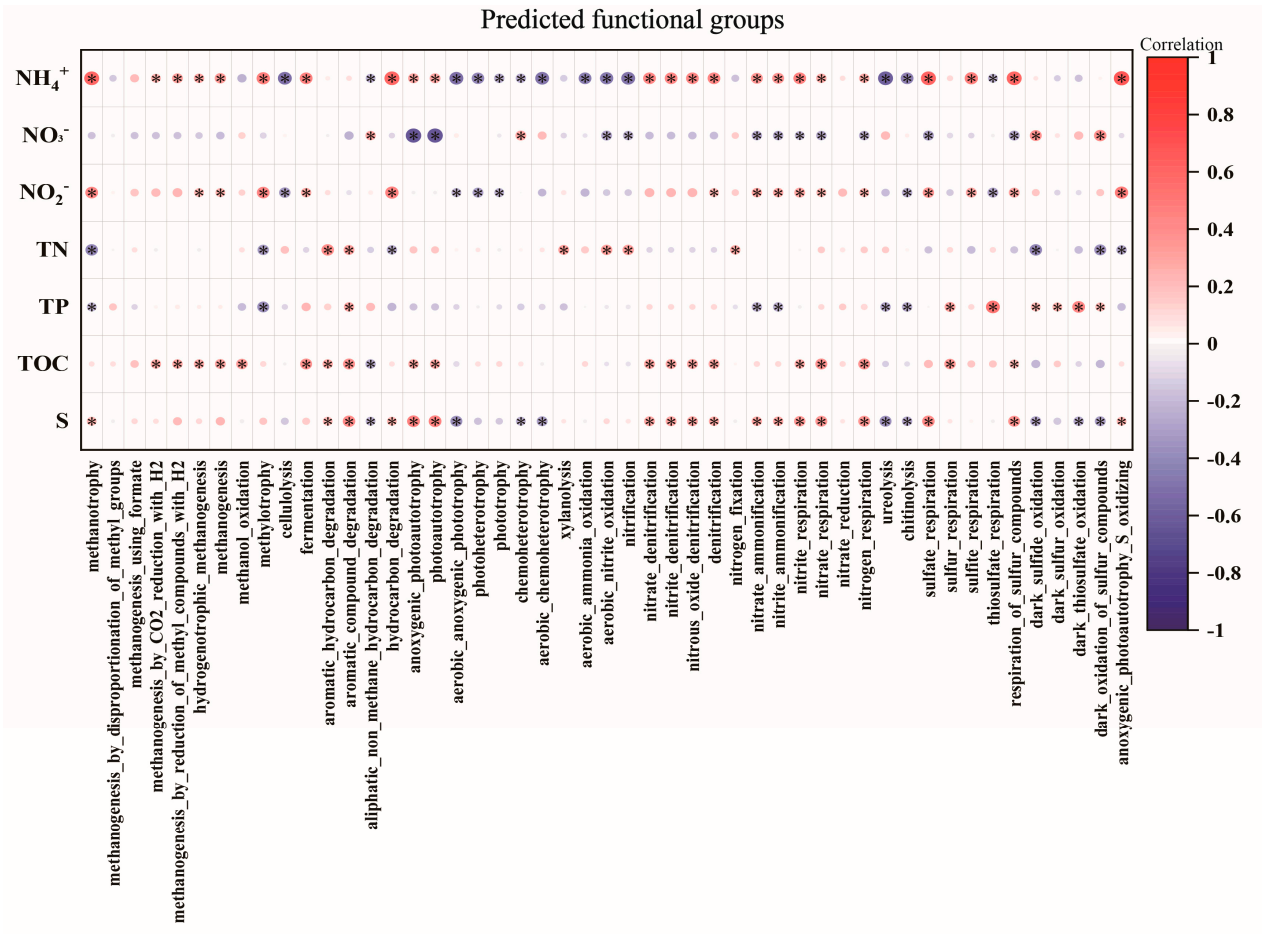

Figure 8. Correlations of the abundance of predicted functional groups. Circle size represents the variable importance as a result of the permutation of a given variable. Colors represent Spearman correlations. *: $p < 0.05$.

4. Discussion

4.1. The Interconnection between Environmental Factors and Aquaculture Activities

In order to assess the trophic levels and changes of the aquaculture water before and after the bottom improvement of different river crab breeding areas, the samples were collected in two aquaculture areas of the Pukou District and the Gaochun District of Nanjing City, and then the concentrations of NH_4^+-N, NO_3^-N, and NO_2^--N of the water samples were determined under laboratory conditions. The results showed that as one of the water environment factors with the greatest impact on Chinese mitten crabs (*Eriocheir sinensis*) farming [20], the comparison results of NH_4^+-N concentrations of water samples before and after sediment improvement in the Gaochun District were completely opposite to those in the Pukou District. The increase in NH_4^+-N concentrations in improved water samples was mainly attributed to river discharge or nutrient enrichment caused by aquaculture [21,22]. Similarly, the change of NH_4^+-N concentration in sediment samples from the Gaochun District after sediment improvement was affected by nutrient enrichment in water, and the results of the two rounds of comparison still displayed the opposite trend with the samples from the Pukou District. After a series of aquaculture practice activities such as dredging and pond drying, the sulfide and TOC content in the improved sediments of the two aquaculture areas have decreased, and the opposite trend of TPK1 and TPK3 was due to the fact that they had not yet completed the above aquaculture practice activities.

The distribution of heavy metals was not homogenous among all sites. With the exception of Ca, Mg, and Cd, there was no consistent increase or decrease in the concentrations of most heavy metals in the improved sediments. The accumulation of heavy metals in sediments was mainly from river inflows, atmospheric sedimentation, and human activities. Among all factors, aquaculture is one of the most critical sources of heavy metals from human activities [23,24]. Chinese mitten crabs (*Eriocheir sinensis*) will complete its metamorphosis development process, and of the entire growth process, periodic molting molting activity is one of the important growth indicators in the developmental stage of crustaceans [25,26]. In the juvenile stage, abnormal molting activity and unhealthy state lead to high mortality of Chinese mitten crabs. Both Ca and Mg are mineral elements necessary for crustacean molting; therefore, in order for crustaceans to complete normal molting activity during metamorphosis, it is necessary to increase their intake of Ca and Mg at the time of molting [27,28]. In order to improve the survival rate of crabs, the two river crab farming areas in this study increased the content of Ca and Mg in the environment and the intake of crabs by sprinkling drugs and increasing the proportion of Ca and Mg in the feed, which explains the higher levels of Ca and Mg in the improved sediments.

4.2. Microbial Diversity in Response to Aquaculture Activities

In this study, an analysis of the diversity and community composition of microorganisms was conducted, and it was found that close interconnections were presented among the physicochemical indicators of surface water and sediments, the microbial diversity of sediments, and aquaculture activities. It was found that the α diversity index (richness and Shannon index) of the sediment samples at each sampling site was conspicuously higher than that of the water samples. This is consistent with previous research that the abundance of bacteria in sediments in culture ponds is significantly higher than that in water [29]. The richness and Shannon index of the pre-improved sediment samples (GCS) of the Gaochun District were significantly higher than those of the improved sediment samples (TGCS), whereas the sediment samples of the the Pukou District showed the opposite trend. These results may be attributed to the different physicochemical characteristics of sediments (such as NH_4^+-N, Ca, and Mg) caused by different aquaculture activities, which perturbate different bacterial communities [30–32].

As the most abundant phyla of the sediments in this study, the relative abundances of Proteobacteria, Bacteroidetes, and Chloroflexi far exceeded other phyla, and the sum of the three phyla exceeded 50%. These bacteria are closely related to the circulation of nutrients and trace metals, and have been widely detected in sediments in aquaculture ponds [33–35]. Most

bacteria in Proteobacteria have the potential for aerobic and anaerobic biodegradation, and some of the genera are species involved in methane production [34], Bacteroidetes has a special role in the absorption of complex organic matter and the degradation of organic pollutants [22]. Chloroflexi contains several species of obligate organochlorine respiration, which plays a crucial role in the degradation of organochlorine pesticides [33]. In aquaculture ponds and lakes, except for the top sediment, the sediments at the remaining depths are anaerobic, which is why the three phyla are highly abundant. Similarly, Actinobacteria, with higher abundance in various groups of water samples, are widely found in aquaculture [36–38]. The variation of abundance in different groups explains the difference in the bacterial community structure. Previous studies had also confirmed the results of this study by showing that Chloroflexi was more common in sediments from aquaculture ponds, whereas Actinomycetes was more common in water [29,33].

4.3. Environmental Factors Affecting Bacterial Community and Function of Sediments

A spearman correlation analysis was conducted to explore the effects of environmental factors on bacterial communities, and the results showed that NH_4^+-N, NO_3^--N, TP, and heavy metals were the main factors affecting the bacterial communities in this study. Similar to the results of the present study, Gan et al. report that microbial diversity is susceptible to eutrophication from nitrate as well as nitrite accumulation [39]. In our study, NO_3^--N and NO_2^--N in the pre-improved samples were significantly negatively correlated with Nitrospirae ($p < 0.05$), while the two were significantly positively correlated in the improved samples ($p < 0.01$). This may be due to fluctuations in NO_3^--N and NO_2^--N concentrations caused by different feeding habits in the two culture areas or differences in river inputs. What's more, the findings of our study showed that Proteobacteria were negatively correlated with NO_3^--N and NO_2^--N, which is consistent with previous studies showing that Proteobacteria play a crucial role in the biochemical processes of sediment [40]. The accumulation of heavy metals in ponds and lakes is an important environmental problem in aquaculture [41], and there is a close relationship between heavy metal pollution and microbial diversity [42]. In our study, the phylum-level bacterial communities of samples prior to sediment improvement exhibited strong associations with heavy metals. Bacteroidetes and Firmicutes were positively associated with Ca, Mg, and Cd ($p < 0.01$), whereas Zn, Cu, Ni, Hg, As, and Cr were negatively correlated ($p < 0.01$), which not only showed the important role heavy metal content played in the abundance of Bacteroides and Firmicutes, but also displayed that the sediment improvement which promoted the consistent upward trend of Ca, Mg, and Cd indirectly affected the microbial community in the culture environment of crabs. Furthermore, Mg was positively associated with the vast majority of bacteria in the improved sediment samples, suggesting that elevated levels of Mg had a crucial impact on microbial diversity. According to the results of the RDA plot, the effects of physicochemical factors on sediment microbial diversity at the two sampling points varied greatly before and after the improvement. Except for Pb, the remaining heavy metals detected in this study showed strong and significant correlations with the microorganisms before and after the improvement of the two culture areas. NO_3^--N was significantly positively correlated with the bacterial community of the modified sample ($p = 0.001$), but significantly negatively correlated with the pre-reformed bacterial community ($p = 0.001$). Previous studies have also presented that the form of nitrogen is affected by Nitrospirae, resulting in a great change in nitrogen concentration, and some bacteria can directly participate in nitrogen metabolism, which in turn affects the concentration of different forms of nitrogen [43,44]. However, total organic carbon (TOC) exhibited a completely opposite correlation result to NO_3^--N, precisely because of the reduction in the content of TOC in the sediment samples due to sediment improvement. In addition, it was further proved that the sediment improvement indirectly affected the bacterial community structure in the crab farming environment.

By classifying and screening the functional groups obtained by FAPROTAX function annotation, the abundance of functional groups involved in C, N, and S cycles in each sample group was quite different. Some functional groups in the sediment involved with the C, N cycles (such as methanogensis, nitrate respiration) changed significantly after treatment

by the relevant aquaculture practice activities such as sediment improvement, indicating that the C and N cycles of the sediment were affected by changes in the composition of the microbial community structure [45]. In the sulfur cycle, most sulfides will be oxidized by oxygen, nitrates, some trace metals, and other oxidants to eventually form sulfates, which also includes the geochemical cycle process and the promotion of microbial degradation. In addition, in most temperate sediments, iron, carbon, and sulfur cycles were closely linked, microbial dissimilatory iron and sulfate reducing agents remineralize organic matter to carbon dioxide and further produce some volatile fatty acids. This may affect the feeding activity of crabs and increase the level of food consumption [46–49]. It was reported by Zhang et al. that aquaculture activities can significantly enhance the microbial sulfur cycle [50], which is consistent with our findings: sediment improvement enhances the metabolic functions of bacteria which are associated with sulfur cycles; for example, sulfide oxidation promotes the relevant geochemical cycle processes in the sediments of crab culture ponds, which further enhances the feeding activities of river crabs and improves the survival rate of crabs and the possible economic benefits of subsequent consequences.

5. Conclusions

Overall, this study revealed the effects of substrate improvement on the structure and function of nutrients, heavy metals, and bacterial communities in river crab cultured areas. The changes in the concentration of nutrients and heavy metals before and after the sediment improvement show that the sediment improvement has a great optimization effect on the crab farming environment. The findings of the study showed that Proteobacteria, Bacteroidetes, and Chloroflexi were the most abundant phyla in the sediments. Variations in diversity among different groups were significant. NH_4^+-N, NO_3^--N, TN, TP, and heavy metals proved to be essential factors in controlling the bacterial diversity and function, and these factors were altered by substrate improvement and indirectly affected the structure of bacterial communities in river crab farming environments. In addition, our study revealed the promoting effect of sediment improvement on related geochemical cycling processes such as sulfur metabolism, and provided a theoretical basis for optimizing the corresponding measures for sediment improvement and healthy aquaculture of crabs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8020098/s1, Figure S1: Concentrations of TN and TP in the sediments; Figure S2: Intergroup comparison of dominant phylum; Figure S3: Comparison of functional abundances of different groups involved in C, N and S metabolism.

Author Contributions: T.G. conceived and designed the research. T.G. and H.L. conducted the sample collection. N.L. and W.X. analyzed the data analyses. N.L. wrote the manuscript with the help from Y.H., W.X. and T.G. All authors have read and agreed to the published version of the manuscript.

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