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The Effect of Artificial Substrate and Carbon Source Addition on Bacterial Diversity and Community Composition in Water in a Pond Polyculture System

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Abstract: The use of artificial substrates and biofloc technology can favor fish culture and improve water quality. The aim of this study was to evaluate whether artificial substrates and carbon source additions modify the microbial activity of water bodies. The diversity and structure of microflora in the water after adding artificial substrates and carbon sources to the ponds were analyzed using high-throughput sequencing based on the V3-V4 region of 16S rRNA genes. The results showed that there was no difference in the richness and diversity of intestinal microflora between the control and experimental groups. Principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) showed that artificial substrate and carbon source addition changed the structure of the microflora. The results of a linear discriminant analysis (LDA) effect size (LefSe) indicated 11 biomarkers in the EG_st group. Spearman correlation heatmap analysis showed that environmental factors affected the bacterial communities, and the results of a redundancy analysis indicated that chemical oxygen demand was a critical factor in controlling the bacterial communities in the water. These results provide an understanding of the effect of artificial substrate and carbon source addition on bacterial diversity and community composition in water.

Keywords: artificial substrate; biofloc; 16S rRNA; high-throughput sequencing; microbial community

Key Contribution: The results demonstrated that combined artificial substrate and carbon source addition can modify microbial activity, thereby regulating pond water bodies.

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1. Introduction

Common carp (*Cyprinus carpio*) is an important species of freshwater fish that is commonly cultured in over one hundred countries throughout the world [1]. In 2022, the annual production of this species in China was approximately 2,831,763 tons, accounting for 10.7% of the output of freshwater fish [2]. However, intensive aquaculture is currently the main production mode of fish, which creates a stressful environmental problem due to high aquaculture density and high feed input [3]. Due to the impact of pollutants (such as ammonia nitrogen and nitrite) generated in intensive aquaculture, pond water needs to be frequently exchanged with external water to ensure the standard survival rate of fish and shrimp [4]. The discharge of wastewater can lead to the entry of nitrogen, phosphorus, plankton, organic matter, and suspended solids into natural water bodies, resulting in

severe eutrophication [5]. To reduce environmental pollution caused by aquaculture, it is urgent to seek technology for treating aquaculture wastewater.

In recent years, some new technologies have been studied and applied in the treatment of aquaculture wastewater, including in situ treatment and off-site treatment technologies. In order to achieve the development of high-quality aquaculture, the Ministry of Agriculture and Rural Affairs of China has started implementing the "Five Actions" for green and healthy aquaculture since 2020 [6], and increasing numbers of environmentally friendly aquaculture models have gradually been explored and promoted. Biofloc technology (BFT) utilizes beneficial microorganisms to convert toxic nitrogen-containing compounds into edible biological flocs for fish, significantly enhancing the recycling and reuse of nutrients and waste, which has been widely applied in various green aquaculture models [7]. At present, BFT can significantly reduce breeding costs and improve the survival rate of breeding objectives in the cultivation of *O. niloticus*, *L. vannamei*, and other species, with great economic benefits [8,9]. Wei et al. [9] found that biological flocs contain essential nutrients such as amino acids and fatty acids for aquatic animals, which can replace some commercial feed and increase feed efficiency. In addition, research has found that the application of biofilm water quality remediation technology in intensive aquaculture can effectively purify aquaculture wastewater [10], providing a new approach for ecological-friendly aquaculture. An eco-substrate is an environmentally friendly material that can serve as a carrier for biofilms [11]; it promotes microbial reproduction by adsorbing suspended solids and fish and shrimp excrement in water and utilizes microbial metabolism to remove pollutants in water, thereby purifying the water environment [12,13]. In aquaculture systems, bacteria and algae are the main absorbers and converters of ammonia–nitrogen compounds [14]. However, the process of bacteria converting ammonia–nitrogen compounds in aquaculture water into bacterial proteins only occurs in environments with a C/N greater than 10.

Previous studies have shown that combining appropriate C/N with artificial substrate can effectively purify water quality, increase yield, and enhance fish immunity, and this technology has been found to be promising [15,16]. For example, AftabUddin et al., (2015) showed that increasing the C/N ratio with corn meal could significantly increase the total heterotrophic bacteria in water and the shrimp yield in biofilm systems [17]. Asaduzzaman et al., (2008) found that carbon source supplementation combined with artificial substrate addition greatly improved the net production of farmed animals, which is related to the reduced nitrogen-containing inorganic compounds and elevated concentration of heterotrophic bacteria [18]. In short, providing a substrate for the development of surrounding organisms and increasing the C/N ratio can increase pond yield. Similarly, in previous studies, we found that the combination of artificial substrates and carbon source addition can improve the water chemical indicators of carp aquaculture ponds [19].

Microorganisms play a crucial role in the transformation and metabolism of organic matter in aquaculture environments [20]. Understanding the characteristics of aquatic microbial communities has important theoretical value for the construction of ecological models in aquaculture [21]. In this study, we analyzed the effect of artificial substrate and carbon source addition on bacterial diversity and community composition in a pond polyculture system to provide a technical basis for the in situ water quality control of a carp aquaculture.

2. Materials and Methods

2.1. Experimental Sites and Design

A culture trial was conducted in six earthen fish ponds with a surface area of approximately 740 square meters and a depth of approximately 2 m at the breeding experimental base of the Heilongjiang River Fishery Research Institute, Chinese Academy of Fishery Sciences in Harbin, Heilongjiang Province, China (Figure 1). The experimental period was 140 days. Before the experiment began, the soil pond was disinfected with commercially available chlorine-containing products [19].

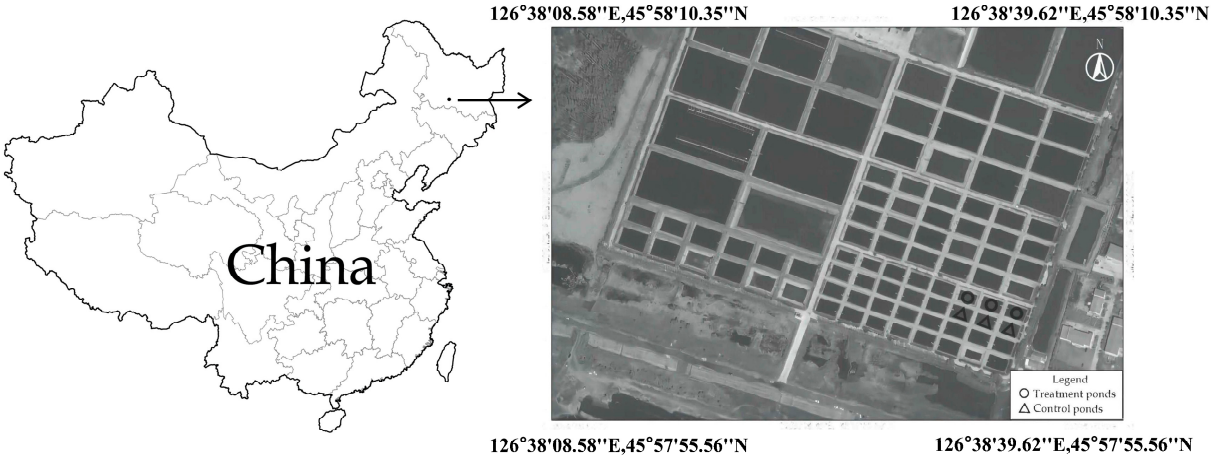

Figure 1. Experimental location of the study.

In the ponds of the experimental group, fifteen eco-substrates (2.0 m × 1.5 m) were fixed in the enclosure through a string and suspended vertically. Due to its unique arrangement structure, geotextile Aquamat™ (Meridian Aquatic Technology, Silver Spring, MD, USA) was used as the eco-substrate, which provided a living environment for microorganisms and was more conducive to microbial growth. As an organic carbon source, corn starch was added to each aquaculture pond every two weeks during the trial period. In the ponds of the control group, neither artificial substrate nor carbon source was added. Each group was set with 3 repeated ponds.

2.2. Experimental Fish and Culture Management

After a week of injecting water into the pond, 1000 young bottom-feeder mirror carp (*Cyprinus carpiospecularis*) (193.11 ± 14.17 g), 70 filter-feeder silver carp (*Hypophthalmichthys molitrix*) (45.52 ± 5.94 g), and 19 big carp (*Aristichthys nobilis*) (34.11 ± 4.48 g) were collected according to the ordinary standard of the stocking ratio and density of the main farmed species and supporting species in mixed culture ponds in Northern China.

Throughout the culture duration, the water of all the ponds was not exchanged. The temperature, dissolved oxygen (DO), and pH of the water were continuously tested. The commercial feed was fed 3 times each day (7:00–7:30, 12:00–12:30, and 17:00–17:30, respectively). The daily feeding amount was 2% of the total body weight and gradually increased to 4% of the total body weight [19].

2.3. Water Quality Indicators

Starting from the hanging eco-substrate, determination was performed every two weeks, and the collection time occurred at 10:00 am; the day after, measurements were obtained for nitrate nitrogen NO_3^- (ultraviolet spectrophotometry), nitrite nitrogen NO_2^- (diazo coupling spectrophotometer), total ammonia nitrogen (TAN) (Nesser reagent colorimetric method), phosphorus (phosphorus molybdenum blue—ascorbic acid spectrophotometry), total phosphorus TP (acid potassium persulfate digestion spectrophotometry), total nitrogen TN (alkaline potassium persulfate digestion ultraviolet spectrophotometry), COD (potassium permanganate method), suspended matter in water (extraction and filtration method), pH (water quality analyzer), and dissolved oxygen DO (water quality analyzer) (SEPA.2002).

2.4. Bacterial DNA Extraction and 16S rRNA Sequencing

From each enclosure, 1.0 L water samples were collected and were then filtered using a mixed cellulose ester microporous membrane with a pore diameter of 0.22 μm. The total bacterial DNA was extracted from water samples using the DNeasy® PowerSoil® Pro Kit (QIAGEN, Hilden, FL, USA), in accordance with the manufacturer's instructions. The

quality of the extracted genomic DNA was detected by 1% agarose gel electrophoresis, and the concentration and purity of the DNA were determined using an ultra-micro spectrophotometer (NanoDrop 2000, Waltham, MA, USA).

Variable regions of the bacterial 16S rRNA gene (V3-V4) were amplified via polymerase chain reaction from the extracted DNA using universal primers primer pairs of 338f/806r (5′-ACTCCTACGGGAGGCAGCAG-3′/5′-GGACTACHVGGGTWTCTAAT-3′). The PCR products of the same sample were mixed, which then were purified using an AxyPrep DNA Gel Extraction Kit. DNA fragment libraries were built for the purification of PCR products with the following criteria: (i) joint connection; (ii) using magnetic beads to screen and remove self-connected fragments of the connector; (iii) enrichment of library templates using PCR amplification; and (iv) recycling PCR products with magnetic beads to obtain the final library. Purified amplicons were sequenced based on an Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA) according to the standard procedures (Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China).

2.5. Statistical Analysis

Nonmetric multidimensional scaling (NMDS) ordination based on an analysis of similarity using the Bray–Curtis dissimilarity statistic was conducted to visualize the differences in microbial community structure in the two treatment groups. Redundancy analysis (RDA) and principal coordinate analysis (PCoA) were applied to study the correlation between environmental factors and microbial community structures. A Venn diagram was used to show the unique and shared OTUs detected in two treatment groups. Bacterial groups with significant differences in abundance from phylum to genus level among different groups were analyzed using Linear discriminant analysis Effect Size (LEfSe). Bioinformatic analysis of the water microbiota was carried out using the Majorbio Cloud platform (https://cloud.majorbio.com) accessed on 24 December 2021. The significant level in water quality indicators between the two treatment groups was analyzed with SPSS 20.0 software by using one-way ANOVA.

3. Results

3.1. Bacterial 16S rRNA Sequencing Data and OTUs

The changes in the water microbial community structure of the two treatment groups were studied using high-throughput sequencing technology. The total number of effective tags obtained from each sample of water was 779,004 sequences, of which the length ranged from 200 to 521 bp, resulting in an OTU range of 781–1189. The mean numbers of reads and OTUs were 47805 and 2058, respectively. The optimized sequences were clustered into operational taxonomic units (OTUs) with a 97% sequence similarity level and then classified into different taxonomic levels, including 55 phyla, 156 classes, 359 orders, 559 families, 946 genera, and 1838 species or varieties.

The rarefaction curve was prepared using randomly selected sequence numbers and their relative number of OTUs from each sample, which indicated that the sequencing depth was sufficient to cover the expected number of OTUs at a 3% sequence difference level in the study (Figure 2a). The Venn diagram showed similarity and overlaps of OTUs in the two groups (Figure 2b). The results revealed that the CG_st and EG_st groups shared 2020 OTUs, and there were 955 and 837 unique OTUs, respectively.

3.2. Analysis of Alpha Diversity and Beta Diversity

The alpha diversity indices of microbial communities, including richness (ACE and Chao) and diversity (Simpson and Shannon), for the two groups are shown in Table 1. There was no significant difference between the two groups. Additionally, beta diversity, PCoA, and NMDS based on the Bray–Curtis dissimilarity statistic were used to examine the community structure of the water microbiota among samples. The distance between symbols in a PCoA and NMDS plot (Figure 3) reflects relative differences in the community structure, with each symbol representing one water sample.

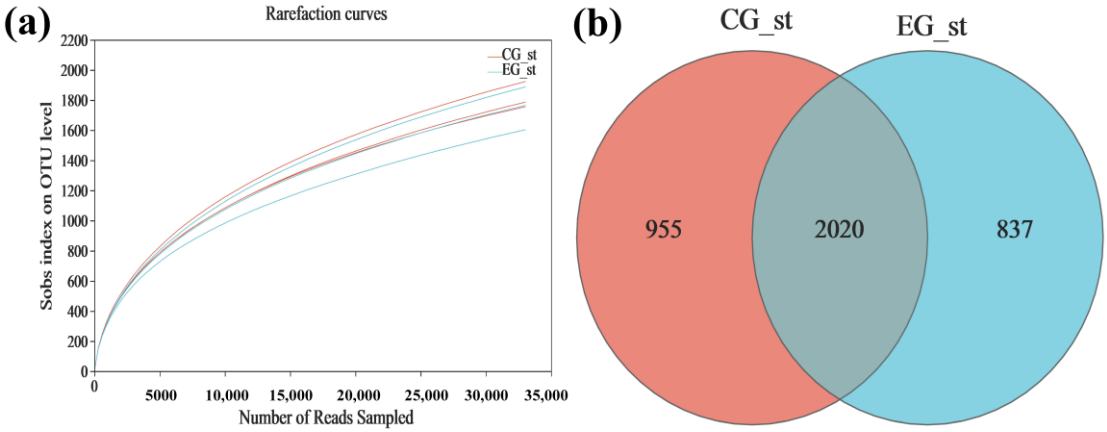

Figure 2. Rarefaction curves and Venn diagram analysis of water microbes with OTUs of the two groups. (**a**) Rarefaction curves; (**b**) Venn diagram.

Table 1. Analysis of richness and diversity of the microbial community.

Sample	Coverage	Richness		Diversity	
		ACE	Chao	Shannon	Simpson
CG_st	0.979 ± 0.001	3055 ± 389	2720 ± 172	5.455 ± 0.048	0.0170 ± 0.009
EG_st	0.978 ± 0.001	3247 ± 90	2678 ± 154	5.318 ± 0.076	0.0266 ± 0.0070

Note: Values represent the mean ± standard deviation (SD; n = 3).

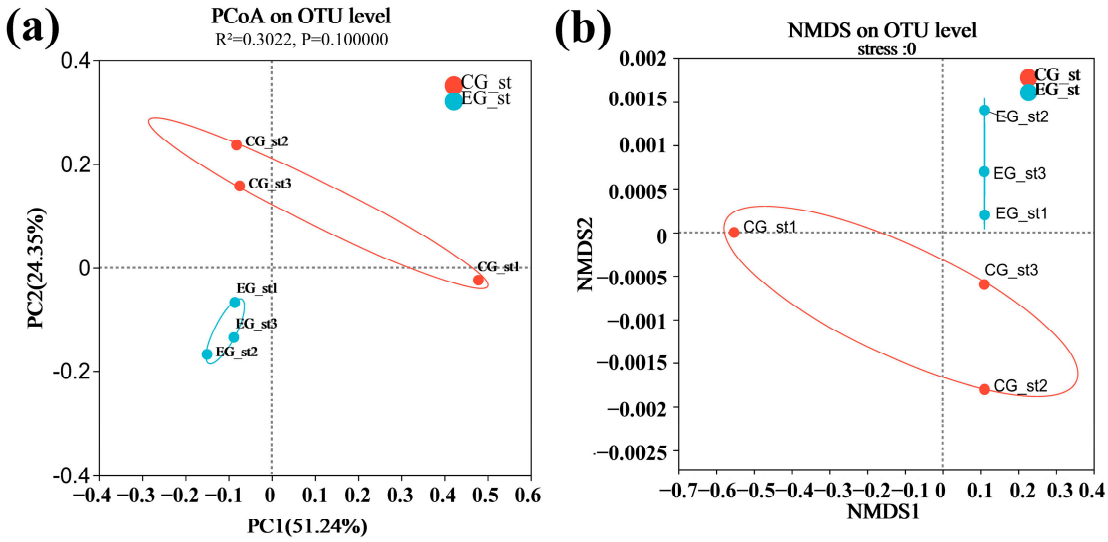

Figure 3. PCoA and NMDS based on the Bray–Curtis dissimilarity statistic of the microbial communities between the two groups. (**a**) PCoA; (**b**) NMDS.

3.3. Bacterial Community Composition

The top 11 phyla and classes were selected from each treatment group, and a bar chart was created to display relative abundances and their proportions at different classification levels. The relative abundance of taxa in the phylum and class are presented in Figure 4a,b. The largest relative abundance among the 5 phyla in the CG_st group was dominated by Proteobacteria (38.4%), Cyanobacteria (15.2%), Bacteroidota (15.1%), Actinobacteriota (7.93%), and Chloroflexi (8.59%). Based on the research results, a change was found in the relative abundance in the EG_st group compared to that in the CG_st group, such as an increase in the abundance of Bacteroidota, Cyanobacteria, and Actinobacteriota and a decrease in the abundance of Proteobacteria and Chloroflexi, but the

main phylum did not change. The EG_st group was dominated by Proteobacteria (35.45%), Bacteroidota (20.37%), Cyanobacteria (18.56%), Actinobacteriota (8.06%), and Chloroflexi (5.57%). The largest relative abundance among the 5 classes in the CG_st group was dominated by Gammaproteobacteria (22.7%), Alphaproteobacteria (15.7%), Cyanobacteriia (15.1%), Bacteroidia (14.1%), and Anaerolineae (7.9%). Compared to the CG_st group, the relative abundance of some classes in the EG_st group showed a decrease, such as Gammaproteobacteria, Alphaproteobacteria, and Anaerolineae, whereas some classes showed an increase, such as Bacteroidia and Cyanobacteriia, but the main phylum did not change. The EG_st group was dominated by Gammaproteobacteria (20.2%), Bacteroidia (18.5%), Cyanobacteriia (18.4%), Alphaproteobacteria (15.3%), and Anaerolineae (5.1%). Figure 4c shows the microbial relative abundance of bacteria at the genus level. The top 5 bacterial genera in the CG_st group were Microcystis_PCC-7914 (5.08%), norank_f__Caldilineaceae (5.60%), norank_f__Sutterellaceae (4.36%), Cyanobium_PCC-6307 (5.64%), and norank_f__norank_o__Chloroplast (3.08%). The top 5 bacterial genera in the EG_st group were Microcystis_PCC-7914 (13.74%), norank_f__Sutterellaceae (4.30%), norank_f__Caldilineaceae (3.92%), Flavobacterium (3.84%), and Ahniella (2.99%). The heatmap indicates that the abundance and composition of water bacteria were affected by the addition of artificial substrates and carbon sources.

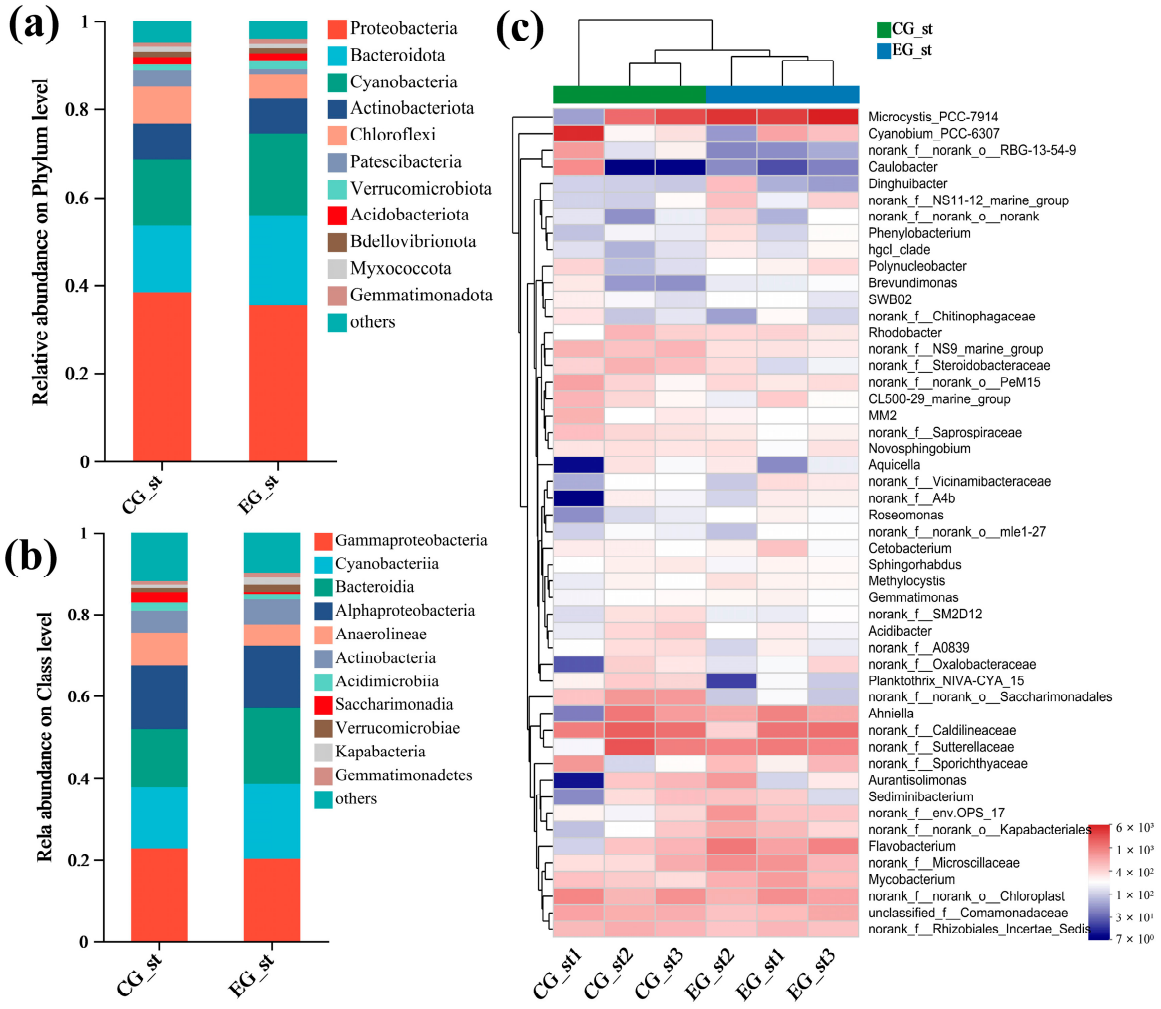

Figure 4. Microbial community composition in the two groups. (**a**) Relative abundance of the major taxonomic composition at the phylum level; (**b**) relative abundance of the major taxonomic composition at the class level; (**c**) heatmap at the genus level.

3.4. Taxonomic Composition Analysis

LefSe was applied to analyze bacterial community data at all taxonomic levels (Figure 5). There were 12 groups of bacteria enriched in the CG_st group, which was significantly higher than the EG_st group: Chloroflexi (at the phylum level), Patescibacteria (at the phylum level), Anaerolineae (at the class level), Saccharimonadia (at the class level), Steroidobacterales (at the order level), Saccharimonadales (at the order level), Phormidiaceae (at the family level), NS9_marine_group (at the family level), Saprospiraceae (at the family level), RBG-13-54-9 (at the genus level), Steroidobacteraceae (at the genus level), and Planktothrix_NIVA-CYA_15 (at the genus level). The bacterial lineages enriched in water from the EG_st group were Verrucomicrobiota (at the phylum level), Verrucomicrobiae (at the class level), Corynebacteriales (at the order level), Cyanobacteriales (at the order level), Microcystaceae (at the family level), Mycobacteriaceae (at the family level), Microcystis_PCC-7914 (at the genus level), Flavobacteriaceae (at the genus level), Flavobacterium (at the genus level), env_OPS_17 (at the genus level), and Mycobacterium (at the genus level).

3.5. Correlation Analysis of Water Quality and Microbial Community

A Spearman correlation heatmap was generated to study the relationships between the microbial phyla of water and water quality indicators such as DO, TP, TN, NH_4^+, NO_3^-, COD, NO_2^-, and PO_4-P (Figure 6). Myxococcota was significantly positively correlated with TN; Chloroflexi was significantly positively correlated with NO_3^-, TN, and NO_2^-; Patescibacteria was significantly positively correlated with PO_4-P, NH_4^+, and NO_3^-; Actinobacteriota and Cyanobacteria were significantly positively correlated with COD; Verrucomicrobiota was significantly negatively correlated with NO_3^-, TN, and NO_2^-.

The RDA supported the findings of Spearman's correlation and illustrated how environmental parameters affect bacterial communities. The dimensions of all variables were reduced, and two principal components were retained. Axis 1 and Axis 2 accounted for 52.45% and 39.14% of the variability, respectively. COD had significant effects on the distribution of sample communities (Figure 7).

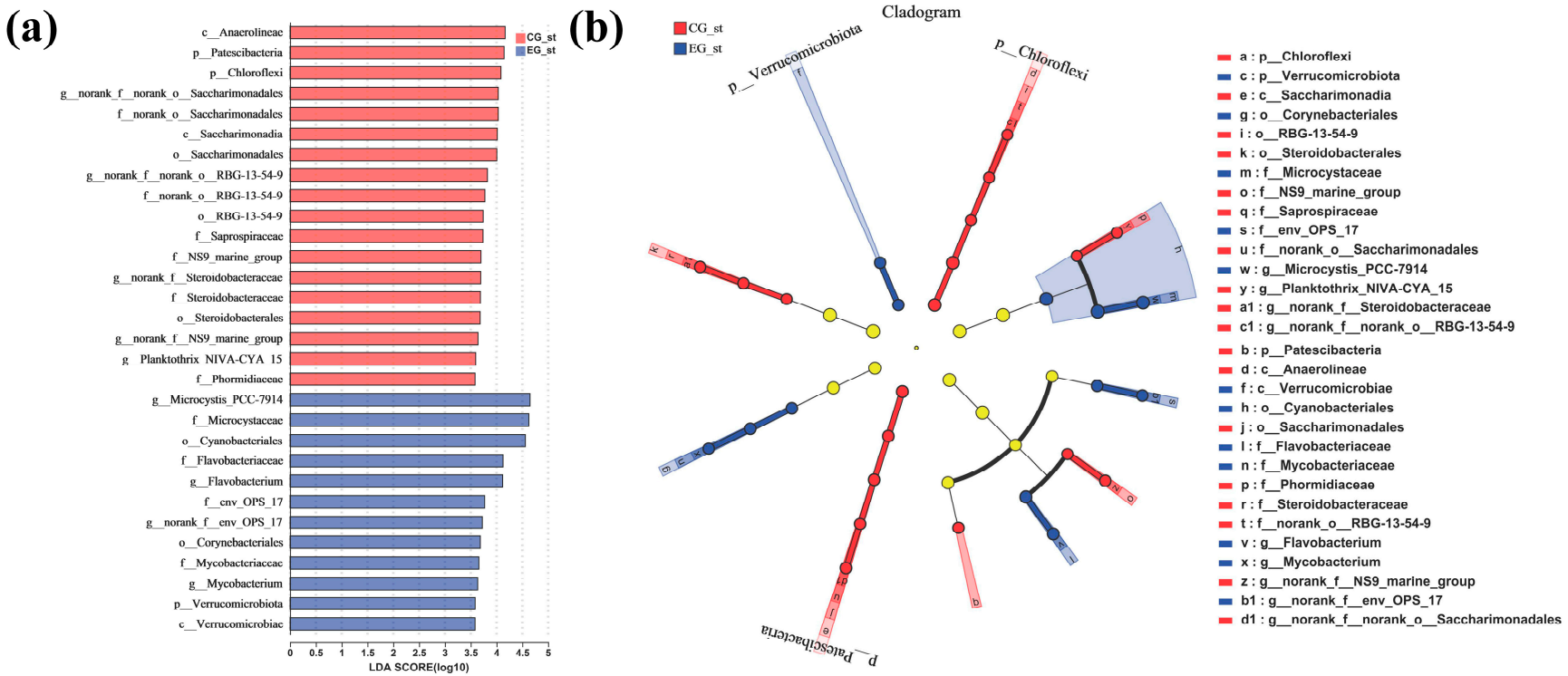

Figure 5. OTU markers in the microbial community of water in the CG_st and EG_st groups through linear discriminant analysis (LDA) (**a**) and effect size (LEfSe) (**b**).

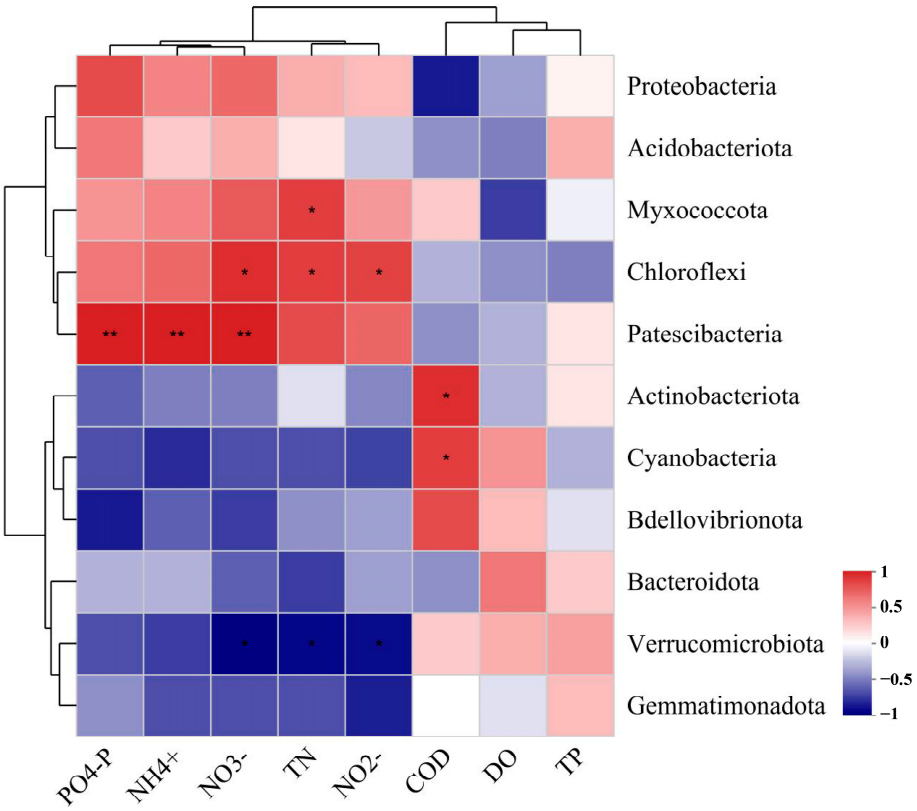

Figure 6. Spearman correlation heatmap between microbial communities based on the relative abundance of bacterial phyla and water quality indicators.* indicates $p < 0.05$ and ** indicates $p < 0.01$.

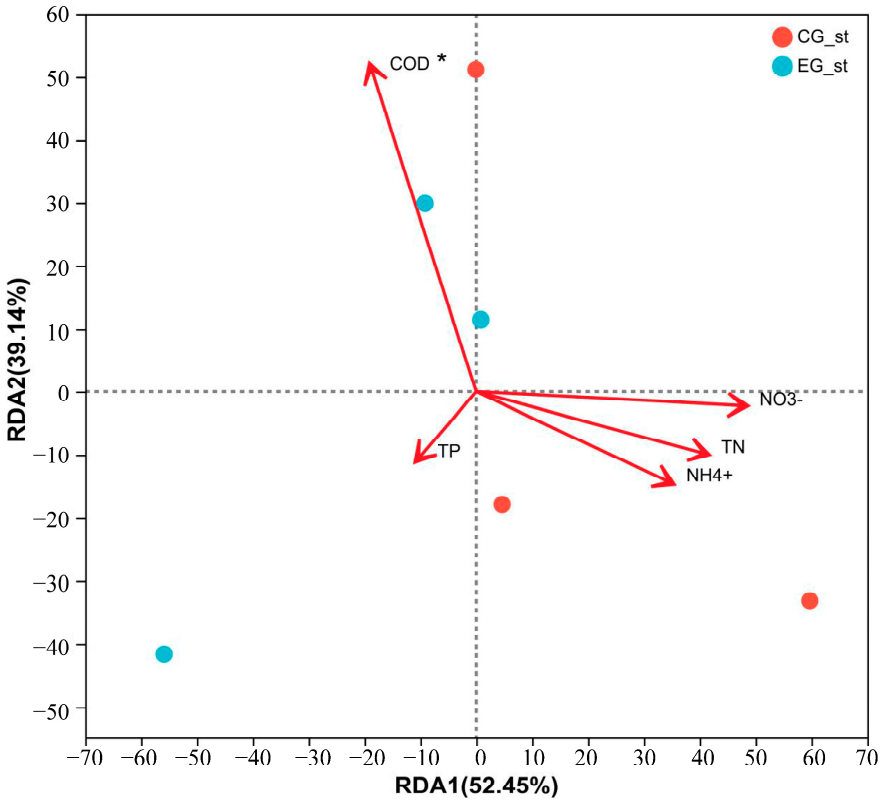

Figure 7. RDA biplot of the distribution of the bacterial community and environmental factors. * $p < 0.05$.

4. Discussion

The stability of aquaculture ecosystems is supported by complex physical and biochemical interactions. With the development of intensive production ponds, the balance of the system has become disrupted, which has restricted the growth and survival of aquatic animals. In recent years, BFT has developed into a mature technology that can achieve zero water exchange and ensure the healthy growth of aquatic animals [9]. Compared with facility-based tail water treatment technology, BFT generally has low operating costs because it requires fewer hardware facilities. In addition, BFT converts ammonia into bacterial proteins, which provides a source of food to farmed animals. However, the management of the system is difficult, and if not properly managed, complex microorganisms may increase ammonia nitrogen in the water [22]. There are three main ways for biofloc systems to remove ammonia nitrogen: namely, heterotrophic, chemoautotrophic, and photoautotrophic biological process [23]. Previous studies have indicated that the microbial composition of BFT systems varies greatly and can be influenced by various environmental factors, such as temperature, dissolved oxygen concentrations, and the C/N ratio [24,25]. In BFT systems, different microorganisms play different roles in the nutrient cycling of water [26]. Microbial communities can be suspended in water or attach to solid surfaces to form biofilms, and they are easily influenced by aquaculture patterns [27]. Under higher breeding density conditions, microbial processes are disrupted when low dissolved oxygen and other indicators of bad water quality occur. Substrates have been applied in the BFT system of aquatic animals, playing a synergistic role in in situ water quality control. In aquaculture environments, different types of substrates can be used as carriers for biofilms to improve water quality and increase the yield of aquaculture species, such as bamboo, nylon mesh, PVC pipes, and commercial products. The substrate provides attachment points for the growth of microorganisms and peripheral organisms. Keshavanath found that the type of artificial substrate has a significant impact on the primary productivity of algae in the study of the impact of artificial substrates on the yield of fish aquaculture ponds [28]. Carlos [29] studied the effects of biofilm on the growth and water environment of juvenile *Litopenaeus vannamei*, using AquaMats as the biofilm attachment substrate, which showed that different microbial communities worked together in the aquaculture system to promote nitrogen conversion in the aquaculture water.

In the composite aquaculture system of BFT and artificial substrates, biofilms may contain microalgae, algae, protozoon, and nitrifying and heterotrophic bacteria, which can absorb and assimilate free nitrogen in water, effectively reducing the ammonia nitrogen content in breeding water [4,10,30]. Microorganisms play a major role in water quality control, and the use of high-throughput sequencing can accurately characterize changes in microbial communities [31].

A total of 50,177 (CG_st) and 53595 (EG_st) high-quality gene sequences were obtained; 2082 and 2036 OTU were generated in CG_st and EG_st group, respectively. The ACE, Chao, Shannon, and Simpson index values ranged from 2525 to 3446, 2466 to 2948, 5.22 to 5.52 and 0.016 to 0.036, respectively. The data showed that richness and diversity changed between the two groups, but there was no significant difference. Bratvoldet et al., (2001) and Ray et al., (2010) confirmed the abundant change in microorganisms during the aquaculture period by adding carbon sources and artificial substrates [32,33]. Beta diversity is often applied to compare microbial community compositions and evaluate differences between microbial communities [34]. The experimental results revealed differences in the bacterial community structure of water, as evidenced by the clear separation of the CG_st group and EG_st group on the PCoA and NMDS based on the Bray–Curtis dissimilarity statistic. Figure 4a–c show that the microbial communities varied in different experimental groups at the phylum, class, and genus levels, respectively.

Water quality can influence or, at most, even disturb bacterial communities, which have a guiding role in water quality evaluation [7,35]. Proteobacteria, Bacteroidota, Chloroflexi, and Actinobacteriota are the main bacteria in phylum in river lakes and aquaculture ponds [36,37]. The results in the experiment were consistent with the research findings

above. Proteobacteria account for a significant proportion of bacteria that participated in nitrification and denitrification [38]. Previous studies have shown that this phylum is widely distributed in freshwater environments and participates in various biogeochemical processes, such as nutrient cycling and organic compound mineralization processes [22,39]. In addition, the majority of microorganisms in biofloc systems for wastewater treatment are these bacteria [40]. This means that they can effectively control the water quality of aquaculture. Bacteroidota mainly exist in hypoxic environments and participate in nitrogen removal, metabolizing large amounts of organic matter and proteins [20]. Chloroflexi is a common anaerobic ammonia oxidation denitrifying bacterium that thrives in autotrophic environments by using carbohydrates or amino acids [41]. The results showed that the abundance of Proteobacteria, Chloroflexi, and Bacteroidota changed, indicating that artificial substrate and carbon source addition changes the nitrification and denitrification process in water bodies, thereby altering nitrogen cycling in aquatic ecosystems.

The results of the Spearman correlation heatmap and RDA suggested that the bacterial composition was related to some environmental factors (Figures 6 and 7). The most significant environmental factor that influenced the dominant bacterial community distribution was COD. The results were similar to those of Tang et al., (2021), who indicated that the bacterial community composition could be affected by environmental factors and that COD was the one of the main environmental factors affecting the bacterial community; it was positively related to the dominant phyla Actinobacteria and Cyanobacteria [42]. Evidence suggests that Actinobacteria exist in various freshwater habitats [43]. It is widely believed that Actinobacteriota, as producers of antibacterial and growth-promoting substances, have the potential to develop new types of aquatic probiotics [44]. In this study, Actinobacteriota was the third most dominant phylum in the water and was significantly positively correlated with COD. This result may indicate that Actinobacteria play an important role in the metabolism of microbial communities in aquaculture water.

5. Conclusions

In the study, 16S rRNA high-throughput sequencing technology was applied to analyze the effect of artificial substrate and carbon source addition on bacterial diversity and community composition in water in a pond polyculture system in Northeast China. Artificial substrate and carbon source addition changed the structure of the microflora. There were 11 biomarkers enriched in the EG_st group: Verrucomicrobiota, Verrucomicrobiae, Corynebacteriales, Cyanobacteriales, Microcystaceae, Mycobacteriaceae, Microcystis_PCC-7914, Flavobacteriaceae, Flavobacterium, env_OPS_17259, and Mycobacterium. Research has shown that the addition of artificial substrates and carbon sources can alter the structure of bacterial communities, thereby regulating pond water bodies. The results of this study have significance as a reference for freshwater aquaculture water quality control technology.

Author Contributions: K.G.: Investigation, Writing—original draft, Project administration. M.S.: Resources, Formal analysis. X.H.: Formal analysis. L.L.: Software, Visualization. S.W.: Resources, Formal analysis. R.Z.: Supervision. W.X.: Software, Visualization. G.R.: Writing—review and editing. Z.Z.: Project administration. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal procedures in this study were conducted according to the guidelines for the care and use of laboratory animals of Heilongjiang River Fisheries Research Institute, CAFS. The studies of the animals were reviewed and approved by the Committee for the Welfare and Ethics of Laboratory Animals of Heilongjiang River Fisheries Research Institute, CAFS. Approval Code: 20210401-001; Approval Date: 1 April 2021.

Data Availability Statement: Data are contained within the article.

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Conflicts of Interest: All the authors declare that there were no conflicts of interest or personal relationships that interfered with this study.

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