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A Study of the System Performance and the Microbial Community Composition of Chemical Wastewater in an AO-MBBR Treatment Process

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Abstract: To improve the nitrogen removal and reduce the chemical oxygen demand (COD) of a full-scale wastewater treatment plant, two sequential batch reactor devices were used to treat chemical wastewater with biocarriers in low carbon-to-nitrogen (C/N) ratio conditions. The results showed that the addition of biocarriers to the anoxic tank reduced the average concentration of COD in the effluent from 98.1 mg/L to 80.7 mg/L and increased total nitrogen (TN) removal by 9.4%. Metagenomic sequencing was performed to study the composition and function of microbial community samples taken from anoxic sludge and anoxic-carrier biofilms in this wastewater treatment plant. The results showed that *Proteobacteria* and *Actinobacteria* were the dominant phyla in the two samples, ensuring their capability for organic matter removal. The anoxic-carrier biofilms were mainly enriched with denitrifying bacteria such as *Thauera* (10.7%) and *Comammonas* (2.2%) and the anammox bacteria *Candidatus Kuenenia* (0.03%). Meanwhile, the nitrogen metabolism pathway was elaborated and the abundance of the functional genes involved in the nitrogen metabolism pathway was quantified. In addition, results from qPCR showed increased copy numbers of denitrification and anammox genes in the anoxic-carrier biofilms compared to those in the anoxic sludge, further confirming the enrichment of functional bacteria.

Keywords: biocarriers; metagenomic sequencing; microbial community; nitrogen metabolism pathway

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1. Introduction

The chemical industry is an integral part of modern industrial chain supply chains; however, wastewater is usually produced by chemical processes. Chemical wastewater has diverse sources and complex compositions. This study focused on the treatment of wastewater from hydrogen peroxide production, which contains a variety of nitrogen-containing pollutants and refractory organic pollutants, including harmful substances such as ammonium salt, 2-ethylanthraquinone, trioctylphosphate, and heavy aromatic hydrocarbons. These pollutants pose serious threats to both the ecological environment of water bodies and public health. Therefore, the treatment of such wastewater is an important challenge for sustainable development and water recycling.

The anoxic/aerobic (AO) process is a popular chemical wastewater treatment method because it is simple, effective, and eco-friendly. Conventional activated sludge (CAS) is usually employed for biological nitrogen removal from sewage [1,2]. However, its performance is often influenced by factors such as temperature fluctuations, sludge bulking, carbon source type, and influent load, which may inhibit the growth activity of microorganisms and lead to poor denitrification efficiency. In light of increasingly stringent wastewater discharge standards [3], some improvements to the AO process have been proposed to boost the efficiency of wastewater treatment. To reduce the requirement for carbon sources, free nitrous acid (FNA) sludge treatment and dissolved oxygen (DO) control have been combined to achieve partial nitrification and denitrification in an assessment of a continuous flow system (aerobic-anoxic-aerobic process) with real wastewater [4]. Some studies have

proposed operation modes for a step-feed anoxic/oxic (AO) process with the distribution of the carbon source from the anaerobic zone in terms of the treatment effects on sewage with low carbon and high nitrogen and phosphorus. At the optimal flow distribution ratio, the highest microbial abundance and treatment removal efficiency were achieved [5]. However, these studies mainly focused on the process performance and optimization side, while the microbial community side was overlooked to a certain extent. Therefore, there is potential for optimizing the AO process for microbial enrichment. The moving-bed biofilm reactor (MBBR) process has attracted considerable attention since its initial development in Norway during the early 1990s for organic matter and nitrogen removal purposes [6]. It has proven to be an effective treatment technology which provides a suitable environment for the adhesion and growth of microorganisms by adding biological carriers to the system. Thus, the MBBR system has increased the number of active microorganisms, leading to excellent treatment efficacy [7] and high shock resistance. The combination of the MBBR and AO processes effectively makes up for the shortcomings of CAS, offering improved flexibility. Consequently, it has found widespread adoption in upgrades of municipal and industrial wastewater treatment plants.

The biocarrier is the core component of the MBBR system, where microorganisms attach and form a complex community, contributing strongly to nitrogen removal [8]. The composition of the microbial community directly affects the nitrogen conversion efficacy. Therefore, it is imperative to study factors affecting microbial composition and function. Currently, sequencing technologies like qPCR and 16S rRNA have provided methods for in-depth analysis of microbial communities [9]. However, 16S rRNA sequencing provides little information on functional genes. Metagenomic sequencing has emerged as an effective tool widely used for analyses of various environments like soil, laboratory-scale wastewater systems, and municipal wastewater treatment plants. Chen [10] employed metagenomic technology to study the mechanism of complete nitrification under conditions of low nitrogen and DO concentrations. Employing metagenomic analysis, Zhu [11] investigated the impact of different biocarriers on municipal sewage treatment using MBBR systems. Therefore, metagenomic analyses are necessary tools for understanding the bacterial composition and functional gene classes of the MBBR system.

In China, the adoption of MBBR is still in the initial stage, and there is a lack of relevant research on both theoretical and practical aspects of the technology. In this study, MBBR technology was applied to the traditional AO treatment process of hydrogen peroxide wastewater to improve the denitrification and COD removal effects of the wastewater, and this proved to be an effective biological method for the efficient treatment of hydrogen peroxide wastewater. A laboratory-scale anoxic/aerobic moving-bed biofilm reactor (AO-MBBR) system was built and studied with real wastewater to simulate industrial-scale nitrogen and organics treatment. Metagenomic sequencing and qPCR were used to study the composition of the bacterial community in different biochemical tanks, functional genes and pathways related to nitrogen metabolism, and their effects on wastewater nitrogen removal, providing reference data and microscopic interpretation for nitrogen removal by AO process.

2. Materials and Methods

2.1. Reactor Setup and Operation

This study focused on the treatment of chemical wastewater from a local hydrogen peroxide production plant (Changzhou, China). Currently, the anaerobic/anoxic/oxic (AAO) process is used in the treatment plant for this type of wastewater. Our laboratory-scale AO-MBBR system consists of an anoxic zone, an aerobic zone, and a sedimentation zone, as shown in Figure 1. The effective volumes of each zone are 25, 20, and 18 L, respectively. In the pilot experiment, biocarriers were only added in the anoxic zone. The carriers used are short polyethylene plastic cylinders with honeycomb structures (as shown in the inset of Figure 1). They have a density of 0.94~0.97 g/cm^3, with a specific surface area greater than 500 m^2/m^3 and a filling rate of 40%. The activated sludge used

in the experiment was from the returned sludge of the AAO process of the wastewater treatment plant, and the anoxic carriers used were first placed in the anoxic tank of the wastewater treatment plant for 180 days to develop mature biofilms. A mechanical agitator promoted biological mass transfer between the activated sludge, the carrier biofilms, and the wastewater. The temperature of the wastewater was not controlled. The experiment lasted for 150 days in two phases. The first phase (0–54 days) was the classic AO process; the core operational phase then began on the 54th day. The anoxic-carrier biofilms were inoculated into the anoxic zone of the reactor in July 2022, and the DO concentrations in the anoxic zone and the aerobic zone were maintained at 0.2–0.5 mg/L and 2.0–3.5 mg/L, respectively. The real nitrogen-containing chemical wastewater was fed into the reactor, and the sludge reflux ratio and nitrifying liquid reflux ratio were set at 100% and 200% respectively, both using pump reflux. The hydraulic retention time (HRT) was about 12 h, comprising anoxic 5.5 h and aerobic 6.5 h, and the sludge retention time (SRT) was controlled at about 16 days. The pH was 7~8, and the temperature range was 19.8~25.2 °C.

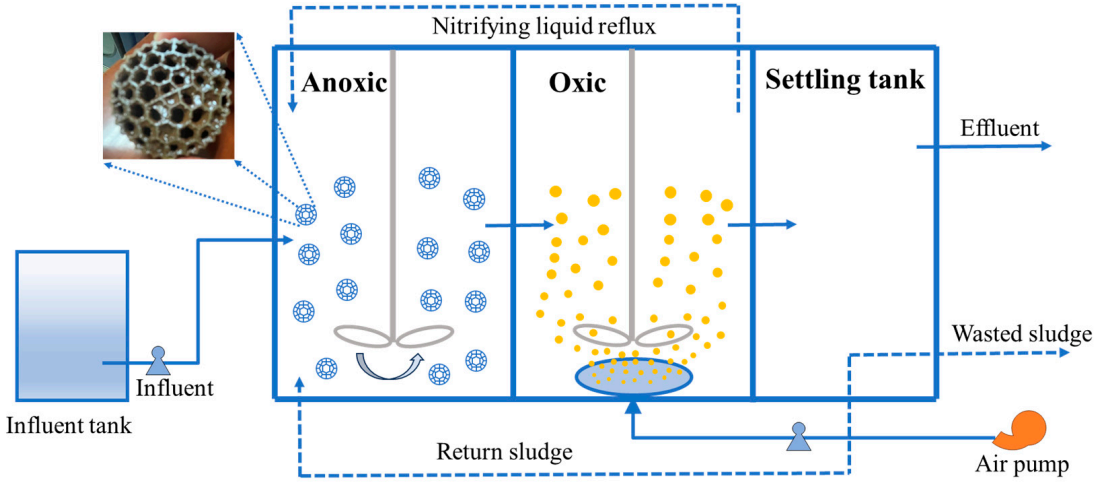

Figure 1. Schematic of the anoxic/aerobic moving-bed biofilm reactor (AO-MBBR) pilot-scale reactor. The inset shows a photo of a biocarrier.

2.2. Analytical Methods

During the operation of the reactor, daily samples were collected at the inlet and outlet. The samples were filtered through a 0.45 μm filter before water-quality tests were performed. The concentrations of COD, NH_4^+-N, TN, NO_3^--N, and NO_2^--N were determined according to the standard method [12]. These indicators were measured by a multi-parameter water-quality analyzer [5 B-3 B (V11), Lianhua Technology, Beijing, China]. Temperature, pH, and DO were measured by a separate sensor. The data obtained were plotted with Origin 2021, and a one-way analysis of variance was performed with SPSS 26.

2.3. Metagenomic Sequencing and Functional Analysis

The biofilm samples were analyzed with metagenomic sequencing to study differences in microbial community composition, nitrogen transformation pathways, and metabolic functions. The metagenomic analysis was carried out following the process of DNA extraction, sequencing, sequence analysis, gene prediction, classification, and functional annotation [13]. The samples of sludge and anoxic-carrier biofilms were taken out and freeze-dried, and then the DNA of microorganisms in the samples was extracted by the CTAB method. After the extraction was completed, the DNA samples were analyzed by 1% agarose gel electrophoresis (AGE). The DNA samples were initially processed with ultrasound into fragments of 350 bp. Subsequently, these fragments were refined at their ends by adding an A-tail and connected through full-length splicing for Illumina sequencing and subsequent PCR amplification. After library construction, the insert size of the library was measured using a bioanalyzer (Agilent 2100, Walnut Creek, CA, USA) and

finally sequenced with an Illumina PE 150 platform. The obtained sequencing readings were compared to various functional databases, such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, for annotation of nitrogen metabolism-related enzymes and genes, and functional prediction.

2.4. qPCR

The different biofilm samples were taken out, and the process of collecting the biomass was repeated three times. The two extracted DNA samples were then mixed to create one uniform DNA sample. The concentrations of the DNA samples were measured using NanoDrop instruments (ND-2000, Thermo, Waltham, MA, USA). To determine the variations in the abundance of denitrifying and anammox bacteria, a real-time quantitative PCR system dyed with fluorescent dye SYBR-Green (MA-6000, Yaro, Qingdao, Shandong, China) was used to determine the gene copy numbers of *narG*, *nirS*, *nosZ*, *norB*, *hzsB*, and *hdh*. PCR amplification was performed in 10.8 μL of reaction mixtures consisting of 10 μL of 2 × SYBR real-time PCR premixtures (Vazyme Biotech, Nanjing, China) and 0.4 μL of forward and reverse primers (10 μmol/L).

3. Results and Discussion

3.1. Performance of Nitrogen and COD Removal in the AO-MBBR System

The reactor operated for over 150 days, and the removal performance of COD, NH_4^+-N, and TN is presented in Figure 2. The results of the 150-day tests show that the influent C/N and NH_4^+-N were in the ranges of 4.1~5.8 and 28.9~38.5 mg/L. In phase I (0~54 d), the reactor was operated using the traditional activated sludge process without the addition of carriers. The removal efficiency of COD and NH_4^+-N remained relatively stable at 64.3 ± 5.7% and 93.7 ± 3.1%, respectively, indicating effective nitrification. However, the effluent TN concentration reached 28.6 ± 6.5 mg/L, with a low removal rate of only 50.6 ± 9.8%. It has been reported that wastewater treatment systems often exhibit reduced nitrogen removal efficiency when the C/N ratio is low (<6) and no external carbon source is provided for denitrification [14]. The nitrogen removal efficiency (NRE) was only 61.39 ± 10.71% in a continuous flow bioreactor with carriers in the anaerobic zone and anoxic zone under an influent C/N ratio of 2.2 [15]. Feng et al. [16] also reported that the NRE of different carrier mixed-membrane bioreactors in the treatment of wastewater with a low carbon–nitrogen ratio was lower than 70%. The NRE without carriers in this study was 50.6 ± 9.8%, which was similar to the previous reports. Moreover, the overall nitrogen removal efficiency of anoxic/oxic (AO) systems can be influenced by the nitrate recycling ratio [17], with two-stage AO systems demonstrating higher TN removal efficiency compared to one-stage AO systems [18]. In phase II (55–150 d), anoxic-carrier biofilms were inoculated into the anoxic zone without altering the operational parameters. The NH_4^+-N removal rate did not change significantly, while the average effluent COD concentration decreased from 98.1 mg/L to 80.7 mg/L, with a corresponding removal rate of 70.1 ± 6.4%. The concentration of NO_3^--N in the effluent decreased to 18.4 ± 5.1 mg/L, and the concentration of NO_2^--N in the effluent stabilized at 1.0 ± 0.9 mg/L, which was significantly lower than that in the first stage. Additionally, the TN concentration in the effluent decreased to 20.8 ± 6.2 mg/L, resulting in a 9.4% increase in the removal rate compared to the first stage. Zhao [19] conducted a pilot study on the MBBR system with real sewage as the research object. The TN concentration in the effluent decreased significantly after the addition of biological carriers, reaching 9.8 ± 1.6 mg/L, and the nitrogen removal efficiency increased significantly to reach 74.4 ± 3.5%. Overall, the addition of anoxic carriers successfully enhanced both the nitrogen and COD removal efficiency of wastewater treatment. To further analyze how anoxic carriers improve these efficiencies, metagenomic sequencing was employed to investigate microbial diversity, microbial community composition, the nitrogen metabolism pathway, and functional genes present in the anoxic sludge and the anoxic carrier.

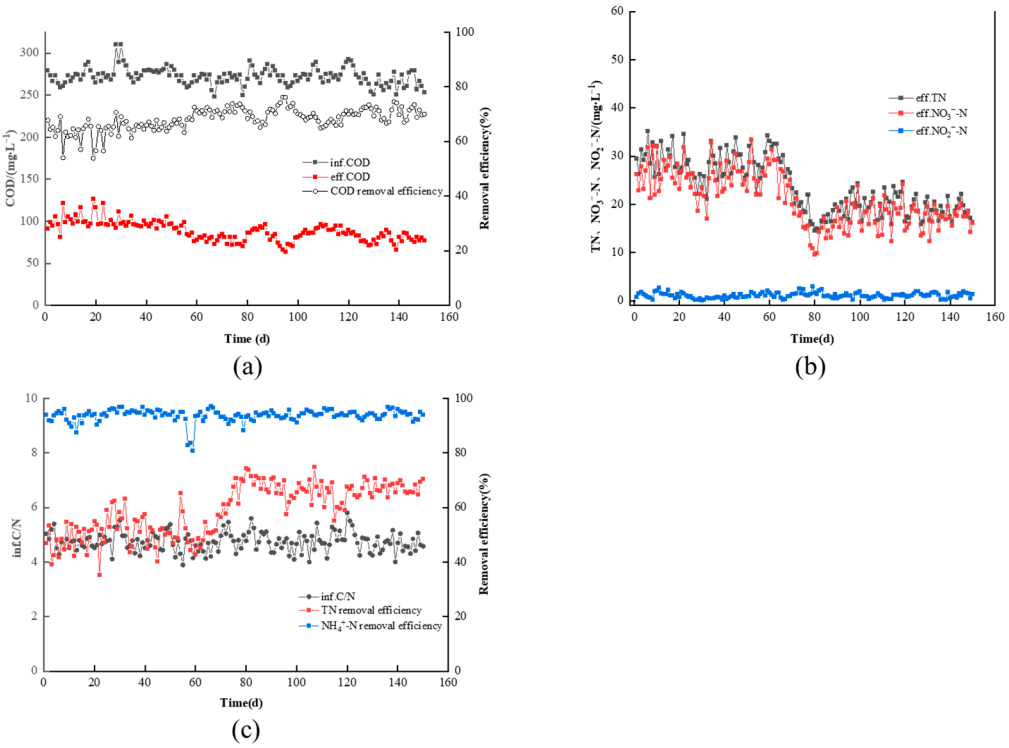

Figure 2. Performance of AO-MBBR: (**a**) Influent COD, effluent COD concentrations, and COD removal rate; (**b**) effluent TN, NO_3^--N, and NO_2^--N concentrations; (**c**) Influent C/N, TN, and NH_4^+-N removal rates.

3.2. Microbial Diversity Analysis

The α-diversity index of the anoxic sludge and the anoxic carriers is presented in Table 1. The library coverage for both samples ranges from 99.89% to 99.95%, indicating the high efficiency and comprehensiveness of the sequencing technology used in representing most bacterial species. The Chao 1 index reflects microbial community richness, while the Shannon index demonstrates a positive correlation with species diversity, and the Simpson index shows a negative correlation with species diversity [20]. Regarding the Chao 1 index relationship among the two samples, it can be said that anoxic carriers > anoxic sludge, suggesting that biological carriers enhance microbial community richness. Regarding the Shannon index relationship among the two samples, it can be said that anoxic carriers > anoxic sludge, which suggests the diversity index of anoxic sludge is comparatively lower due to its longer microbial adaptation period. Conversely, the diversity index of anoxic carriers is higher, indicating that the addition of biological carriers increases both species diversity and richness.

Table 1. Comparative analysis of α diversity index.

Sample	Sobs	Chao 1	Coverage	Shannon	Simpson
anoxic sludge	2067	2287	99.95	3.165	0.109
anoxic carriers	2529	2778	99.89	4.454	0.035

Figure 3 shows a Venn diagram of common or endemic species of anoxic carriers and anoxic sludge, which can further explain their common microbial status. Despite the species differences between the anoxic carriers and anoxic sludge samples, the total number of OTUs reached 1256. In addition, the OTUs of the anoxic carriers were higher than those of the anoxic sludge, reaching 3125. The main reason is that the diversity of microorganisms changes significantly after anoxic-carrier biofilms mature.

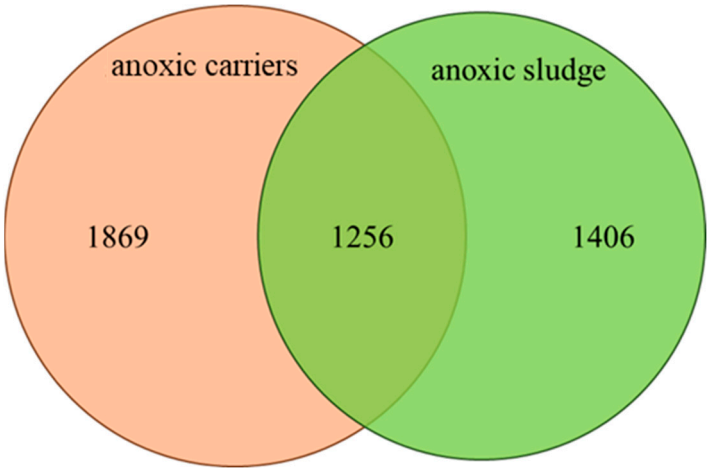

Figure 3. Venn diagram of common or endemic species.

3.3. Microbial Community Analysis

Metagenomic analysis of microbial communities in the two samples showed that the microorganisms in the two samples covered 51 phyla, 70 classes, 152 orders, 351 families, and 1180 genera. The differences in the microbial communities of the two samples were further studied at phylum and genus levels, and the effects of the biocarriers on microbial community structure and wastewater treatment efficiency were analyzed.

Similar to findings from other studies [21], *Proteobacteria* and *Actinobacteria* were identified as the dominant phyla in both samples at the phylum level (Figure 4a). The relative abundance of *Proteobacteria* was found to be approximately 83.6% in the anoxic sludge and 67.5% in the anoxic carriers, respectively, and plays a key role in denitrification and COD reduction. *Proteobacteria* are widely distributed in various laboratory and industrial wastewater treatment systems and have been identified as the dominant bacteria [22]. Many denitrification microorganisms belong to this phylum. *Actinobacteria* belong to the second most abundant phylum, with relative abundance of 12.6% and 10.8% in anoxic sludge and anoxic carriers respectively, mainly degrading organic matter [23]. The high abundance of these two phyla in anoxic sludge and anoxic carriers ensures the basic capability for COD and nitrogen removal in the process. In this study, the relative abundance of *Firmicutes*, *Bacteroidetes* and *Planctomycetes* in the anoxic carriers were 5.1%, 4.5% and 0.4% respectively, which were higher than those in the anoxic sludge. *Firmicutes* can degrade carbohydrates and proteins through hydrolytic enzymes [24]; Bacteroides are beneficial for nitrogen element absorption in sewage and play a crucial role in wastewater denitrification; Anammox bacteria, as a subordinate genus of *Planctomycetes* [25], may contribute to denitrification processes within anoxic tanks. These findings indicate that anoxic carriers enhance nitrogen removal efficiency by enriching microorganisms associated with nitrogen metabolism.

Further analysis is performed on microbial community characteristics at the class level (Figure 4b). At the class level, the two samples showed relatively high abundance of *Gammaproteobacteria*, *Actinomycetia*, *Alphaproteobacteria*, and *Betaproteobacteria*. All of them except the Actinomycetes belong to the *Proteobacteria*. Actinomycetes belong to the phylum actinomycetes, and their abundance was higher in the anoxic sludge, which was consistent with the phylum-level study. The higher abundance of *Gammaproteobacteria* in anoxic sludge was 28.5%. The abundance of α-*Proteobacteria* in anoxic carriers was higher at 15.9%. Some studies have confirmed that α-*Proteobacteria* were the dominant bacteria in some biofilm samples, and their main function was the biodegradation of pollutants in sewage, including COD removal, sulfur metabolism, and nitrogen removal [7]. The abundance of β-*Proteobacteria* in the anoxic sludge and the anoxic carriers was 12.2% and 26.3%, respectively. Previous studies have found that β-*Proteobacteria* are the dominant class of bacteria in wastewater treatment reactors and are closely related to denitrification processes [26]. This finding suggests that the high denitrification activity of anoxic-carrier

biofilms enhances the denitrification effect of the MBBR process. Overall, these bacteria exhibit a high diversity in environmental adaptation and metabolism, which contributes to the removal of various pollutants.

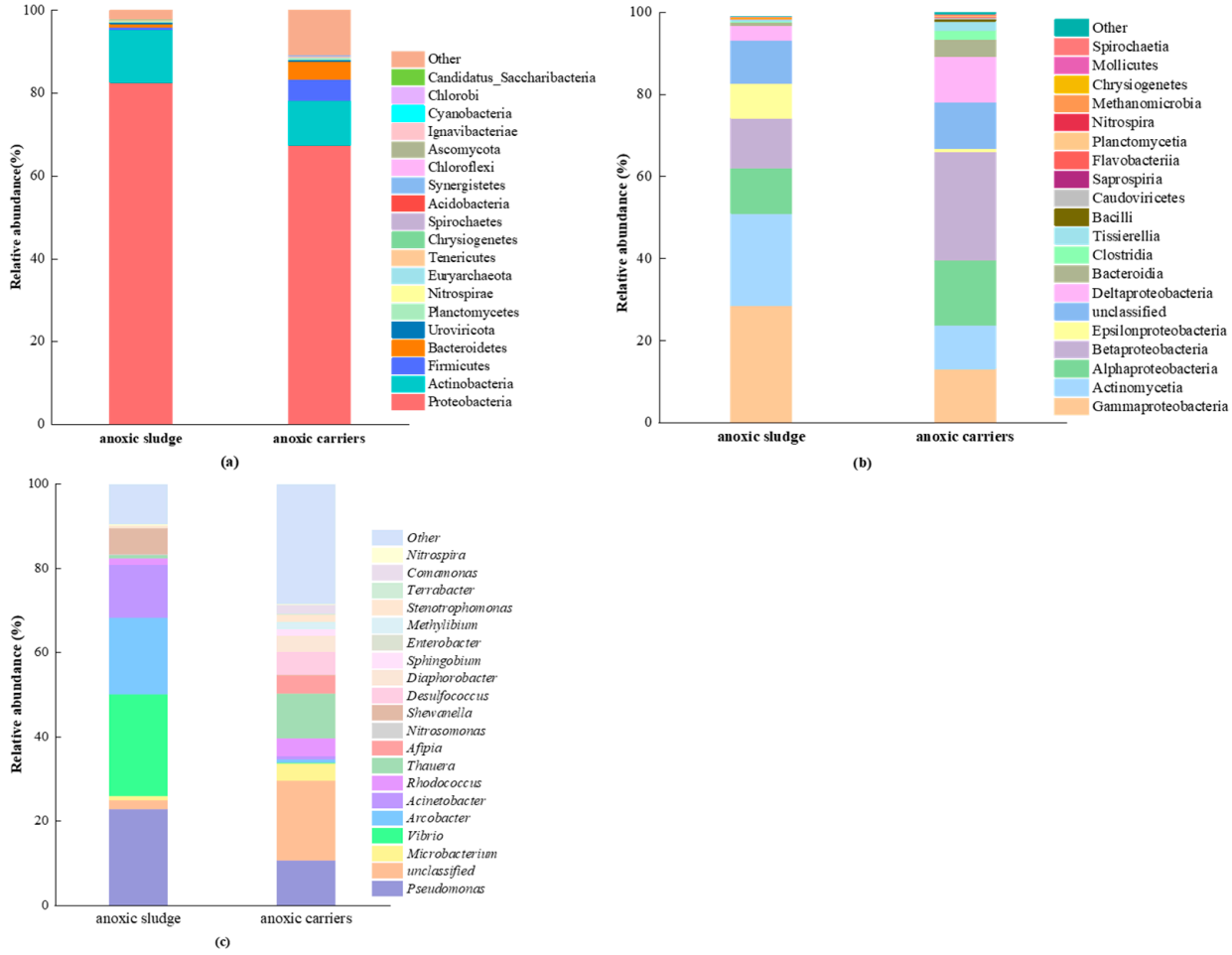

Figure 4. Microflora structure composition of different samples at phyla level (**a**), composition diagram of the top 20 classes in relative abundance (**b**), microflora structure composition of different samples at genus level (**c**).

Microbial species associated with nitrogen metabolism and organic degradation were analyzed at the generic level (Figure 4c). Denitrifying bacteria are the dominant microbial communities in anoxic sludge and anoxic carriers, mainly including *Thauera* and *Comamonas*, which are classified into *Proteobacteria* and *Bacteroides*. *Thauera* is a typical genus of denitrifying bacteria, ubiquitous and dominant in many wastewater treatment plants, which are involved in carbon and nitrogen metabolic pathways that enable them to remove carbon and nitrogen from wastewater [27]. *Comamonas* is an effective heterotrophic denitrification bacterium for nitrate removal in carbon-rich environments with low oxygen levels [28]. The relative abundance of *Thauera* and *Comamonas* in the anoxic carriers was significantly higher at 10.7% and 2.2%, respectively, compared to the anoxic sludge ($p < 0.05$), indicating that efficient enrichment of denitrifying bacteria within the anoxic carriers is crucial for improving COD degradation efficiency as well as the total nitrogen removal rate. In addition, *Pseudomonas* and *Acinetobacter* with high abundance were detected in the anoxic sludge, accounting for 22.8% and 12.5%, respectively. *Pseudomonas* has the ability to decompose organic compounds such as phenols and polycyclic aromatic hydrocarbons [29]; *Acinetobacter* is considered to be a potential phosphorus-accumulating bacteria (PAOs), which plays a significant role in phosphate removal [24].

In summary, most of the bacteria present within the anoxic tank exhibit organic matter degradation abilities which ensure excellent denitrification performance along with COD degradation capacity; however, functional bacterial enrichment related to carbon and nitrogen metabolism through the utilization of anoxic carriers further enhances both the total nitrogen removal rate as well as COD degradation efficiency.

3.4. Functional Annotation

The functional gene annotation of the biofilm samples was achieved by comparing the sequenced reads with the KEGG database. The results of the annotation of the two samples mainly comprise six pathways. The metabolism of microorganisms accounts for 69.10~73.49%, and the remaining 26.51~30.90% is shared among five pathways: genetic information processing, environmental information processing, cellular processing, biological systems, and human diseases. These six pathways were further expanded in the KEGG database (Figure 5), where amino acid metabolism dominated, accounting for 12.38% to 13.01%, followed by carbohydrate metabolism, accounting for 9.24% to 11.07%. The carbohydrate metabolism subsystem consists mainly of the tricarboxylic acid cycle, glycolysis, and common pathways of carbohydrate transport, which are widely observed in activated sludge and utilized for microbial energy production and cellular synthesis processes [30]. Additionally, cofactors and subpathways of vitamin metabolism, nucleotide metabolism, energy metabolism, translation, membrane transport, and signal transduction are also critical. In conclusion, the complex metabolic pathway is consistent with the diversity of microorganisms in wastewater treatment plants, and the addition of anoxic carriers does not change the metabolic pathways of microorganisms.

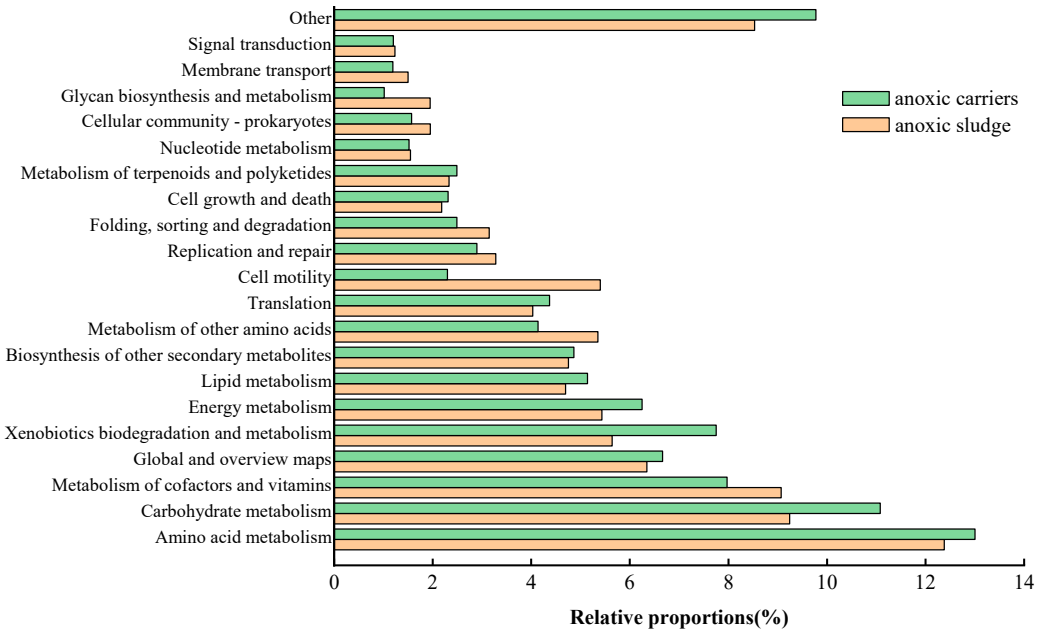

Figure 5. Functional annotation of genes in three samples according to the KEGG database.

3.5. KEGG Nitrogen Metabolic Pathways and Functional Genes

By summarizing the genes related to nitrogen metabolism reported in the literature, the corresponding nitrogen cycle pathway was constructed [31] (refer to Figure 6). In the AO process, the nitrogen removal process is mainly divided into two parts: nitrification and denitrification. Nitrification includes ammonia oxidation and nitrite oxidation; denitrification mainly involves the step sequence NO_3^--N→NO_2^--N→NO→N_2O→N_2 [32,33], where each step is controlled by different reductases.

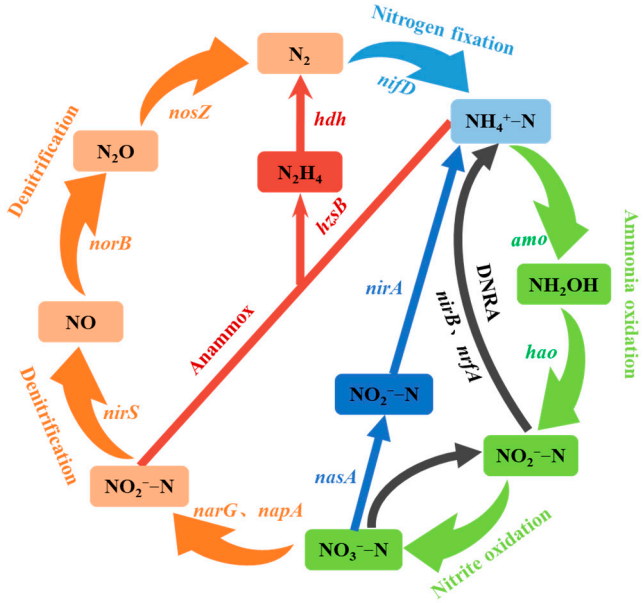

Figure 6. Nitrogen metabolism pathways under the action of different microorganisms.

To better understand nitrogen transformation through different pathways, the relative abundances of key enzyme genes were obtained by metagenomic sequencing. The assembled sequence data were imported into the KEGG database for functional annotation at the KO (KEGG Orthology) level. Subsequently, the annotated genes were compared with those encoding key metabolic enzymes in the nitrogen metabolic pathway map00910. A total of 788 genes were identified to be functionally associated with nitrogen metabolism, accounting for 0.107% of the anoxic sludge and 0.111% of the total genes. Finally, the relative abundance of functional genes involved in nitrogen metabolism was calculated (see Table 2). The results showed that the relative abundance of denitrifying genes (*narG*, *napA*, *nirS*, *norB*, *nosZ*) was the highest, which was consistent with the high abundance of denitrifying bacteria obtained by sequencing. The next genes were nitrogen-fixing genes (*nifD*) and nitrification genes (*amo*, *hao*). Finally, nitrate reduction genes (*nirB*, *nrfA*) and anammox genes (*hzsB*, *hdh*) were identified. Among them, the enzyme genes controlling denitrification were highly enriched in the anoxic-carrier biofilms, and nitrate reductase and nitrite reductase had a high abundance (*narG* 2011 hits, *nirS* 1345 hits), which was consistent with the results of qPCR (see Figure 7) and explains the cause and mechanism of the decrease in NO_3^--N concentration in the effluent. Besides, a low abundance of ammoxidation enzyme genes (*amo* 232 hits, *hao* 325 hits) was detected in the anoxic sludge because the growth of nitrifying bacteria was inhibited in the anoxic environment; similar patterns of functional genes were also observed by Guo et al. [31].

In addition to the denitrification genes, *Candidatus Kuenenia* and its functional genes (*hzsB*, *hdh*) were also detected in the anoxic sludge and the anoxic carriers. Metagenomic analysis showed that the relative abundance of anammox bacteria in the anoxic carriers (0.03%) was higher than that in the anoxic sludge (0.0016%), which was consistent with the difference of *Planctomycetes* found at the phylum level and the gene abundance measured by qPCR (see Figure 5). Gong et al. [34] successfully initiated the anammox process using flocculated sludge as the inoculum and biocarriers for the rapid cultivation and enrichment of anammox bacteria, achieving a TN removal rate of 66.7% on day 64. Furthermore, qPCR results showed that the copy number of the nitrate reductase gene (*narG*) in the anoxic carriers was significantly higher than that in the anoxic sludge, which may promote the enrichment of anammox bacteria in anoxic carriers. Nitrite nitrogen, as the matrix for anammox, probably originates from denitrification and dissimilatory nitrate reduction processes, as it is difficult to accumulate by nitrification in an anoxic zone. To sum up, these findings suggest that adding biocarriers to the anoxic tank enriched denitrification

bacteria and anammox bacteria effectively, thus improving the removal rate of COD and TN. The analysis of the nitrogen metabolism pathway suggested that the nitrogen removal efficiency of the wastewater treatment plant could be further improved by strengthening the partial denitrification with anammox (PD/A) coupling technology.

Table 2. Abundance of key enzymes associated with nitrogen metabolism.

Pathway	Gene	Anoxic Sludge	Anoxic Carriers
Nitrogen fixation	*nif*D	1092	915
Nitrification	*amo*	232	102
	hao	325	189
Denitrification	*nar*G	497	2011
	*nap*A	371	634
	*nir*S	360	1345
	*nor*B	315	1745
	*nos*Z	113	550
Dissimilatory nitrate reduction	*nir*B	598	665
	nrf A	298	230
Assimilatory nitrate reduction	*nas*A	114	102
	*nir*A	0	0
Anammox	*hdh*	38	156
	*hzs*B	63	278

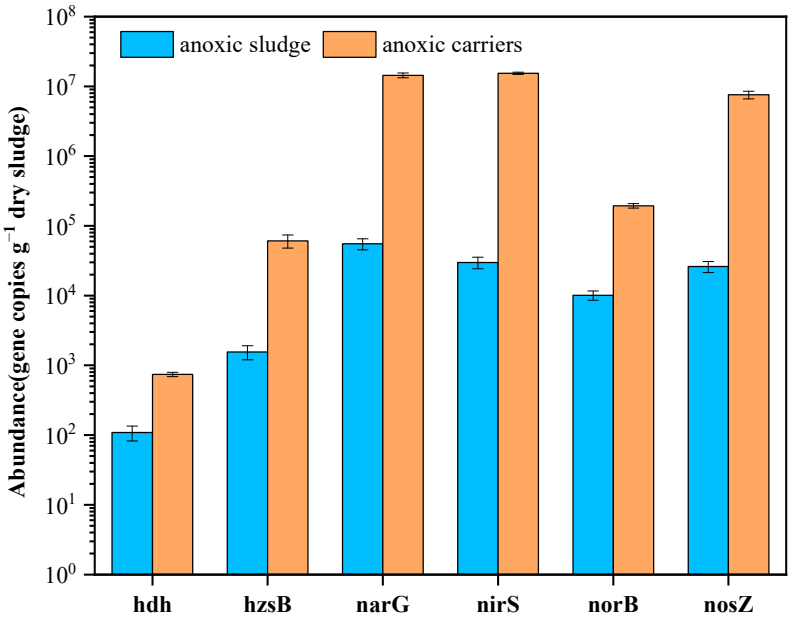

Figure 7. Comparison of the abundance of anammox and denitrification genes in anoxic sludge and anoxic carriers.

3.6. Engineering Potential and Future Trends of AO-MBBR System

Previous studies of the treatment of chemical wastewater using the AO-MBBR process have mainly focused on the aerobic zone, as it has shown higher rates of organic matter removal and nitrification efficiency [35,36]. In contrast, there have been few studies that have applied carrier biofilms to the anoxic zone to enhance the nitrogen removal efficiency of industrial wastewater treatment plants. Furthermore, the microbial composition and the mechanism of nitrogen conversion in anoxic-carrier biofilms are not fully understood. This study aimed to address these gaps by combining the AO process with the MBBR system to evaluate nitrogen metabolism in anoxic carriers. A comprehensive analysis of microbial

communities, nitrogen metabolism pathways, and functional genes was conducted. The results demonstrated that anoxic-carrier biofilms facilitate efficient nitrogen and organic matter removal. Additionally, this study highlights the presence and potential contribution of anammox. Considering the comprehensive nitrogen removal requirements of wastewater treatment plants, the combination of PD/A and AO-MBBR is a promising option for facility upgrades; however, further research is needed to address controllable issues. Key factors affecting PD/A include carbon source type, nitration reflux ratio, and C/N ratio in AO-MBBR systems. Moreover, studying microbial pathways such as phototrophic nitrite oxidation and complete ammonia oxidation (comammox) should also be considered when investigating denitrification reactions [37].

4. Conclusions

This study proposed the use of an AO-MBBR system in the treatment of chemical wastewater from the production of hydrogen peroxide. A comparative analysis of the diversity and composition of the microbial community, and the abundance of functional bacteria in anoxic sludge and anoxic carriers, was performed using metagenomic sequencing and qPCR. Additionally, quantitative analysis was performed on the functional genes involved in nitrogen metabolic pathways. Based on these findings, we draw the following conclusions:

(1) The presence of anoxic carriers in the AO-MBBR system alters the microbial community composition and improves the abundance of microorganisms associated with carbon and nitrogen metabolism in anoxic sludge. This enhances the reduction of both nitrogen and COD. The main dominant phyla in the MBBR system are Proteobacteria, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*. The dominant classes are *Gammaproteobacteria*, *Actinomycetia*, *Alphaproteobacteria*, and *Betaproteobacteria*. The dominant genera include *Pseudomonas*, *Thauera*, and *Comamonas*.

(2) Metagenomic sequencing analysis showed that the relative abundance of functional genes that control the denitrification pathway is the highest among the six nitrogen cycle pathways, indicating that denitrification plays a dominant role in systematic nitrogen removal. In addition, metagenomic analysis revealed significantly higher abundances of denitrification functional genes (*narG*, *napA*, *nirS*, *norB*, *nosZ*) and anammox genes (*hzsB*, *hdh*) in the anoxic carriers compared to those found in the anoxic sludge, indicating the presence of anammox processes.

(3) Through enrichment of the denitrifying and anammox bacteria, anoxic carriers effectively increase total nitrogen removal. However, further investigation is required to determine the contribution from each of the bacteria types. We hope that the results of this study will provide important data for a better understanding of nitrogen removal mechanisms within AO-MBBR systems, paving the way for industrial adoption of this technology in the treatment of chemical wastewater.

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