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Pilot-scale Evaluation of an Anaerobic/Anoxic/Oxic Process for Nitrogen Removal from Sewage Using Metagenomic Sequencing

Dongyang Hu [1], Jie Zhao [2], Jiancheng Wang [1], Shenghu Yan [1], Chengrui Wang [3], Qing Qing [1], Jie Zhu [1], Wenping Tao [2], Liqun Wang [1, *]

[1] Department of Biological Engineering, School of Pharmacy, Changzhou University, Gehu Rd, Wujin District, Changzhou 213164, China
[2] Changzhou Chemical Co., LTD, China Salt Group, Changzhou 213200, China
[3] Wekemo Tech Group Co., LTD, Liuxian Rd, Nanshan District, Shenzhen 518000, China
[*] **Corresponding author**: Liqun Wang (Email: wlq@cczu.edu.cn)

Abstract: A modified pilot plant with two sequencing batch reactors on the strength of utilizing the inner carbon supply and adding suspended carriers was operated for 280 d to analyze nitrogen removal performance and microorganism community. Despite a low chemical oxygen demand (COD)/total nitrogen (TN) ratio of 3.5, the TN in the effluent decreased from 37.2 to 10.4 mg N/L. High-throughput sequencing indicated significant differences in the abundance of the phylum Actinobacteria ($p<0.0001$), Firmicutes ($p<0.0001$), Bacteroidetes ($p<0.001$), Uroviricota ($p<0.0001$) and Planctomycetes ($p<0.0001$) between the anoxic-carrier biofilms and flocculent sludge. Quantitative PCR revealed that denitrification and anammox genes were additional abundant in the anoxic-carrier biofilms than flocculent sludge (*narG*: $p<0.0001$; *nirS*: $p<0.01$; *narH*: $p<0.01$; *norB*: $p<0.0001$; *hzsB*: $p<0.01$; *hdh*: $p<0.01$). Thus, enrichment with denitrification and anammox bacteria might improve nitrogen removal; this was supported by KEGG pathway annotation. Collectively, this study suggests that anoxic-carrier biofilms might enhance nitrogen removal through partial denitrification and anammox process in CWWTPs.

Keywords: Bacterial Modified Anaerobic/Anoxic/Oxic Process; Metagenomic Sequencing; Microbial Community Structure; Metabolic Pathways; Nitrogen Removal.

1. Introduction

With the rapid development of the chemical industry, pollution from chemical manufacturing processes will cause significant harm to humans and the environment [1]. During the production of hydrogen peroxide, the treatment of effluents that contain a large amount of inorganic and organic pollutants, such as ammonium salts, 2-ethylanthraquinone, 1,3,5-trimethylbenzene, and trioctyl phosphate, is challenging [2]. Traditional biological treatment does not meet the new local standards; chemical oxygen demand by dichromate (CODcr <60 mg/L, NH4-N <8.0 mg/L) for direct discharge [3]. Consequently, several biological processes, such as anaerobic-anoxic-aerobic (AAO), anaerobic-anoxic-aerobic-aerobic (AAOO), and anaerobic-aerobic-anoxic-aerobic (AOAO), have emerged [4].

The AAO process, a system combining the traditional activated sludge, nitrification, denitrification, and biological phosphorus removal processes, has been widely used for chemical wastewater treatment plants (CWWTPs) in China owing to its effectiveness and low cost compared to other biological treatment strategies [5,6,7]. The removal of nitrogen in the AAO process is commonly related to low chemical oxygen demand/total nitrogen (COD/TN) ratio, biomass, temperature, influent load fluctuation, and sludge bulking [8]. First, a lower COD/TN ratio in CWWTPs results in unsatisfactory performance because of it lacks sufficient carbon for the denitrification process [9,10]. Second, the process conditions to satisfy the requirement for biodegradation has been reported to be related to the mass of sludge in the reactor, as well the sludge age [11]. In addition, a combination of high impact resistance with a membrane bioreactor process with high sludge age is more effective concerning reduction of sludge production than with a conventional activated sludge process at lower sludge ages [12].

On this basis, some improvements were made to the AAO process to improve the efficiency of wastewater treatment. Using an internal reflux system in the deoxidation zone, a step-feed AAO process for treating low-carbon and high-nitrogen wastewater by distributing carbon sources from the anaerobic zone has been developed to improve the treatment response [13]. To reduce the carbon source requirement, the practicability of mixing free acid sludge treatment and dissolved oxygen (DO) management to achieve partial nitrification–denitrification in a continuous flow system (an aerobic-anoxic-oxic process) using real wastewater was assessed [14]. These studies only targeted process performance and improvement, whereas the microorganism community was majorly overlooked. However, modifications in control and operating conditions have also been reported, such as extended hydraulic retention time (HRT) and solid retention time (SRT) [15]. Nonetheless, the longer HRT would diminish the sewage sludge treatment efficiency per unit of time, and the longer SRT would reduce the nitrogen removal efficiency. Therefore, the AAO process requires a modification to allow for low temperatures and carbon/nitrogen (C/N) inflow.

Significant nitrogen loss may occur in the anoxic zone owing to the concentrations of organic carbon and favorably low DO level [16,17]. Biofilms effectively enrich low-growth biomass, thereby enhancing retention; this is attributed to the specific bacterial niche [18]. In addition, the enrichment of bacteria might improve the overall nitrogen removal performance of CWWTPs, especially for wastewater with an insufficient influent carbon source. [19]. Thus, it is more significant to explore the nitrogen removal effect of suspended carriers with mature biofilms in anoxic zone in pilot scale installations.

This study integrated two sequencing batch reactors (SBRs) with anoxic-carrier biofilms into the AAO process. They were observed for over 280 days with a low COD/TN ratio of actual chemical industry wastewater. We aimed to (1) investigate the performance of the AAO system and modified pilot-scale plant for treating low C/N chemical wastewater; (2) use metagenomic sequencing and quantitative reverse-transcription PCR (qPCR) to analyze the abundance and composition of the microbial community structure in flocculent sludge in the anoxic and oxic zones and anoxic-carrier biofilms, and (3) to elucidate the basic mechanism of nitrogen removal. Compared with assembly-based species annotation, reads-based metagenomic species annotation methods are more comprehensive and accurate. We believe our study would provide novel insights into potential strategies for optimizing chemical sewage treatment plants.

2. Materials and Methods

2.1. Pilot-scale Experimental Reactor Operation

This study investigated the CWWTP located in Changzhou City, southeast China. The typical AAO process was used as the primary treatment for removing organic matter and nutrients. The pilot-scale experimental reactor contained two tandem SBRs (40L) simulating anoxic and oxic zones, named SBR-anoxic and SBR-oxic, respectively. Anoxic carriers were added into the anoxic zone to form mature biofilms and ensure that the suspended carriers and sewage water were fully in contact. The experiment lasted 280 days and was divided into two phases. In Phase I (days 1–52), the activated sludge was inoculated into the SBR according to the frequency of inoculated activated sludge entering the AAO process (activated sludge was collected from a water treatment plant in Changzhou). The real sewage contained NO_3^--N, NO_2^--N, NH_4^+-N and complex organic salt. The HRT was approximately 12 h, including 2 hours, 3 hours, and 7 hours under anaerobic, anoxic, and oxic conditions, respectively. The SRT was set to approximately 16 days. The temperature ranged from 10.7 °C to 25.2 °C. In Phase II (days 53–280), the suspended carriers from the anoxic zone were added into the SBR-anoxic reactor with a filling ratio of 40%. Influent and effluent samples of pilot-scale experimental reactor were collected daily.

2.2. Analytical Methods

All samples were filtered through 0.45-μm filters. The COD and concentrations of ammonium and total nitrogen were measured according to standard methods [20]. The MLSS and MLVSS from different zones were measured following standard methods [20].

2.3. High-throughput Sequencing and Microbial Community Analysis

Flocculent sludge and anoxic carriers were taken from the anoxic and oxic zones in August 2022 and freeze-dried (Free Zone 2.0; Labconco Co., Kansas City, MO, USA). The extraction was carried out using the cetyltrimethylammonium bromide method. The purity and integrity of the extracted DNA samples were analyzed using agarose gel electrophoresis for metagenomic sequencing. DNA purity was assessed by Nanodrop (OD 260/280 ratio). DNA concentration was accurately quantified with Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA). The extracted DNA samples were fragmented by sonication and then end-polished, A-tailed, and ligated with a full-length adaptor for Illumina sequencing with further PCR amplification. After the library preparation was completed, an Agilent 2100 bioanalyzer system (Santa Clara, CA, USA) was used to detect the size of the inserts. Then, sequencing was performed using an Illumina PE150 platform (San Diego, CA, USA) [21]. KneadData software was used to conduct quality control (based on Trimmomatic) and dehosting (based on Bowtie2) of the original data [22]. Before and after using KneadData, Fast QC was used to test the rationality and effect of quality control. Kraken2 and the self-built microbial nucleic acid database, which contains the screened sequences belonging to bacteria, fungi, archaea, and viruses in the NCBI Nucleotide and RefSeq genome-wide database, were used to calculate the number of sequences of species in the sample [23]. Bracken was then used to estimate the actual abundance of species in the sample [24].

2.4. Quantitative Reverse-Transcription PCR

Anoxic carriers and flocculent sludge were collected in August 2022 from the anoxic zone and freeze-dried (Alpha 2-4 LSCbasic, Christ, Osterode am Harz, Germany). The biomass was collected thrice. Extracted triplicate DNA samples were pooled together to create one single DNA sample. The concentrations of the pure DNA samples were measured by a NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA, USA). To determine the variations in the abundance of denitrification and anammox bacteria, the gene copy numbers of narG, nirG, narH, norB, hzsB, and hdh were measured by qPCR using an MA-6000 Real-Time PCR system (Agilent, Stratagene, Santa Clara, CA, USA) stained with fluorescent SYBR-Green. PCR amplification was performed in 10.8 μL reaction mixtures, consisting of 10 μL of 2× SYBR real-time PCR premixture (Vazyme Biotech, Nanjing, China) and 0.4 μL of both the forward and reverse primers (10 μmol/L).

2.5. Statistical Analyses

All data were expressed as mean ± SD analyzed by GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). A T test was used to evaluate the significance of all pairs. The following terminology denotes the statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

3. Results and Discussion

3.1. Chemical Oxygen Demand Concentration and Nitrogen Removal Performance

The SBRs were fed with real chemical sewage and made consistent with AAO without changing the Phase I (Days 1–53) operational parameters; this included the HRT, SRT, and nitrifying liquid reflux ratios. Maintaining stability between the water output index of the two tandem SBR devices from day 33 resulted in an average ammonium removal efficiency of 96.1%, with an average effluent ammonium concentration of 2.6 mg/L (Fig. 1B). These results indicated optimal nitrification performance during this process. However, the average effluent TN was 37.1 mg/L, with an average TN removal efficiency (NRE) of only 51.4% owing to the low C/N ratio, resulting in incomplete denitrification (Fig. 1C). Meanwhile, the average effluent COD concentration was 50.5 mg/L with a removal efficiency on average was 81.2% (Fig. 1A). In Phase II (days 54–280), The carriers were inoculated in SBR-Anoxic without changing the operational parameters on day 54. During days 54–108, the anoxic biofilm activity gradually recovered; however, no noticeable effects were observed. During days 54–280, in the following stable operating period, the average effluent ammonium concentration was stable at 5.4 mg/L, and TN decreased from 37.2 to 13.4 mg/L. The NRE was stable at 82.6%, and the effluent COD concentration was 48.3 mg/L. Adding anoxic carriers could increase the NRE from 51.4% to 82.6%.

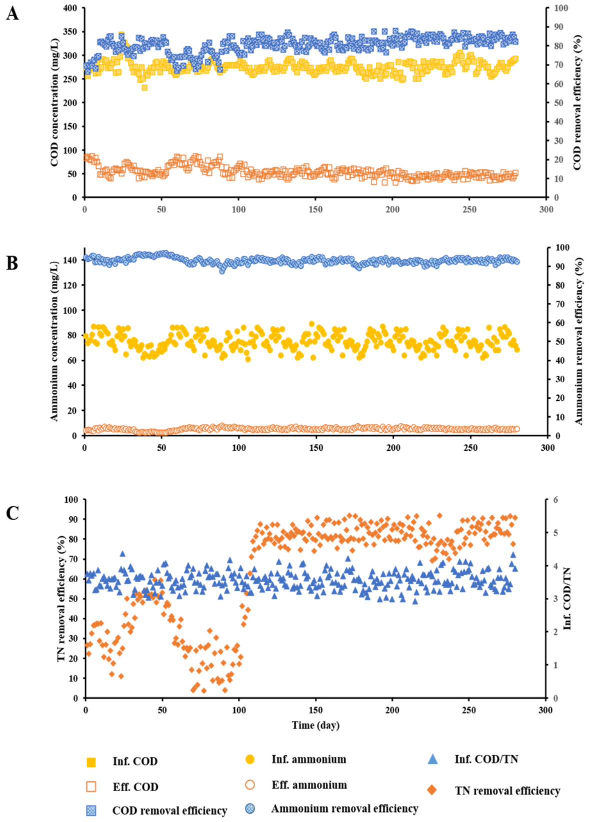

Figure 1. Performance of the process during the 280-day operation: characteristics of (A) influent (inf.) chemical oxygen demand (COD), effluent (eff.) COD, and COD removal efficiency; (B) inf. ammonium, eff. ammonium, and ammonium removal efficiency; (C) inf. COD/total nitrogen (TN) ratio and TN removal efficiency

3.2. Analysis of Phyla and Class of Bacteria in Anoxic and Oxic Zones

The microbial community structure and diversity composition in the anoxic zone, anoxic-carrier biofilms, and oxic zone biofilms of the AAO system were investigated using the Illumina PE150 sequencing platform. A total of 6000 effective sequences were obtained by quality filtering steps and producing approximately 31000 sequences in all samples within the finish. These results confirmed the reliability and repeatability of sequencing.

The structures of the microbial community in biologically activated sludge systems were closely associated with the performance of the wastewater treatment system [25]. Nine and six main phyla were identified in the anoxic and oxic zones, respectively. A detailed description of microbial community structure is depicted in Fig. 2A. Proteobacteria was the most abundant phylum (relative abundance, 91.2–95.2%; average: 93.2%) in the anoxic zone. This phylum is prominent in pharmaceutical, petroleum refinery, wood preservation, pet food and coking wastewater; the highest relative abundance reached approximately 90.38% [26]. The other major phyla in the anoxic zone included Actinobacteria (average 3.49%, each parallel sample 3.13–3.85%), Bacteroidetes (1.086%, 0.95–1.223%), Firmicutes (0.646%, 0.614–0.678%), Uroviricota (0.259%, 0.228–0.29%), Tenericutes (0.135%, 0.103–0.167%), and Chrysiogenetes (0.198%, 0.176–0.221%), which were also extensively distributed in other CWWTPs systems [27]. Proteobacteria, Bacteroidetes, and Firmicutes are vital bacteria involved in heterotrophic nitrification [28]. Actinobacteria, with the highest abundance in the oxic zone (average: 53.6%), is involved in carbon degradation processes, such as glucose, xylan, and cellulose assimilation [29]. At the class level, γ-Proteobacteria is the most abundant (62.3–67.1%) in the anoxic zone, reflecting their excellent adaptability to various environmental factors such as temperature, pH and nitrogen concentration. γ-Proteobacteria have been identified as highly versatile in all degradation strategies and are typically discovered among many biotreatment systems, such as those for coke-oven, phenol-containing, and domestic wastewater [30]. Other subdivisions of the Proteobacteria phylum in the anoxic zone were Epsilonproteobacteria (average: 22.82%), β-Proteobacteria (1.9%), and δ-Proteobacteria (3.19%). Except for the subdivisions of Proteobacteria, other major classes contained Actinomycetia (average: 2.34%), Bacteroidia (0.78%), Clostridia (0.36%), Tissierellia (0.28%), Caudoviricetes (0.23%). However, differences were evident in the oxic zone. Actinomycetia was the largest class, with sequence percentages ranging from 50.78–53.97%. The percentages of α-Proteobacteria were 19.7–21.38%, γ-Proteobacteria were 7.48–8.77%, and β-Proteobacteria were 6.33–6.85%. Most Actinomycetia can decompose many organic substances, including complex compounds such as aromatic compounds, paraffin, cellulose, and some highly toxic compounds such as cyanide [31]. Therefore, Actinomycetia play a vital role in the material cycle in nature and the biological treatment of sewage and organic solid waste [32,33].

3.3. Analysis of Bacterial Community at Genus Levels in Anoxic and Oxic Zones

As displayed in Fig. 2C, Vibrio (average: 24.09%), Pseudomonas (22.79%), Arcobacter (18.3%), Acinetobacter (12.51%), Shewanella (6.09%), Rhodococcus (1.42%), Desulfomicrobium (1.41%), Desulfovibrio (1.18%), Salmonella (1.1%), Microbacterium (0.88%), and Thauera (0.82%) were the 11 most abundant genera in the anoxic zone. Vibrio is present in coastal water samples with a high winter iron concentration, indicating a good resistance to low temperatures and might play a crucial role in iron metabolism [34]. Nitrate is the best inorganic nitrogen source for Pseudomonas, which exhibits a highly versatile metabolic capacity and broad potential for adaptation, enabling them to transform various kinds of nitrogen compounds and organic complex compounds [35].

Microbacterium was the most abundant in oxic zones, suggesting its critical role in hydrolyzing different types of organic matter. Notably, Pseudomonas (average 4.32%) and

Acinetobacter (2.14%) can hydrolyze some organic matter, particular proteins, and high-saline wastewater. They were present in the activated sludge of Chemical wastewater treatment plants based on the AAO process [36,37].

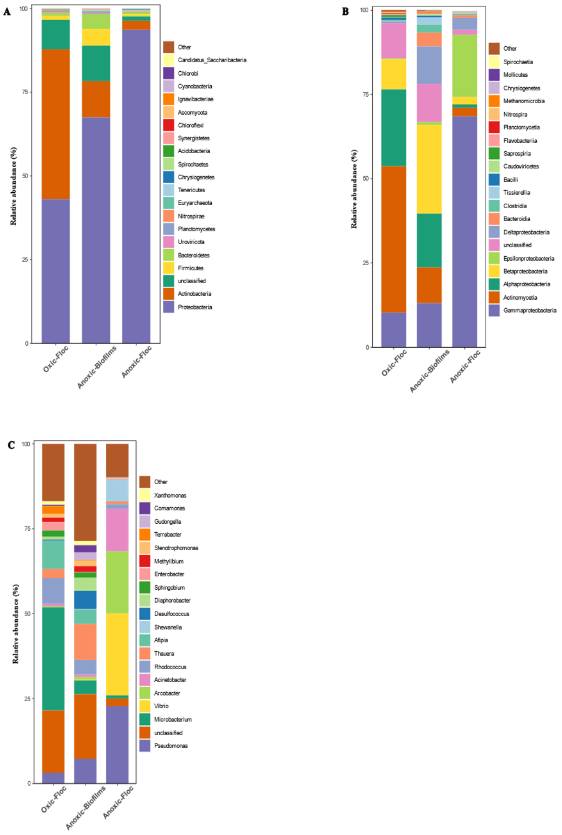

Figure 2. Bacterial community structures in the anoxic zone, oxic zone, and anoxic- carriers: (A) Phylum level; (B) Class level; (C) Genus's level

3.4. Analysis of Bacterial Community in Anoxic-Carrier Biofilms and flocculent Sludge

The top eight most relative abundance of anoxic-carrier biofilms and flocculent sludge were compared (Fig. 3). Proteobacteria was the dominant bacterial phyla in the anoxic-carrier biofilms and flocculent sludge, accounting for 67.54% and 93.27%, respectively; this is consistent with the findings of a previous study showing that it was the most abundant phylum in most CWWTPs [38]. The microbial community of anoxic-carrier biofilms contained a significantly higher abundance of Actinobacteria ($p<0.0001$), Bacteroidetes ($p<0.001$), and Firmicutes ($p<0.0001$). Relevant studies have found that Actinobacteria play a vital role in the carbon and nitrogen cycle, especially in sewage treatment and aeration, where the abundance increases significantly [39]. Bacteroidetes and Firmicutes play a crucial role in nitrogen removal; the genera in these phyla have the functions of nitrification, denitrification, and even phosphorus removal [40]. The increased abundance of related phyla strengthens the ability of microorganisms to degrade nitrogen and organic matter in sewage. During the biological nitrogen conversion in the CWWTPs, typical microorganisms include sulfate-reducing bacteria (e.g., Desulfococcus) and microalgae aggregating bacteria (e.g., Microbacterium); some denitrifying bacteria (e.g., Thauera) (Fig. 4) were observed in this study [41]. Although these bacteria were found to be lower in abundance, they were found to be significantly higher in the anoxic-carrier biofilms than in the flocculent sludge. Furthermore, the metagenomic sequencing revealed the key microorganisms and related carbohydrate-active enzymes. Anammox bacteria (e.g., Planctomycetes ($p<0.0001$)) were observed in anoxic-carrier biofilms (Fig. 3); these favorably metabolize without DO or an organic carbon supply [42].

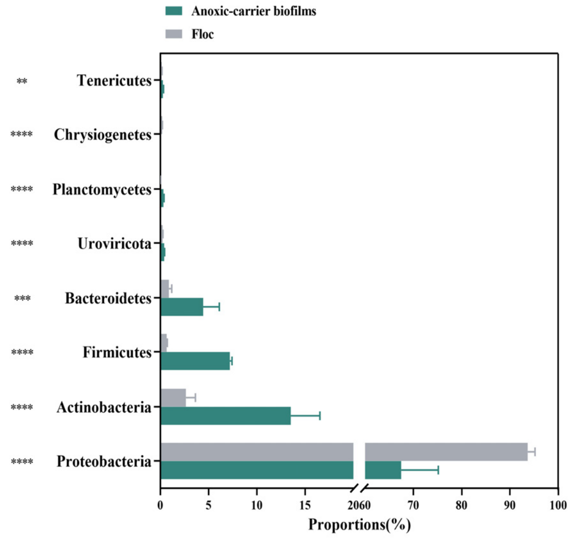

Figure 3. Difference analysis in phylum level (the top eight) between the flocculent sludge and anoxic-carrier biofilms using metagenomic sequencing based on the 16S rRNA gene

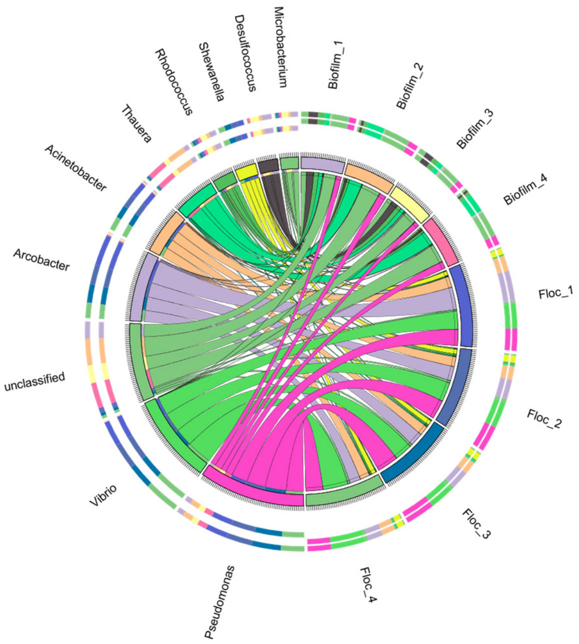

Figure 4. Collinear relationship between the samples (anoxic-carrier biofilms and flocculent sludge, n = 4) and the top ten bacterial groups at the phylum level

3.5. Potential Mechanism of Nitrogen Conversion Via Anoxic-carrier Biofilms

A LEfSe difference comparison analysis at the KEGG functional module level revealed 164 pathways that were significantly different in anoxic flocculent sludge and anoxic biofilms. Among these pathways, 97 were enriched in anoxic flocculent sludge and 67 in anoxic biofilms. Notably, genes related to nitrogen metabolism-related pathways (map00910) were significantly enriched in anoxic biofilms, suggesting enhanced nitrogen metabolism function (Fig. 5).

To further study the genes associated with nitrogen treatment in wastewater, we generated datasets related to nitrogen metabolism, including all genes in the nitrification, denitrification, and anammox pathways, and compared them with our metagenomic gene abundance results. “Deep” genetic mining revealed that the abundance of narG, nirS, narH, norB, hzsB, and hdh in anoxic-carrier biofilms was much higher than in flocculent sludge, the enrichment of denitrifying bacteria and anammox bacteria was proved successfully. qPCR results also confirmed that the key denitrification and anammox gene abundance in anoxic-carrier biofilms was significantly higher than that in flocculent sludge (narG: $p<0.0001$; nirS: $p<0.01$; narH: $p<0.01$; norB: $p<0.0001$; hzsB: $p<0.01$; hdh: $p<0.01$) (Fig. 6). This indicated the potential rates of denitrification and anammox significantly increased in anoxic-carrier biofilms than in flocculent sludge, which was probably due to the enrichment of typical denitrifying bacteria and anammox bacteria on the anoxic carrier biofilm. Moreover, the functions of several anammox bacteria to facilitate a nitrite loop might be essential for enhancing overall nitrogen removal performance in the bioreactor [43]. Thus, the results of this study corroborate previous findings that nitrate-to-nitrite conversion and anammox process could play a vital role in nitrogen metabolism [44].

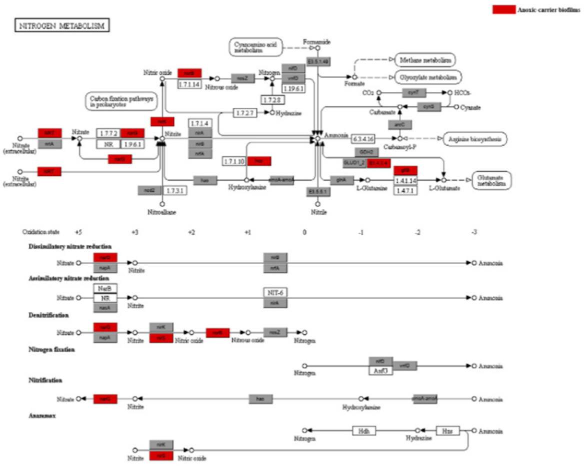

Figure 5. Enrichment of genes related to the nitrogen metabolism pathway (map00910) in anoxic-carrier biofilms

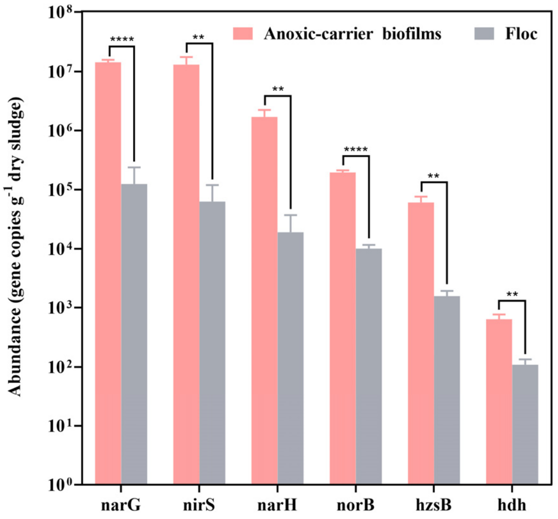

Figure 6. Abundance of the key denitrification and anammox genes targeted by qPCR in anoxic-carrier biofilms and flocculent sludge

3.6. Potential Mechanism of Nitrogen Conversion Via Anoxic-carrier Biofilms

For the treatment of chemical wastewater via moving bed biofilm reactor (MBBR), most previous studies have confirmed it is an efficient technology for removing organic matter and nitrogen from industrial and urban wastewater. However, most have focused on the oxic zone owing to its high efficiency for ammonium oxidation and organic matter removal [45,46]. Fewer studies have applied suspended carriers to the anoxic zone, compared with the oxic zone, to improve the nitrogen removal efficiency of CWWTPs [47,48], especially at low C/N ratio [49]. Consequently, the microbial community composition and functional transformation of anoxic-carrier biofilms remain poorly understood. Thus, we evaluated the removal of COD and nitrogen in modified SBRs during long-term operation and comprehensively analyzed the microbial community, metabolism-related pathways, and functional genes. The results suggest that abundant denitrifying and anammox bacteria were immobilized on the anoxic-carrier biofilms, which positively contributed to nitrogen removal.

Furthermore, the anaerobic zone is worth investigating due to the similar environmental conditions (e.g., pH, salinity, temperature, and DO). In the future, the effect of carrier biofilms in the anaerobic zone (e.g., microbial community composition, metabolic pathways, and the shift of microbial community composition) deserves further detailed research [50]. The modified system integrating AAO with SBRs exhibited considerable nitrogen removal. Although this case study shows the potential contribution of anoxic-carrier biofilms, further research on other possible microbial pathways, including nitrite ammonification, nitrous oxide denitrification, and their contribution to nitrogen removal [51] should be considered. The relationship among the physicochemical and biological processes, physicochemical characteristics, and environmental parameters in the biosystem and the effect on nitrogen removal must be further assessed using dynamic simulation [52].

4. Conclusion

The enhanced nitrogen removal in a CWWTP with anoxic-carrier biofilms, an AAO system, and SBRs was investigated for 280 days. Some characteristic bacteria were detected in the anoxic-carrier biofilms, and the significant inner associations were revealed. Our microbial community structure analysis indicates that some functional bacteria in the anoxic-carrier biofilms partially contribute to nitrogen removal. The abundance pattern of narG, nirS, narH, norB, hzsB, and hdh indicates a higher potential for denitrification and anammox pathways in anoxic-carrier biofilms than in the flocculent sludge. Overall, this study demonstrated a successful combination of SBRs with the AAO system. However, further efforts are required to clarify their complex interactions and potential metabolic pathways.

Acknowledgments

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