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Original Research

Mechanism of Nitrogen Removal Enhancement in Low Carbon/Nitrogen Municipal Sewage by AAO Process with Activated Sludge-Biofilm Composite System

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Abstract

To address the issue of insufficient carbon sources in urban sewage, which leads to poor nitrogen removal performance in sewage treatment systems, an anaerobic/anoxic/aerobic (AAO) pilot-scale reactor was established. The reactor aimed to treat low C/N (chemical oxygen demand; COD/total nitrogen; TN) municipal wastewater (C/N<5). To enhance nitrogen removal and investigate the mechanism in the AAO process, a Pall ring modified biological suspended filler was introduced to the aerobic zone after achieving partial nitrification and denitrification (PND). The results revealed that the activated sludge-biofilm composite system can be successfully formed within 40 days, with a stable loaded biomass on the membrane at 40.06 mg/g (measured by volatile suspended solids (VSS)/filler). The aerobic zone of the activated sludge-biofilm composite system demonstrated an increase in nitrite accumulation rate (NAR) and simultaneous nitrification and denitrification efficiency (SND), from 60.46% and 19.42% in the initial stage (stage 1) to 69.62% and 46.47% in the stable forming stage (stage 3), respectively. By promoting both PND and SND pathways for nitrogen removal, the effluent from the system exhibited decreased concentrations of ammonia nitrogen (NH_4^+-N) at 0.11 mg/L and total nitrogen (TN) at 4.55 mg/L, indicating the significant synergistic effect of the biofilm on nitrogen removal. 16S rRNA amplification and sequencing analysis revealed that Proteobacteria was the dominant microorganism in the 60-day biofilm, accounting for 76.12% of the relative abundance. The main ammonia oxidizing bacteria (AOB) were *Nitrosomanas* (1.77%) and *Nitrosococcus* (1.69%). Meanwhile, denitrification microbial species were found to have a substantial proportion (29.11%), along with a small amount of Anammox bacteria (*Anammoxoglobus*,

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0.35%) within the biofilm. These sequencing results were consistent with the macroscopic performance of the reactor. Overall, these findings establish a theoretical foundation for enhancing nitrogen removal in the AAO system.

Keywords: AAO process, activated sludge, low-C/N municipal wastewater, enhanced nitrogen removal, microbial community structure

Introduction

Nitrogen pollutants in municipal sewage primarily exist in the form of ammonia nitrogen (NH_4^+-N) and organic nitrogen [1]. The primary treatment, which mainly targets suspended solids removal, only has a significant effect on NH_4^+-N adsorbed onto the surface of some granular organic nitrogen and particulate matter. Conventional advanced treatment methods, such as coagulation and ozonation, have limited effectiveness in removing dissolved inorganic nitrogen and are associated with high economic costs, making them less suitable for application in municipal sewage treatment systems [2]. In the research field of wastewater treatment, biological nutrient removal (BNR) has long been recognized as the most effective and economical treatment, thus becoming a crucial area of focus [3]. Currently, wastewater treatment plants (WWTPs) in China commonly face influent carbon source deficiencies, indicated by influent C/N ratios below 5. This scarcity of carbon source poses challenges in achieving satisfactory nitrogen removal performance in most WWTPs in China [4, 5]. With the steady progress of wastewater treatment, water quality standards will inevitably necessitate increased nitrogen reduction. Therefore, the development of new BNR processes that can reduce the carbon source demand has emerged as research priority in recent years.

The advancement of molecular biology and the refinement of mathematical models have led to further exploration of BNR processes, resulting in the proposal of new theories and the development of numerous BNR processes by researchers. For instance, some microorganisms can perform denitrification under aerobic conditions [6], and nitrification and denitrification can occur simultaneously within the aerobic environment of the same reactor [7]. Furthermore, NH_4^+-N has been found to react with nitrate (NO_3^--N) or nitrite (NO_2^--N) to produce nitrogen in an anoxic environment [8]. These findings surpass the traditional theoretical limits of BNR. At present, research in the field of process application mainly focuses on partial nitrification and denitrification (PND) [9], simultaneous nitrification and denitrification (SND) [10], and anaerobic ammonium oxidation (Anammox) [11].

Currently, the majority of studies on the new BNR process are conducted using laboratory-scale sequencing batch reactor (SBR), with artificial simulated municipal sewage as the experimental water [12-14]. These studies have yielded significant research achievements in realizing and implementing PND, SND, and Anammox processes. However, about 90% of WWTPs in China currently use continuous flow processes, with the anaerobic/anoxic/aerobic (AAO) process accounting for over 50% of these plants [15]. Therefore, studying the new BNR process within the AAO system holds great practical significance. In this study, actual low-C/N (C/N<5) municipal wastewater was selected as the research object, and biological fillers were introduced into the aerobic zone of a pilot-scale reactor operating under the AAO process, which had already achieved PND. The aim was to explore the nitrogen removal and enhancement mechanisms of the activated sludge-biofilm composite system within the AAO system. The results from this study can lay a theoretical basis for the advanced nitrogen removal in AAO systems.

Material and Methods

AAO Reactor and Biological Suspension Filler

The pilot-scale bioreactor (Fig. 1) was employed to implement the AAO process. Constructed using 10 mm thick welded steel plates, the bioreactor had an approximate working volume of 7.8 m^3 (L × W × H = 3.7 m × 1.5 m × 2.0 m). A series of baffle slots were arranged between the anoxic and aerobic zones, allowing for adjustable partition proportions by changing the baffle's position. To ensure the desired flow pattern of sewage inside the bioreactor, openings were incorporated above and below the baffle. Additionally, the aeration pipe with micropores was situated at the bottom of the aerobic zone, to meet the requirements of stirring and aeration. The flow rates of the influent, internal and external recycle were controlled using electromagnetic flowmeters, while the aeration rate was adjusted using a high-precision gas rotor flowmeter.

The biological suspension filler selected in this study was the Pall ring modified biological suspension filler, measuring φ 25 × 10 mm and possessing an effective specific surface area exceeding 500 m^2/m^3. The Pall ring modified biological suspension filler offers several advantages, including facilitating the solidification, proliferation, and metabolism of microorganisms on its surface. Furthermore, it exhibits strong adhesion and favorable surface hydrophilicity. The filler's specific gravity was measured to be 0.96-0.98 before the formation of the biofilm, increasing to 1.0 g/cm^3 afterward. Overall, its comprehensive

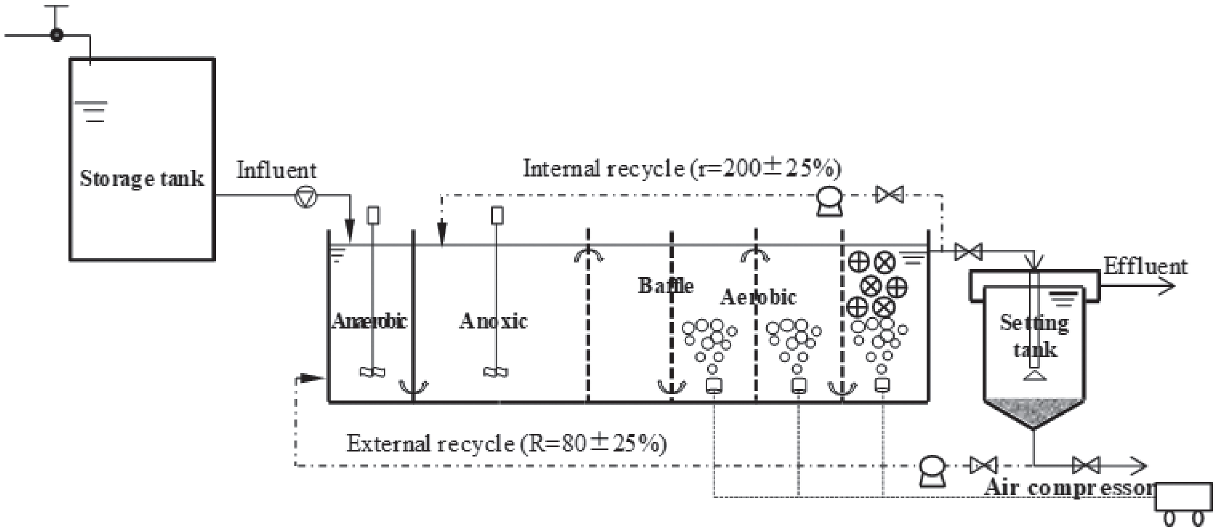

Fig. 1. Schematic plan of the pilot-scale bioreactor with AAO process.

performance aligns with the requirements of this study. The mixed liquor suspended solids (MLSS) of system was maintained at 3500±200 mg/L, and the biological suspension filler was added to the aerobic zone at a filling rate of 15%. According to the system's operating parameters, effective volume of the aerobic zone, and size of the biological suspension filler, calculations indicated the addition of approximately 55,000 pieces of the filler, equivalent to a mass of approximately 25 kg.

Wastewater and Sludge

The influent of the pilot-scale bioreactor was the effluent from the aerated sand sedimentation tank of a 200,000 m^3/d WWTP in Xi 'an, China, which followed a traditional AAO process. The quality of the influent is presented in Table 1. The activated sludge used in the bioreactor was obtained from the aerobic tank of the WWTP and exhibited high activity. Following a 30-day period of acclimation and adaptation, the system demonstrated a stabilization trend in the removal performance of pollutants.

Experimental Methods

The concentrations of NH_4^+-N, NO_3^--N, NO_2^--N, and TN were determined using standard Chinese State Environmental Protection Administration methods [16]. Monitoring of dissolved oxygen (DO), pH, and temperature was conducted using a Hashi WTW multifunctional automatic tester. The extraction and sequencing methods for microbial DNA followed a previous study [3].

For the analysis of biofilm samples, a scanning electron microscope (SEM) model JSM-6510LV was utilized. The pretreatment method involved cutting a 5 mm × 5 mm determination sample piece from the central region of the carrier filler. The sample, along with the attached biofilm, was placed in a glass petri dish and fixed with a 4% paraformaldehyde solution for 6 h. Subsequently, the fixed biofilm was eluted with ethanol solutions of varying concentration gradients, including 30%, 50%, 70%, 80%, 90%, and 100%, with each gradient lasting for 15 min. The eluted biofilm was then replaced with isoamyl acetate:ethanol in a 1:1 for 5 min, followed by 100% isoamyl acetate solution for an additional 5 min. The supernatant was removed, and the replaced biofilm was transferred to a clean petri dish for air drying. Finally, the air-dried biofilm was observed using SEM.

Bioreactor Operation and Calculation Formula

The pilot-scale bioreactor was operated for a period of 80 d, which was divided into the following three stages: stage 1 (0-15 d), the initial stage of membrane hanging; stage 2 (16-40 d), biofilm rapid growth; and stage 3 (41-80 d), the stable formation of the activated sludge-biofilm composite system. During the entire operational period, the influent flow rate was controlled

Table 1. Influent characteristics of the pilot-scale bioreactor.

Item	pH	COD (mg/L)	TN (mg/L)	TP (mg/L)	NH_4^+-N (mg/L)	NO_3^--N (mg/L)
Range	7.34-7.62	201.21-388.73	40.13-54.80	4.91-7.66	24.57-39.02	0.07-1.30
Mean	7.53	249.52	49.62	6.62	33.24	0.72

Table 2. Operational conditions for this study.

Stage	Time (d)	DO in aerobic zones (mg/L)	$V_{anaerobic}:V_{anoxic}:V_{aerobic}$	SRT (d)	Internal recycle (%)	External recycle (%)
1	0-15	0.5-0.8	1:3:3	12	200±25	80±25
2	16-40					
3	41-80					

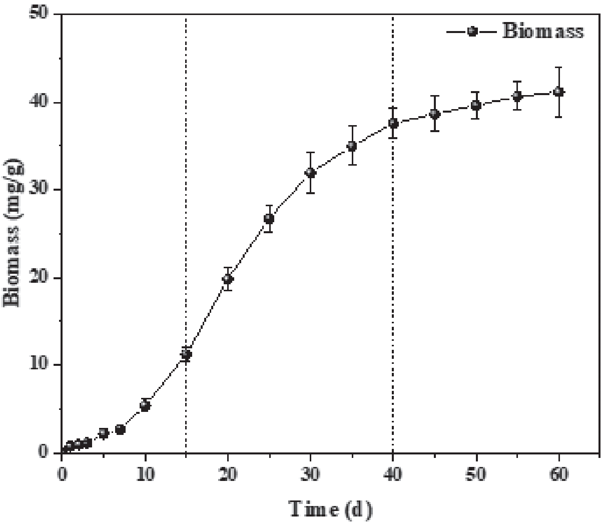

Fig. 2. Variation of biomass on the carrier with time.

at 0.5±0.05 m^3/h, the hydraulic retention time (HRT) was set at 15.6 h, and the temperature was maintained at 25±1°C using a thermostat. Additional operating parameters of the reactor are shown in Table 2.

The efficiency of partial nitrification is commonly assessed through the nitrite accumulation rate (NAR), which represents the percentage of NO_2^--N concentration in the effluent from the aerobic zone in relation to the sum of NO_3^--N and NO_2^--N concentrations [9]. The formula is as follows:

$$NAR = \frac{NO_2^-{}_{(Aer-eff)}}{NO_2^-{}_{(Aer-eff)} + NO_3^-{}_{(Aer-eff)}} \times 100\% \quad (1)$$

Where, $NO_2^-{}_{(Aer-eff)}$ and $NO_3^-{}_{(Aer-eff)}$ refer to the concentrations of NO_2^--N and NO_3^--N in the effluent of the aerobic zones (mg/L), respectively.

SND efficiency was used to characterize the performance of simultaneous denitrification in the aerobic zone [9]. To simplify the determination process, it is generally assumed that the ammonia process of nitrogen is fully completed in the anoxic area, implying no organic nitrogen is present in the influent of the aerobic area, and NH_4^+-N does not exhibit an increasing trend. In this case, the calculation formula for SND efficiency is as follows:

$$SND = [1 - \frac{NO_{X(Aer-eff)}^- - NO_{X(Aer-inf)}^-}{NH_{4(Aer-inf)}^+ - NH_{4(Aer-eff)}^+}] \times 100\% \quad (2)$$

Where, $NO_x^-{}_{(Aer-inf)}$ and $NH_4^+{}_{(Aer-inf)}$ refer to the concentrations of NO_x^--N and NH_4^+-N in the influent of the aerobic zones (mg/L), respectively, and $NO_x^-{}_{(Aer-eff)}$ and $NH_4^+{}_{(Aer-eff)}$ represent the concentrations of NO_x^--N and NH_4^+-N in the effluent of the aerobic zones (mg/L), respectively.

Results and Discussion

Study on Attachment of Biological Suspension Filler

Due to the small size of individual microorganisms and their weak metabolic impact, significant macroscopic effects can only occur when they reach a certain threshold and form clusters [17]. Therefore, it is crucial to investigate the variation of microorganism biomass attached to the surface of suspended fillers over time. To minimize sampling contingencies and experimental errors, biological fillers were collected three times from different positions in the aerobic zones, with 10 random samples selected from each batch. After mixing the 30 biological fillers, the attached biomass was measured,

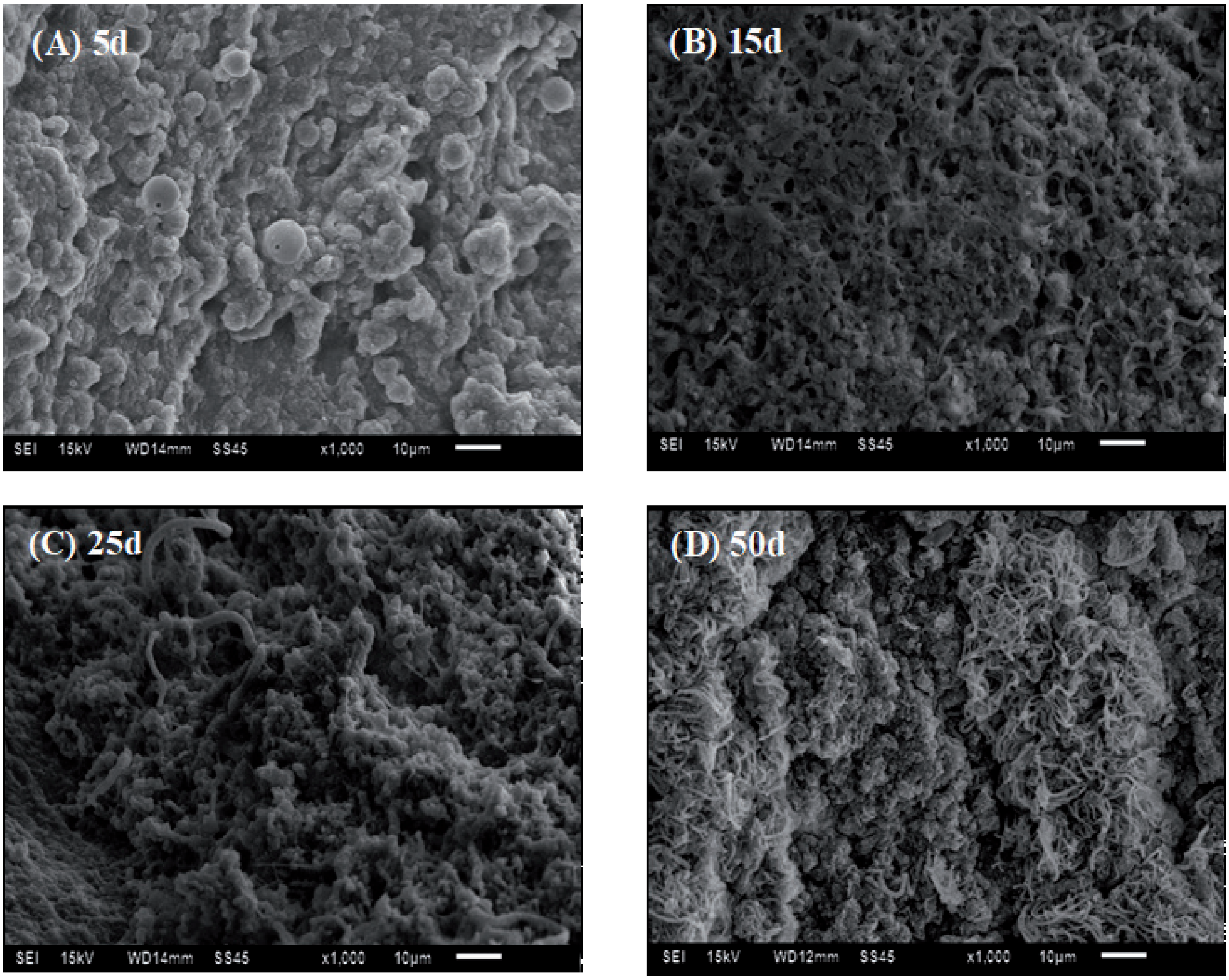

Fig. 3. Variation of biomass on the carrier with time.

as shown in Fig. 2. The biomass attached to the surface of the suspended fillers exhibited an S-shaped curve over time. Three days prior to the addition the fillers, there was almost no microorganism attachment, resulting in close to zero biomass. Subsequently, within 3 to 7 days, microorganisms gradually accumulated on the filler's surface, leading to a slight increase in biomass. On the 7th day, the biomass attached to the filler reached 2.73 mg/g. From the 7th day onward, the biomass began to increase rapidly, with a growth rate of 1.11 mg/g·d during the 7th to 15th day. Biomass measurements were taken every 5 days after the 15th day, dividing the period from 15 to 40 days into 5 sampling cycles. The daily growth rates of biomass for each sampling cycle were 1.67, 1.31, 1.14, 0.55, and 0.47 mg/g·d, respectively. In terms of biomass growth rate, the period from 15 to 40 days represented the rapid growth period of the biofilm, which was also the key period for SND generation in the system. After 40 days, the biomass growth rate gradually slowed down. Although there was a slight increase, the growth effect was not significant. The biomass on the filler only increased by 3.22 mg/g from 40 to 60 days, and eventually stabilized at 40.06 mg/g.

To directly investigate the growth of the biofilm on the filler's surface from a microscopic perspective, SEM was used to analyse the changes in biofilm structure, as presented in Fig. 3. It was observable that the growth of microorganisms attached to the filler's surface intensified significantly as time progressed, aligning with the macroscopic biomass measurements. Concurrently, during the early stage of biofilm growth, microorganisms were sparsely distributed in isolated island clusters on the filler's surface (Fig. 3a). In the subsequent stage, as microorganisms continued to adhere and grow, the biofilm rapidly enriched, resulting in an irregular network structure with interconnecting overlaps (Fig. 3d). This led to a substantial increase in the specific surface area, enhancing the probability of contact with the liquid phase of the activated sludge and reducing the limiting effect of mass transfer resistance on microbial growth.

Study on the Nitrogen Removal Performance

Based on a previous study conducted on the bioreactor, PND was achieved within 36 days under

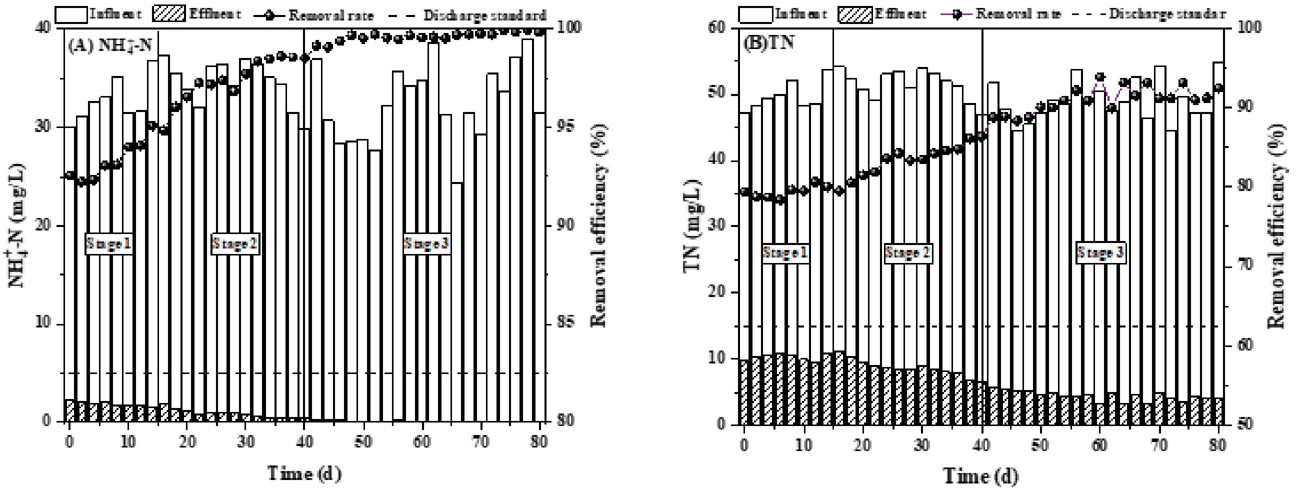

Fig. 4. Nitrogen removal of the system.

the conditions of V_{anoxic}:$V_{aerobic}$ = 1:1, DO = 0.5-0.8 mg/L in the aerobic zone, and SRT=12 d. Once the system stabilized, the maximum NAR reached 62.64%. Under these conditions, the system exhibited NH_4^+-N and TN removal efficiencies of 95.1% and 83.9%, respectively, with effluent concentrations of 1.46 and 9.00 mg/L [18]. Building upon these conditions and the nitrogen removal performance, the activated sludge-biofilm composite system was established by adding biological suspension fillers into the aerobic zone of the AAO system, aiming to achieve deep nitrogen removal. The NH_4^+-N and TN removal efficiencies of the system are presented in Fig. 4. As observed in Fig. 4a), during stage 1, the average NH_4^+-N removal rate of the system was 93.47%, resulting in an average effluent concentration of 1.72 mg/L. At this point, the microbial population responsible for NH_4^+-N degradation consisted mainly of bacterial flocs in the activated sludge. Due to the limited SRT of the system, improving the activity of nitrite-oxidizing bacteria, which requires an adequate SRT, becomes challenging, thus restricting further improvement in NH_4^+-N removal efficiency. In stage 2, the average NH_4^+-N removal rate increased to 96.54%, and the average effluent concentration decreased to 1.13 mg/L. With the enrichment of microorganisms on the surface of the biological fillers, the biofilm actively participated in the biochemical reaction process, extending the SRT of the activated sludge. This enabled the enrichment of nitrifying bacteria and further enhanced the nitrification performance of the system. With the formation of the activated sludge-biofilm composite system in stage 3, the nitrification performance became stable. At this stage, the system exhibited an average NH_4^+-N removal rate of 99.15% and an average effluent concentration of 0.11 mg/L.

Fig. 4b) clearly demonstrates the significant improvement in TN removal performance of the system due to enhanced NH_4^+-N removal efficiency and the gradual establishment of anoxic microenvironments within the biofilm. During stages 1 to 3, the average TN removal rates were 78.51%, 82.77%, and 91.84%, respectively, with corresponding average effluent concentrations of 11.13 mg/L, 8.92 mg/L, and 4.55 mg/L. Following the formation of the activated sludge-biofilm composite system in stage 3, the effluent TN concentration stabilized below 5.0 mg/L, indicating a remarkable achievement in deep nitrogen removal. The presence of an ample anoxic microenvironment within the composite system, using the large surface specific area of the biofilm filler to extend the HRT of the anoxic zone without affecting the aerobic zone's HRT, resulted in more efficient denitrification. Moreover, compared to simply increasing the volume of the anoxic zone, the activated sludge-biofilm composite system offered the advantage of directly utilizing nitrification products, streamlining and expediting the denitrification process. In addition, denitrification within the biofilm mainly depended on cell derivatives and internal carbon sources released through aging and degradation of the biofilm's own microorganisms. Consequently, this approach reduced reliance on external carbon sources present in the wastewater, leading to improved overall denitrification efficiency and reduced sludge yield within the system.

Study on Mechanism of Enhanced Nitrogen Removal

Fig. 5 displays the concentration variations of NH_4^+-N and NO_x^--N at different stages in the system. As observed in Fig. 5a, NH_4^+-N removal primarily occurred in the aerobic zone, excluding reactor dilution. N balance calculations indicated that NH_4^+-N removal in the aerobic zone accounted for 16.53%, 19.22%, and 24.66% of the total removal amount during stages 1 to 3, respectively. Compared to the previous study [18], where NH_4^+-N removal in the aerobic zone was 14.39 %, the activated sludge-biofilm composite system exhibited

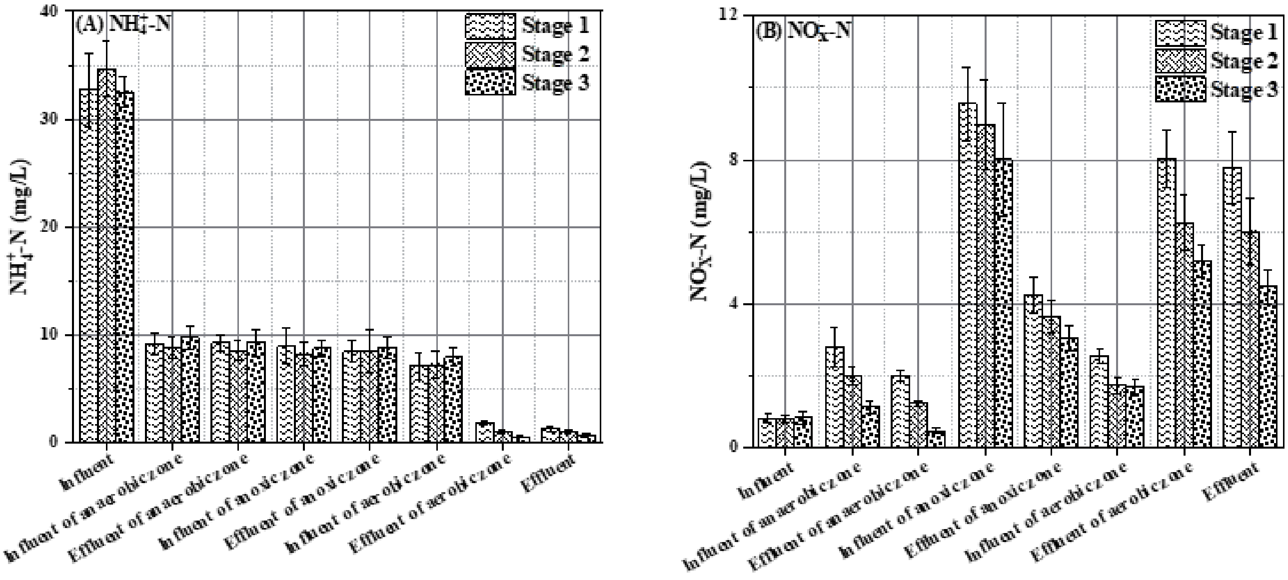

Fig. 5. Variations of NO_x^--N and NH_4^+-N concentrations in different sections of the reactor in each stage.

a 71.37% increase in NH_4^+-N removal, indicating improved nitrification performance. Fig. 5b) indicates that NO_x^--N removal mainly occurred through traditional denitrification in the anoxic zone. The concentration variation of NO_x^--N in the aerobic zone demonstrated a decreasing trend from stage 1 to 3, with values of 77.34%, 65.21%, and 52.06%, respectively. This can be attributed to the increased presence of anoxic microenvironments within the aerobic zone, facilitated by the stable formation of the activated sludge-biofilm composite system. These conditions favored SND, where a portion of NH_4^+-N was converted into NO_x^--N through nitrification, followed by denitrification within the anoxic microenvironment of the biofilm, resulting in the conversion of NO_x^--N into N_2 and subsequent discharge. This phenomenon contributed to the observed decrease NO_x^--N production rate.

To explore the nitrogen removal pathway of the activated sludge-biofilm composite system, the study focused on the aerobic zone throughout the experiment, analyzing the influent and effluent concentrations of NH_4^+-N and NO_x^--N (Fig. 6). Fig. 6a) demonstrates that the addition of biological suspension filler in the activated sludge-biofilm composite system promoted PND. During stage 1, the average NAR of the system was 60.46%, slightly lower than the value of 62.64% in the previous study [18]. Subsequently, with the rapid biomass growth, the average NAR of the system in stage 2 increased to 63.14%, mainly due to the improved adaptability of the activated sludge microbial flora to the combined system. The NAR gradually recovered and surpassed the previous study value. In stage 3, the proportion of biofilm microorganisms involved in nitrogen transformation increased. Within the

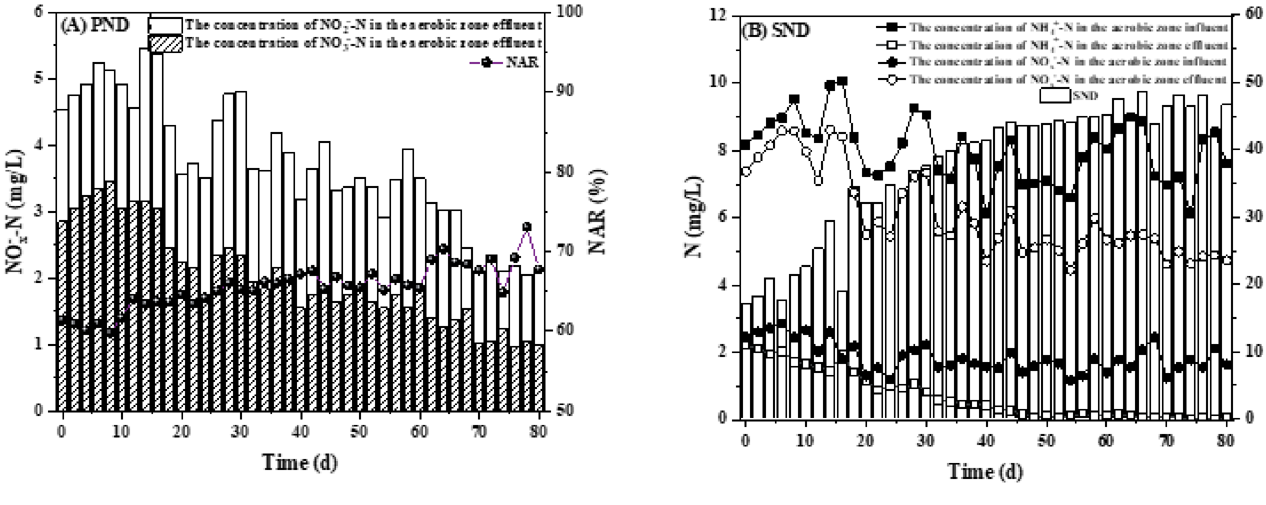

Fig. 6. Variations of NAR and SND in the system.

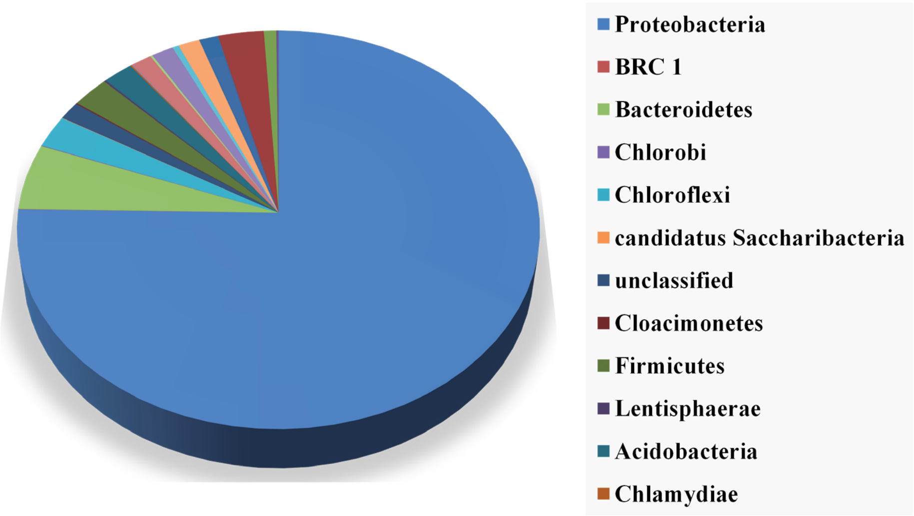

Fig. 7. Relative abundance of microorganisms from the 60th day biofilm at phylum level.

biofilm, the mass transfer resistance resulted in a more pronounced uneven distribution of DO. This led to a selective distribution of microbial communities within the biofilm, resulting in the enrichment of ammonia-oxidizing bacteria (AOB) with lower DO requirements and greater inhibition of nitrite-oxidizing bacteria (NOB) compared to the main bacterial flocs in the liquid phase. These factors contributed to the enhanced PND effect of the system. Towards the late stage of the experiment, the NAR of the system exhibited significant fluctuations but maintained an overall stability of 69.62%.

From Fig. 6b), it is evident that the average SND efficiency in the aerobic zone of the system during stage 1 was about 19.42%. This was due to the low DO concentration (0.5-0.8 mg/L) in the aerobic zone, creating an internal anoxic microenvironment within the activated sludge micelles, which contributed to part of the SND. Subsequently, SND efficiency showed a rapid increase trend during stage 2, with an average value of 36.77%. The main reason for this increase was the transfer of NO_x^--N produced by nitrifying bacteria on the biofilm's surface, to the interior of the biofilm under the action of concentration gradients. Additionally, some nitrifying products in the liquid phase diffused into the biofilm's interior. As the DO concentration decreased inside the biofilm, the microorganisms primarily consisted of obligate or facultative anaerobic bacteria, including many denitrifying bacteria. NO_x^--N served as a substrate for anaerobic respiration. In the absence of sufficient carbon source content in the biofilm environment, denitrifying bacteria consumed their own internal carbon source to complete denitrification [19], resulting in a significant increase in SND at stage 2. During stage 3, with the formation of the activated sludge-biofilm composite system, the SND efficiency tended to stabilize and ultimately reached 46.47%. Theoretical analysis [20] suggested that, at this time, the biofilm created a broad anaerobic environment with matrix conditions such as NO_2^--N and NH_4^+-N. This environment may have facilitated the growth of Anammox bacteria, contributing to the improved nitrogen removal performance of the system. In conclusion, the formation of the activated sludge-biofilm composite system in the AAO process can further improve NAR after successful PND, resulting in improved SND efficiency and deep nitrogen removal.

Study on Microbial Population Structure

To further investigate the mechanisms underlying enhanced biological nitrogen removal in the activated sludge-biofilm composite system within the AAO process, the microbial population of biofilm samples was analyzed using a high-throughput sequencing method. After achieving system stability and biofilm formation (60 d), samples were collected from the biofilm on the biological suspension filler and subjected to 16S rRNA amplification and sequencing.

Analysis of the microbial community structure at the phylum level revealed the relative abundance of different microorganisms in the 60-day biofilm (Fig. 7). Proteobacteria accounted for the majority (76.12%) of the microbial community, followed by Bacteroidetes (4.27%), Planctomycetes (2.88%), Chloroflexi (2.41%), and Firmicutes (2.23%). The dominance of Proteobacteria aligns with findings from previous studies on urban domestic sewage treatment systems [21, 22]. The prevalence of Proteobacteria can be attributed to its significant population base and structural characteristics. Proteobacteria, being gram-negative bacteria, possess an outer membrane rich in lipopolysaccharide, facilitating their adhesion and growth on the biological suspension filler [23]. In addition, Proteobacteria, being facultative or obligate anaerobic heterotrophs, thrive in the biofilm microenvironment with low DO concentrations caused by solid mass transfer resistance. Previous research

Table 3. Relative abundance of microorganisms from the 60th day biofilm at Genus level.

Genus	Relative abundance/%	Genus	Relative abundance/%	Genus	Relative abundance/%
Thauera	11.15	*Thermothrix*	3.11	*Pirellula*	0.55
Comamonas	7.36	*Denitratisoma*	2.04	*Aquisphaera*	0.48
Zoogloea	6.52	*Nitrosomanas*	1.77	*Anammoxoglobus*	0.35
Dechloromonas	4.31	*Nitrosococcus*	1.69	Others	56.55
Gemmatimonas	2.99	*Gemmata*	1.13		

has indicated the significant role of Proteobacteria, especially β-Proteobacteria, in denitrification and phosphorus removal processes in wastewater treatment, with certain strains directly involved in denitrification [24].

To further explore the advanced nitrogen removal microorganisms present in the activated sludge-biofilm composite system, a genus level analysis was conducted on the microbial population, and the results are presented in Table 3. Notably, *Thauera* (11.15%), *Comamonas* (7.36%), and *Zoogloea* (6.52%) exhibited relative abundances above 5% in the biofilms. Genera such as *Dechloromonas* (4.31%), *Gemmatimonas* (2.99%), *Thermothrix* (3.11%), and *Denitratisoma* (2.04%) displayed relative abundances ranging from 2% to 5% in the biofilms. Additionally, there were 11 bacterial types at the genus level with relative abundances of 1% to 2% (not listed in the table), mainly belonging to Proteobacteria, Firmicutes, and Bacteroidetes. Regarding specific functional bacteria, *Nitrosomanas* (1.77%) and *Nitrosococcus* (1.69%), two common AOBs, were detected in the system. However, the relative abundance of NOBs in the biofilms was negligible (less than 0.2%). These results showed that the activated sludge-biofilm composite system selectively retained AOBs while washing out NOBs through a low DO screening mechanism, thereby extending the bioreactor HRT and enhancing the system's short-cut nitrification.

Thauera [25], *Dechloromonas*, *Comamonas*, *Zoogloea* [26], and *Denitratisoma* were identified as the main denitrifiers in the system, accounting for a combined relative abundance of 29.11%. This composition ensured the removal of the short-cut nitrification product, NO_2^--N, in the biofilm, thus improving the SND efficiency of the system. Furthermore, studies have suggested that *Gemmata* (1.13%), *Pirellula* (0.55%), and *Aquisphaera* (0.48%) can contribute to biological mineralization and nitrogen removal processes [27], indicating their potential for enhanced nitrogen removal in the system. In addition, the presence of *Anammoxoglobus* (0.35%), an Anammox bacterium [28], in the biofilm suggests the possible occurrence of anaerobic ammonia oxidation, further enhancing the system's nitrogen removal performance.

Conclusions

Under the specific operating conditions of a DO range of 0.5-0.8 mg/L, a HRT of 4.5 h, and a MLSS concentration of 3500±200 mg·L-1 in the aerobic zone of the AAO pilot-scale reactor, the activated sludge-biofilm composite system can be steadily formed within 40 days, according to a 15% filling rate, and reached a stable biomass level of 40.06 mg/g. The implementation of the activated sludge-biofilm composite system led to notable enhancements in the NAR and SND in the aerobic zone, reaching values of 69.62% and 46.47%, respectively. By promoting both PND and SND pathways, the system achieved reductions in the concentrations of NH_4^+-N and TN in the effluent, reaching 0.11 and 4.55 mg/L, respectively, showcasing its significant synergic effect on nitrogen removal. Upon the stable formation of the activated sludge-biofilm composite system, Proteobacteria emerged as the main dominant bacterium on the biofilm, accounting for 76.12% of the microbial community. Further analysis at the genus level revealed the enrichment of AOB within the biofilm system, primarily represented by *Nitrosomanas* (1.77%) and *Nitrosococcus* (1.69%). Additionally, a significant proportion (29.11%) consisted of typical denitrification microorganisms, while a small amount of Anammox bacteria (*Anammoxoglobus*, 0.35%) was also detected within the biofilm. The enrichment of these functional bacteria constituted the primary internal factor contributing to the enhanced nitrogen removal capacity of the system.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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