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Nitrogen Removal Mechanism and Microbial Community Changes of the MBR Bioaugmented with Two Novel Fungi *Pichia kudriavzevii* N7 and *Candida tropicalis* N9

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Abstract: Ammonia nitrogen wastewater causes dissolved oxygen concentrations to decrease and the content of harmful substances to increase. To characterize the application properties of two novel strains of highly efficient ammonium transforming fungi—*Pichia kudriavzevii* N7 and *Candida tropicalis* N9—this study used both as compound microbial agents to treat nitrogenous wastewater. Here, we investigated the bioaugmentation effect of compound fungi N7 and N9 in the MBR bioreactor and the effect of N7 and N9 on the fungal and bacterial microbial communities in the system. The results revealed that in the first week after inoculation of N7 and N9, the average removal rate of ammonium in the experimental and control groups were 89.43% and 82.86%, respectively, and the NO_3^--N accumulation concentrations were 12.56 mg·L^{-1} and 17.73 mg·L^{-1}, respectively. The average transformation rate of total nitrogen in the experimental and control groups were 46.32% and 30.6%, respectively. ITS sequencing results indicated that N9 could be a dominant fungus in the complex MBR system. The results of 16S rRNA sequencing showed that the dominant bacterial communities in the system were changed by the inoculation of compound fungi. Therefore, the compound fungi can be applied to strengthen the treatment of nitrogenous wastewater due to its compatibility.

Keywords: bioaugmentation; nitrogenous wastewater; fungus; microbial community

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1. Introduction

Increasing economic development and urbanization have prompted humanity to enjoy unprecedented material satisfaction. At the same time, however, sustained population growth and rapid industrial development in many countries have put increasing pressure on the environment. Multiple pollutants produced by industrial activities are often harmful and even carcinogenic and mutagenic [1]. Although ammonium is a nutrient in nature, excessive amounts of it in water—which is discharged directly with domestic sewage into sewers, rivers and oceans in some areas—poses a serious threat to the entire ecosystem [2,3]. This kind of pollutant is toxic to some aquatic plants and fish and also rapidly consumes dissolved oxygen in water that is then used by algae, leading to eutrophication of water bodies [4,5]. Excessive nitrogen in the environment also causes changes in the nutrient cycle in nature [6,7]. Therefore, it is necessary to remove ammonium from the environment.

Presently, denitrification systems, nitrification, biosorption, biofiltration, ion exchange and absorption are applied for ammonium elimination from wastewater. The biological treatment systems are always considered to be economically and efficiently in the existing denitrification method for ammonium removal [8]. The current mainstream processes of biological treatments are activated sludge and biofilm. However, the limited types of indigenous microorganisms capable of transforming ammonium in a treatment system or

polluted environment can lead to unsatisfactory effluent quality, especially for livestock and poultry wastewater because of its large amount of excess nutrients. Bioaugmentation—a technique to introduce microorganisms with specific functions into bioreactors or contaminated areas to help remove specific pollutants [9]—makes up for this shortcoming. Bioaugmentation technology has been widely researched and applied in various environmental remediation. Han et al. summarized the main strategies of accelerating the formation of aerobic granular sludge, wherein bioaugmentation technology showed great potentials in accelerating granulation [10]. Yang et al. used bacterial consortium targeting NPEO and NP for bioaugmentation to control endocrine disrupting toxicity; here, enriched bacterial consortia acclimated using NPEO and NP were added into activated sludge to investigate the toxicity controlling effect. The results showed the bacterial consortia could remove more than 98% of NPEO and NP (both 10 mg L^{-1}) after 48 h [11]. Boonnorat et al. compared the biodegradation efficiency of organic compounds in two-stage activated sludge systems with (bioaugmented) and without aged sludge bioaugmentation (non-bioaugmented). They reported that both systems had good capacity of degrading the organic compounds but that the efficiency of bioaugmented system was higher than that of the non-bioaugmented one [12]. It is suggested bioaugmentation is a feasible and reasonable strategy to enhance the treatment of ammonium-contaminated wastewater.

Most of the microorganisms used for bioaugmentation are screened out from the environment. Ammonium transformation bacteria, with the advantages of wide distribution and easy isolation from natural environment, have previously been focuses of bioaugmentation, such as Rhodococcus [13], *Acinetobacter* [14], *Bacillus methylotrophicus* [15], *Pseudomonas* and *Aeromonas* [16]. Moreover, their diversity and function in wastewater treatment have been investigated intensively.

However, the ecology, abundance and role of fungi have often been neglected; only a limited number of studies have been carried out to research the application of fungi for bioaugmentation. Djelal et al. discovered that both removal of COD and the wastewater biodegradability were enhanced through bioaugmentation of fungal consortia containing *Galactomyces geotrichum*, *Mucor hiemalis* and *Aspergillus niger* [17]. Compared with bacteria, fungi have the merits of strong adaptability to the unfavorable environment (such as low pH, fluctuating hydraulic load, etc.) as well as a faster reaction rate [18,19]. They can also secrete large amounts of enzymes function to degrade a wide variety of environmental pollutants [20]. Therefore, it is demonstrated that bioaugmentation of fungi can effectively promote treatment efficiency and adaptability of an external environment, such as activated sludge. However, due to the limited technologies of screening optimal fungi and keeping them active in the complex wastewater environment, fungi still cannot be applied widely in practical wastewater treatment. Meanwhile, the mechanism relevant to the target pollutant removal is also in its infancy [21]. Lately, bioaugmentation of constructing fungi-bacteria mixed consortia was proposed, which could solve and improve nitrogen removal efficiency in MBR by embedding selected strains or mixed cultures into reactors to enhance the catabolism of specific compounds [22–24]. Therefore, it is necessary to study the biological enhancement of fungi-bacteria mixed consortia in nitrogenous wastewater treatment.

In this study, two novel high-efficiency ammonium-transforming fungi, N7 and N9, were inoculated into the nitrogen-containing wastewater in the MBR system for bioaugmentation through immobilizing on the polypropylene materials, with the aim of enhancing the nitrogen removal efficiency of the whole system. This was the first time embedding mixed yeasts of N7 and N9 into the MBR system to improve the efficiency of nitrogen removal while their feasibility and bioaugmentation performance in this complex system were examined. Moreover, the changes of microbial communities due to extraneous organisms during the experiment were also monitored. In fact, there is still a long way to apply a specific function and efficient strain to wastewater treatment by biofortification. Therefore, it is of great practical significance to study the processing properties of these new strains and their adaptability in indigenous microbial communities.

2. Materials and Methods

2.1. Inoculum Sludge and Synthetic Wastewater

The activated sludge used in this experiment was collected from a municipal wastewater treatment plant (WWTP) in Weihai, China. The samples of activated sludge were cultivated in a sequencing batch reactor (SBR) to increase biomass content.

The experimental wastewater used in the MBR systems was synthetic wastewater containing the following: 0.4124 g glucose, 0.25 g $(NH_4)_2SO_4$, 0.03 g KH_2PO_4, 0.045 g NaCl, 0.05 g $NaHCO_3$, 0.11 g Na_2CO_3, 0.055 g $MgSO_4{\cdot}7H_2O$, $0.2{\cdot}10^{-3}$ g $ZnSO_4{\cdot}7H_2O$, $0.2{\cdot}10^{-3}$ g $FeSO_4{\cdot}7H_2O$, $0.2{\cdot}10^{-3}$ g $CuSO_4{\cdot}5H_2O$, pH 7.5–8.5 and 1 L tap water. The main reagents used in the study were produced by Sinopharm Chemical Reagent Co. Ltd., Shanghai, China.

2.2. MBR Systems Description

Two lab-scale MBR devices, each with a volume of 11 L, were built in the laboratory (Figure 1). Firstly, the experimental water was supplied in an inlet tank and pumped into the MBR reactors by submersible pumps. Then, the simulated wastewater was treated by microorganisms in the MBR systems before finally being extracted by peristaltic pumps (Baoding Chuangrui Pump Industry Co., Ltd., Baoding, China) through the membrane modules. MBR systems adopted the intermittent operation mode of 8 min operation and 2 min suspension to maintain the membrane modules [25]. An aerator was arranged at the bottom of the bioreactor to provide dissolved oxygen (DO). Polyvinylidene fluoride (PVDF) hollow fiber membrane was used as membrane modules in the reactor while a pressure gauge for monitoring the transmembrane pressure (TMP) was being installed. When the value of it reached 30 KPa, the membrane was washed. Samples from the influent and effluent were taken daily to measure the concentration of ammonium, nitrate, nitrite and total nitrogen. The operational parameters adopted in the MBR system are presented in Table 1. Additionally, polypropylene carrier was determined to be seeded into the MBR system together with the compound strains for forming biofilm to promote the removal rate of ammonium nitrogen.

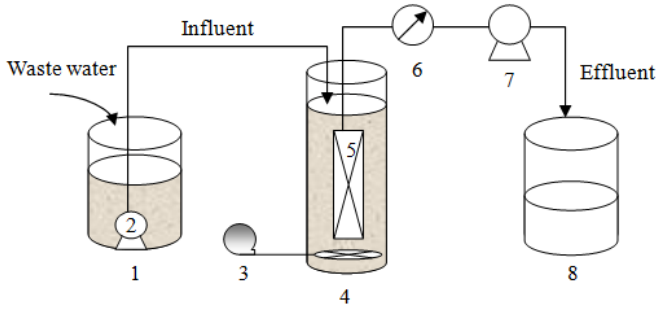


Figure 1. Schematic diagram of the MBR reactor. (1) Influent tank; (2) submersible pump; (3) aerator; (4) MBR; (5) membrane module; (6) pressure gauge (7) peristaltic pump; (8) effluent tank.

Table 1. Operating parameters in MBR systems.

Parameter	Units	Value
DO	$mg{\cdot}L^{-1}$	3–4
HRT	h	12
COD	$mg{\cdot}L^{-1}$	400
NH_4^+-N	$mg{\cdot}L^{-1}$	50
MLSS	$mg{\cdot}L^{-1}$	4500
Temperature	°C	30

2.3. Screening and Identification of Ammonium Transforming Strains

Two sediment samples were collected in July 2022 from the sewage outlets of the international beach in Weihai, China (E 122.0749°/N 37.5407° and E 122.0750°/N 37.5407). In the enrichment medium, 5% ammonium sulfate solution was added every day to eliminate the strain that could not utilize NH_4^+. After enrichment, the optimal enrichment culture solution was used for strain isolation, wherein a single colony was purified and obtained on the ammonia nitrogen-isolation solid medium. Then, the Nessler's reagent colorimetric method was used to calculate the ammonium nitrogen transformation rate of the strain [26].

According to comparison of the ammonium nitrogen transformation efficiency, 3 bacterial strains with the highest capacity were selected. After DNA extraction, PCR amplification (ITS1: TCCGTAGGTGAACCTGCGG as forward and ITS4: TCCTCCGCTTATTGATATGC as reverse primer) and sequencing were completed by Shanghai Sangon Biotech company (Shanghai, China). The sequences were contrasted to available sequences in the EzBioCloud database and GenBank database to find model strains with the highest proportion of similarity and completeness.

2.4. Construction of Compound Bioflocculant and Preparation of Inoculating Strains

Three strains with the best ammonium nitrogen removal performance were selected, mixed and co-cultured at the volume ratios of 1:1 and 1:1:1 in the same medium. After 2-day cultivation in a shaker at 28 °C, 120 rpm, the optical density (OD600) of the culture reached 1.0, and the number of the strains was calculated using the colony counting method. The best combination of strains was obtained by contrasting their final ammonium nitrogen removal rates. The optimal ratio of this combination was investigated in the same way as the former.

To remove the existing nitrogen, the cell suspension of the compound strains from their 2-day-cultivation mixed-culture medium was centrifuged at 3000 rpm for 10 min, followed by washing with normal saline and centrifuged for 3 times. Finally, the above cell suspension was seeded into the MBR reactors. The polypropylene carrier was seeded into the MBR system together with the compound strains for forming biofilm to promote the removal rate of ammonium nitrogen.

2.5. Analytical Methods for Water Quality

The daily samples of influent and effluent were collected and analyzed directly. The concentrations of nitrogen species (total nitrogen, NH_4^+-N, NO_3^--N and NO_2^--N) and chemical oxygen demand (COD) were monitored in accordance with the standard methods [27], while DO and pH were determined using a pH meter (STARTER 3100, Ohaus Instruments Co., Ltd., Shanghai, China) and a DO meter (Multi 3620 JDS, German WTW Instrument Co., Ltd., Munich, Germany), respectively. MLSS and SV_{30} were measured according to the weight method and 30-min settlement method [26].

2.6. Microbial Community Analysis

The community structure of bacteria and fungi was analyzed by 16S rRNA and ITS gene amplicon sequencing. Five sludge samples were collected from the reactors on day 2 and day 16 to determine the effect of the addition of fungi N7 and N9 on the evolution of community structures. The samples were marked as C2 and C16 (from control MBR), E2 and ES16 (suspended biomass from experimental MBR), and EA16 (attached biofilm from experimental MBR). All sequencing was conducted by Shanghai Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China.

Different levels of the microbial community structure were analyzed on a platform of Majorbio I-Sanger Cloud Platform (www.i-sanger.com) on 1 March 2023. The question sequences were eliminated using the default parameters and the 16S rRNA and ITS sequences were clustered into operational taxonomic units (OTUs) by setting a distance limit of 3%. The alpha diversity (including Shannon, Simpson, Ace, Chao and Coverage,

etc.) was used to reflect the richness and diversity of microbial community and the sequencing date depth [28]. The similarity and diversity of microbial composition structure was evaluated based on beta diversity, which was visualized through principal coordinate analysis (PCoA) [29]. The minimum sequence number from samples was chosen as the normalization standard to compare the alpha and beta diversity of different microbe population [30].

3. Results and Discussion

3.1. Strain Screening and Identification

In total, 62 strains were isolated from the coastal zone sediment and screened for the ammonium nitrogen removal ability. Among them, three strains with the best ammonium nitrogen removal performance were selected to construct the compound strains in further study, which were named N6T, N7 and N9, respectively. The DNA fragments of these strains were sequenced and submitted to NCBI GenBank Database under the accession numbers MH368485–MH368487. As contrasted in the EzBioCloud database, the strains N7 and N9 shared 100% sequence identity with *Pichia kudriavzevii* (KY014444.1) and *Candida tropicalis* (MG599234.1) separately, while the strain N6T shared 99% sequence identity with *Candida* sp. (JF896569.1).

3.2. Construction of Compound Strains

The ammonium nitrogen removal rates of strains are largely influenced by the culture environment, which means that mixed culture may increase the microbial diversity in a culture medium, with the possibility of the promotion of the adaptive capacity as well as the activity of these strains [31]. Therefore, it is essential to investigate the symbiotic combination of various microbes to gain the optimal combination of mixed culture with the best ammonium nitrogen removal performance in the MBR system [32,33].

Table 2 presents the ammonium nitrogen removal rates of single strain and compound strains. It shows that when the strain N6T and the strain N7 were mixed in the medium, their compound strains presented lower ammonium nitrogen removal rates than any single strain, probably because of the antagonism or mutual competition among these organisms, which affected their growth and metabolism. Meanwhile, the combination of N7 and N9 showed the best performance, which could be ascribed to the mutual promotion among them as previous study [34]. It has been reported that in the co-culture environment, some strains would present the capacity of synthesizing the nutrients for other organisms while secreting some enzymes to contribute themselves to meet their nutrient demands in the complex culture medium, reaching a higher level of biological activity than single strain [32].

Table 2. Ammonium nitrogen removal rates of single strains and mixed-culture strains.

Strains	Ammonium Nitrogen Removal Rate (%)	Compound Strains (N9:N7)	Ammonium Nitrogen Removal Rate (%)
N6T [a]	75.2 ± 0.8 **	1:1	87.9 ± 1.6 **
N9 [b]	82.1 ± 1.3 **	1:2	86.3 ± 0.9 **
N7 [c]	80.3 ± 0.1 **	1:3	83.9 ± 1.5
N6T + N9	80.8 ± 0.8	3:1	90.2 ± 1.6 **
N6T + N7	79.7 ± 1.7	2:1	94.0 ± 0.6 **
N9 + N7	88.1 ± 0.4 **		
N6T + N9 + N7	82.7 ± 1.6 **		

Notes: [a]: The strain N6T identified as *Candida* sp. [b]: The strain N9 identified as *Candida tropicalis*. [c]: The strain N7 identified as *Pichia kudriavzevii*. **: The data is significant at the 0.01 level.

Based on the former experimental results, the combination of N7 and N9 was selected and their optimal ratio of mixed culture was also explored (Table 2). It was found that the ammonium nitrogen removal efficiency was lower with the increasing proportion of

the strain N7, and the best efficiency was obtained with the ratio of 2:1 (N9:N7), which would be chosen as the compound strains to be inoculated into a MBR system as a kind of functional organisms of bioaugmentation.

3.3. Ammonium Nitrogen

The influent ammonium concentration was 49–52 mg·L^{-1}. From Figure 2A, it can be seen that, within the first week, the ammonium nitrogen removal rate of the experimental system through bioaugmentation was enhanced compared with the control system. Therefore, the process of bioaugmentation was divided into two stages and the first week was determined as stage 1. After the application of the bioaugmentation, the favorable ammonium removal rate was monitored in the experimental system during stage 1, with an average of 90.11%, resulting in the ammonium nitrogen concentration ranging from 1.14 to 7.14 mg·L^{-1}. The enhancement of ammonium nitrogen removal could be connected to the addition of the inoculated compound strains (N7 and N9). Therefore, it was reasonable to conclude that these compound strains, along with the strategy of biofilm carriers, effectively improved the ammonium nitrogen transformation of the system. Similarly, previous studies illustrated that the application of bioaugmentation in various reactors obtained a better rate of ammonium nitrogen removal than the control systems [35–37].

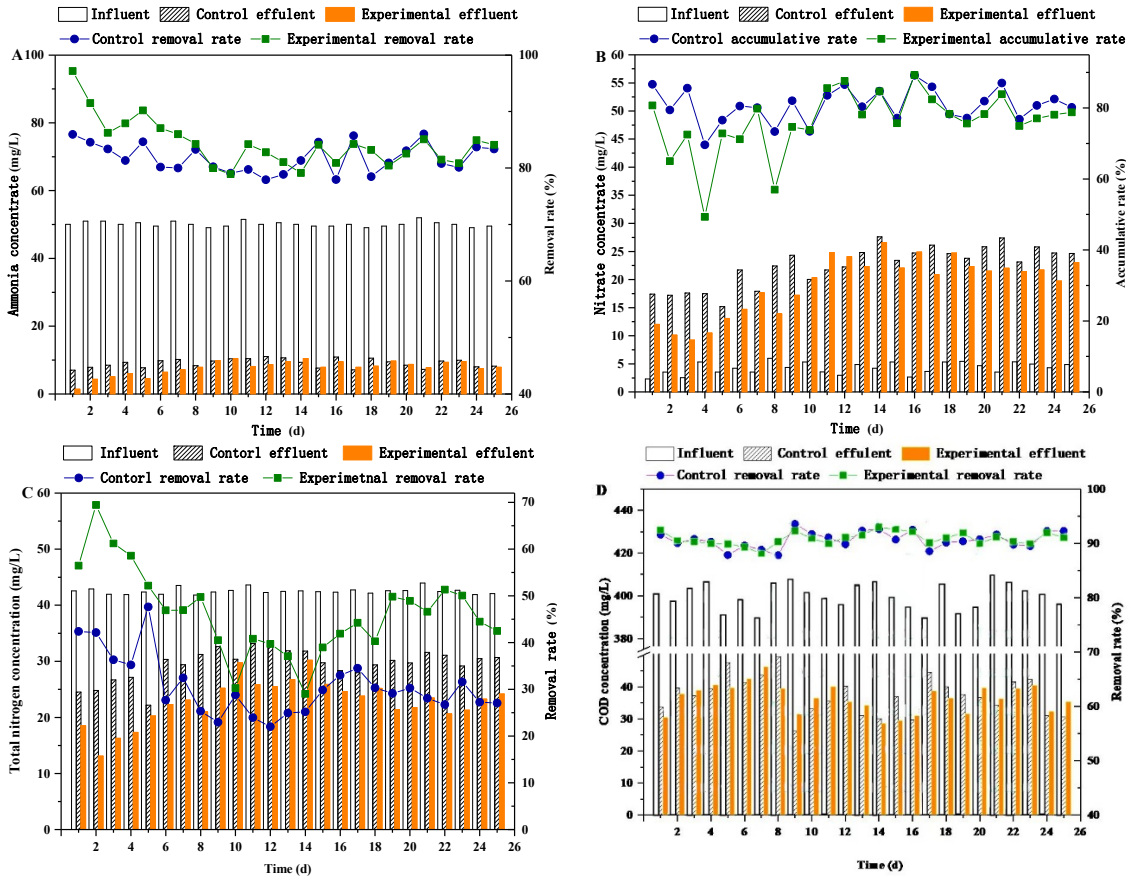


Figure 2. Characteristics of (**A**) ammonium, (**B**) nitrate, (**C**) total nitrogen and (**D**) COD in the MBR systems.

In stage 2, although the performance of the experimental group was still better than control group, there was little difference between their ammonium nitrogen rates, with an average of 82.35% and 81.39%, respectively. Li et al. built complex fora with ammonium nitrogen transforming bacteria and flocculant producing bacteria; the ammonium nitrogen transformation rate was 85.43–89.84% [38]. Similar results indicated that bioaugmenta-

tion with ammonium nitrogen transforming bacteria was an effective method to remove nitrogen from wastewater [39–41].

Through the figures of this experiments from the 10th to 14th day of the experimental group, it was found that the ability of ammonium nitrogen transformation reduced gradually. It could be inferred that the weakening of the inoculated strains also happened gradually, which might be relevant to the competition among the inoculated strains [42] and indigenous organisms, as well as the loss of dominant strains, leading to the transformation performance being leveled off. Therefore, it is suggested that dosage of the compound strains every 8 days may be helpful to keep the activity of MBR system.

3.4. Nitrate Nitrogen

Although low concentration of NO_3^--N was measured in the effluent, ranging from 2 to 6 mg·L^{-1}, Figure 2B illustrates upward trends in both systems because of the accumulation of nitrate, demonstrating the existence of denitrification. For instance, the average accumulation concentration of nitrate in the whole experiment and in stage 1 was about 19.71 mg·L^{-1} and 12.56 mg·L^{-1}, respectively. The advantageous capacity of the inoculated strains to mitigate the accumulation of NO_3^--N decreased gradually, which totally disappeared in the 10th day. Therefore, we considered that the bioaugmentation strains might be answerable for the higher NO_3^--N removal rate to some extent in stage 1. To date, massive novel microorganisms with great ability to remove NO_3^--N in wastewater have been researched, such as *Janthinobacterium* [43], *Pseudomonas nicosulfuronedens* D1-1 [44] and *Pseudomonas tolaasii* Y-11 [45], but the species of yeasts were relatively less. Thus, the results of this study could provide a certain reference value. The mechanism of yeast denitrification is also worth further investigation.

3.5. Total Nitrogen

According to Figure 2C, the total nitrogen in both systems has a certain fluctuation, but the treatment effect of the experimental group was generally better than that of the control group, probably because the inoculated fungi had the capacity of a nitrification-promoted NH_4^+ removal rate so that the accumulation of NO_3^--N simulated the efficiency of denitrification process [46]. On the other hand, the biofilm formed by compound fungi and indigenous microorganisms also played a major role in removing total nitrogen. The depth of biofilm limited the dissolved oxygen (DO) and resulted in the formation of DO gradients. Therefore, DO concentration in the inner layer was lower than that of the outside system, providing excellent anaerobic environment for denitrification [47]. However, the distribution of DO in MBR system was not uneven, leading to unstable anaerobic zone that correspondingly caused dissimilar levels of denitrification and led to violent fluctuation, as illustrated in Figure 2C [48]. Moreover, the effluent concentration and transformation rate of total nitrogen in the experimental group were approximately 22.82 mg·L^{-1} and 46.32%, respectively, while the average total nitrogen removal rate was 30.6% in the control group. Combined with Figure 2A, the total nitrogen efficiency in the experimental system might result from N7 and N9's rapid transformation of ammonium nitrogen. The effluent of both the systems had not reached the first-class requirement of wastewater (15 mg·L^{-1}). Therefore, it is considered that the addition of a special anaerobic zone might tackle this problem because this MBR system was an aerobic reactor.

3.6. COD

The degradation of COD by the experimental system was satisfactory as shown Figure 2D. Whether the compound strains were seeded into the MBR systems, the effluent could meet the first-class standard (50 mg·L^{-1}), indicating that the indigenous microorganisms and the membrane had the capacity of removing organic pollutants. Moreover, the performance of COD elimination in the experimental system was slightly better than in the control system, which could be induced that carbon source was vital to degrade nitrogen

so that the higher ammonium removal performance correspondingly caused better COD removal rate.

3.7. Microbial Community Analysis

3.7.1. Diversity of Fungal and Bacterial Communities in the MBR Systems

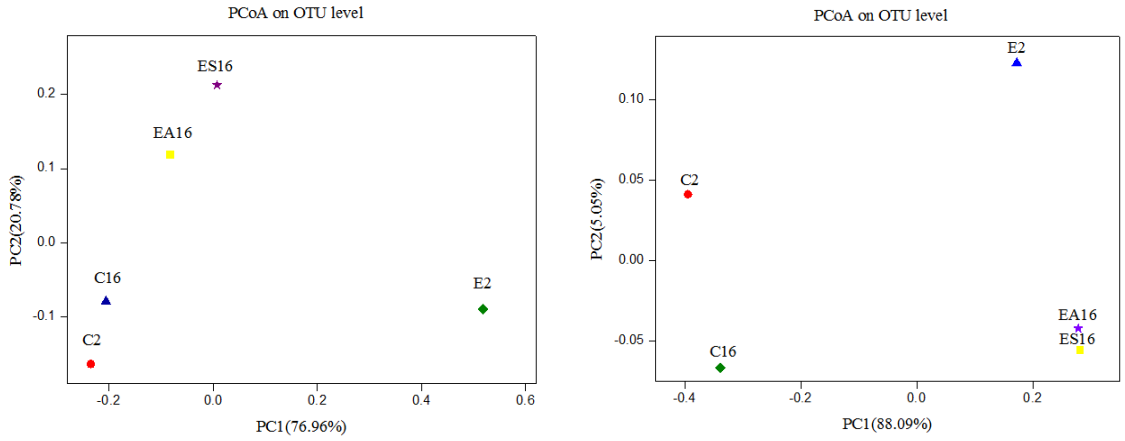
To obtain the diversity of fungal and bacterial communities, 16S rRNA and ITS gene amplicon sequencing of five samples (C2, E2, C16, ES16 and EA16) collected from two MBR systems were conducted. Additionally, 1861 operational taxonomic units (OTUs) were obtained at 97% sequence similarity, containing 326 OUTs from 16S rRNA sequencing and 1535 OTUs from ITS OTUs. Coverage indices of all samples were more than 0.99, which demonstrates favorable coverage of the sample libraries (Table 3).

Table 3. Alpha diversity of the fungal and bacterial communities.

Fungi	Chao	Ace	Shannon	Simpson	Coverage
C2	63.91	67.56	1.07	0.63	0.99
E2	68.67	73.12	1.09	0.52	0.99
C16	97.50	125.30	1.37	0.51	0.99
ES16	71.86	72.72	2.09	0.27	0.99
EA16	62.50	62.95	1.66	0.41	0.99
Bacteria	**Chao**	**Ace**	**Shannon**	**Simpson**	**Coverage**
C2	313.55	312.12	3.38	0.10	0.99
E2	344.63	342.90	3.76	0.06	0.99
C16	331.89	324.40	3.83	0.05	0.99
ES16	320.75	316.18	3.40	0.10	0.99
EA16	319.32	319.71	3.56	0.07	0.99

With the addition of N7 and N9, the Chao and Ace indices rose in the fungal community at the beginning before decreasing after 16 days. This indicates that inoculated fungi N7 and N9 competed with native microbes in a relatively poorer position so that the richness of wastewater was damaged, which was similar to the decrease of microbial community diversity and richness in a constructed wetland through bioaugmentation [49]. This might correspondingly prove why the former assume that ammonium nitrogen transformation tends to be balanced in the system, as shown in Figure 3a. The indices of bacterial community also demonstrated the same changes, which was similar to the findings of Ou et al. [50] and Liang et al. [51] despite the inoculated microorganisms being fungi. Previous studies assumed that certain fungi would interfere with some bacterial growth [52] and, therefore, they induced a kind of interaction between bacteria and inoculated strains in the experimental system. However, the Shannon Diversity Index illustrated a distinct trend in the fungal and bacterial communities: upward and downward, respectively. This means that the abundance of fungi species was distributed evenly while the bacterial abundance presented the opposite. Moreover, the diversity and richness of biofilm was lower than the suspended biomass, probably because of the aerobic and anoxic region on the biofilm carrier [53] filtered certain microorganisms to subsist.

As shown in Figure 3a the PCoA of fungal communities illustrated that there was a significant difference in the fungal communities (samples E2 and E16) after inoculation with N7 and N9 in contrast to the control group's communities (samples C2 and C16). In the experimental group, the communities of suspended fungi were similar to the attached fungal communities (samples ES16 and EA16). Among the five samples, the bacterial community was significantly different except for samples ES16 and EA16, as shown in Figure 3b. The composition of the bacterial communities in suspended and attached organisms was similar, which accounted for how they were located close together in the coordinates. These distinguished fungal and bacterial communities indicated that the strategy of inoculating strains had a great impact on the whole community structure in this bioreactor.

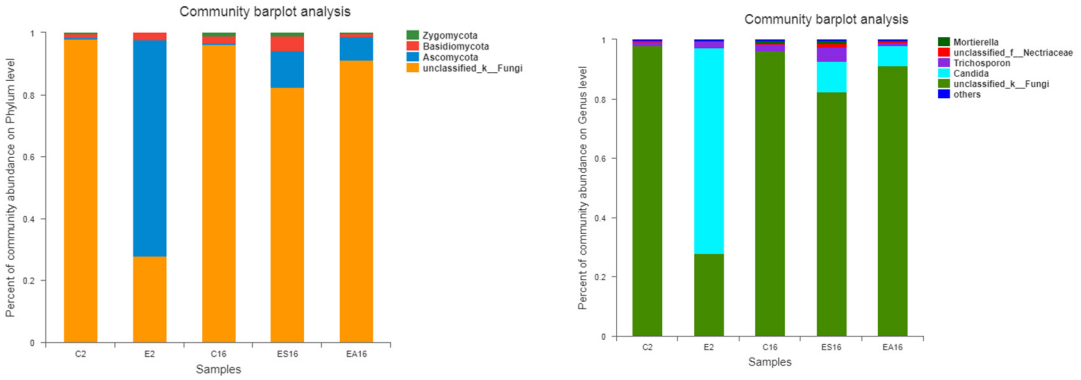


(**a**) (**b**)

Figure 3. PCoA analysis of communities: (**a**) fungal community; (**b**) bacterial community.

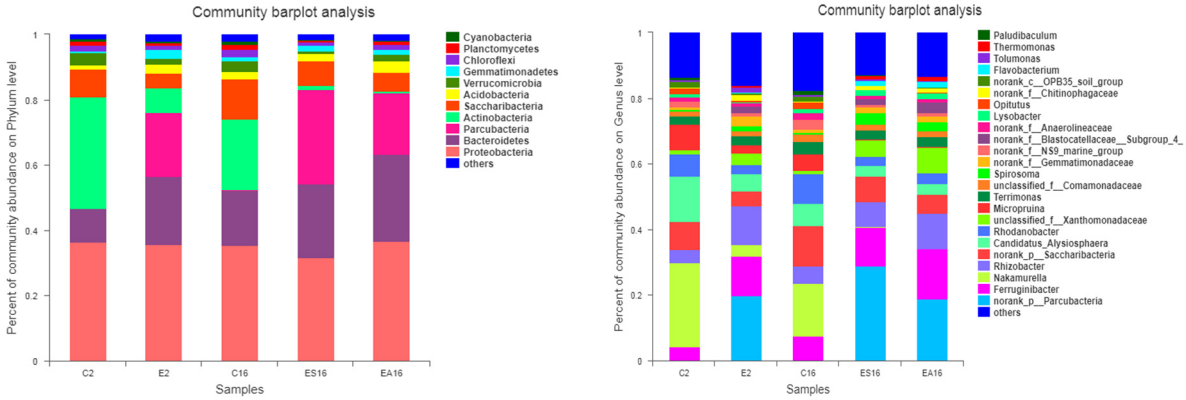
3.7.2. Microbial Community Composition in the MBR Systems

The community structure of the five samples (including fungi community and bacteria community) collected from the experimental and control MBR bioreactors were studied using Illumina high throughput sequencing. The results are shown in Figures 4 and 5.



(**a**) (**b**)

Figure 4. The fungal communities at the class level (**a**) and the genus level (**b**).



(**a**) (**b**)

Figure 5. Taxonomic classification of bacterial communities at the phylum (**a**) and genus (**b**) level.

ITS gene sequences identified 4our major fungi phyla and 11 classes in the five samples. The phyla with a richness greater than 1% are exhibited in Figure 4a. At the phylum level, the unclassified sequences took the first place in most of these samples, especially in C2 and C6 (more than 90%), and *Basidiomycota, Ascomycota* and *Zygomycota* accounted for the remaining proportion. Specifically, *Basidiomycota,* reported as a key bacteria group in conventional wastewater treatment, occupied the first place in the classified fungi class in the control system; it was only in the second place in experimental group, where *Ascomycota* dominated the classified fungi class [54]. This might be directly caused by the introduction of the compound fungi, indicating that the alien microorganism N9 can grow and reproduce well in the system. Moreover, possibly due to the large proportion of indigenous microorganisms and their competitive advantages, *Basidiomycota* decreased over time. This was consistent with the trend of ammonium transformation over time in the experimental MBR system displayed in Figure 2A. Therefore, in combination with Figures 2A and 4a, it can be concluded that increasing the inoculation amount of N7 and N9, or inoculating at regular intervals, can be considered in practical applications.

It can be seen from Figure 4b that most fungal genera sequences were not classified at the genus level. ITS gene sequencing detected 26 fungal genera in all samples. Of the sequences that have been classified, only *Candida*, *Tichosporon*, *unclassified-Nectriaceae* and *Mortierella* exhibit a relative abundance over 1%. Furthermore, there were also some differences in the abundances among these five samples. For example, *Tichosporon*, which possesses the outstanding capacity of degrading complex containments through using extracellular enzymes [55,56] and is frequently isolated from a wide range of substrates, such as the active sludge, biofilms and soil [57–60], was the dominant classified species in the system without embedding bioaugmentation. Moreover, an increase of this genera was observed after a two-week culture. However, *Candida*, as another one of the most common identified fungal pathogens in activated sludge, occupied the most proportion of the whole system in E2, ES16 and EA16. Although many reports have indicated that the relative abundance of *Candida* in activated sludges may mainly derive from municipal wastewater [53], it is reasonable to hypothesize that its presence in the experimental system was largely because of bioaugmentation and its ability to reduce ammonium and nitrates that consisted of higher ammonium transformation rates at early stages of this system (Figure 2). *Trichosporon* occupied a relatively high proportion in the control MBR system. It seems that *Pichia kudriavzevii* has not been found through ITS gene sequences, probably because N7 contributes to the transformation of N9. Still, it fails to compete with indigenous microbes in the system and thereby cannot become a dominant genus. It can be inferred from Figure 4 that the fortified microorganisms can also adhered to the carriers to form biofilm together with the indigenous microorganisms. It is further verified by the relative abundance at the genus level of fungi in the experimental system that N7 and N9 can adapt to the complex MBR system and exert its transformation ability, which has the potential to the treatment of nitrogenous wastewater.

It can be analyzed from Figure 5 that there is a notable difference in the sample's bacterial community. A total of 25 different bacterial phyla was found in the five samples, with relative abundance greater than 1%, including *proteobacteria*, *Bacteroidetes*, *Parcubacteria*, *Actinobacteria*, *Saccharibacteria*, *Acidobacteria*, *Verrucomicrobia*, *Gemmatimonadetes*, *Chloroflexi*, *Planctomycetes* and *Cyanobacteria*. There were obvious differences in the bacterial communities in the MBR system between the control and experimental groups at the phylum level (Figure 5a). The bacterial phyla *Proteobacteria* and *Bacteroidetes* were dominant in all systems, which almost accounts for more than 50% in all samples. *Proteobacteria* and *Bacteroidetes* are common dominant categories in wastewater treatment. It has been reported that *Proteobacteria* plays an important role in the removal of organic substances [61]. *Bacteroidetes* was conducive to promoting the hydrolysis of macromolecules into small molecules [62]. *Actinabacteria* (about 20–30%) was also a dominant phylum in the control MBR system, while accounting accounts for less than 10% of the experimental system. *Parcubacteria* was the unique dominant bacteria at the phylum level in the experimental system. The addition

of alien microbes presumably promoted the growth of the *Parcubacteria* and inhibited the *Actinabacteria* from the bacterial community bar plot at the phylum level.

At the class level, 46 different bacterial classes were identified in all samples. Among the classes, *Sphingobacteriia*, *Actinobacteria*, *Gammaproteobacteria*, *Beteproteobacteria*, *Alphaproteobacteria* and no rank *Saccharibacteria* had the highest relative abundance in the five samples. In particular, no rank *Parcubacteria* was a unique class in the experimental MBR system. *Actinobacteria* accounted for a small component in the experimental group.

A total of 208 different bacterial genera were detected in the five samples. The bar plot of bacterial communities at the genus level is presented in Figure 4b. Samples C2, E2, C16, ES16 and EA16 discovered 176, 193, 181, 172 and 178 genera, respectively, via 16s rRNA gene sequences. This suggests that biofortification with N7 and N9 may stimulate increased diversity of bacterial communities in water treatment. However, how long the influence of compound fungi on the bacterial community can be maintained needs to be further studied. The dominant genera were different between the experimental system and control system. The most abundant genus in the experimental MBR system were *norank-Parcubacteria*, *Ferruginibacter*, *Rhizabacter* and no rank *Saccharibacteria*, while *Nakamurella*, no rank *Saccharibacteria*, *Candidatus-Alysiosphaera* and *Rhodanobacter* were the most abundant in the control MBR system.

4. Conclusions

Through bioaugmentation, the compound fungi N7 and N9 have a positive application value in the treatment of nitrogen-containing wastewater. In this study, the transformation of ammonium and total nitrogen in the MBR bioreactor was accelerated by the novel compound fungi N7 and N9, and the accumulation of nitrate could be controlled. The results demonstrate the feasibility of N7 and N9 treatment of nitrogenous wastewater using bioaugmentation.

(1) In the first week of the experiment, the ammonium removal rate of the MBR system inoculated with N7 and N9 increased and the effluent concentration of NO_3^--N was reduced compared to the MBR system without inoculation of the compound fungi.
(2) Through ITS gene sequencing, it was observed that N9 could survive in MBR-containing activated sludge for a long time, Moreover, N9 was also observed to form biofilm with other microorganisms and maintain the population advantage, demonstrating good adaptability.
(3) High throughput sequencing results illustrated that the addition of N7 and N9 caused the original community structure to be altered, enriching the fungal communities. It should not be ignored that N7 and N9 also have an important impact on the change of bacterial community structure in the MBR system.
(4) Combined with the results of ITS gene sequencing and ammonium transformation characteristics, it is speculated that the compound fungi N7 and N9 inoculated every 8 days might achieve better results in practical application.

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Abbreviations

MBR	membrane bioreactor
DO	dissolved oxygen
HRT	hydraulic retention time
COD	chemical oxygen demand
MLSS	mixed liquor suspended solids
ITS	internal transcribed spacer

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