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Effects of organics on an ANAMMOX biofilm reactor: nitrogen removal efficiency and microbial community

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ABSTRACT

For the purpose of investigating the effect of different organic concentrations on nitrogen removal efficiency in anammox biofilm reactor, the denitrification efficiency of the reactor under the different concentrations of organic matter was tested after the successful start-up of the anammox biofilm reactor. In this study, MPN (most probable number) method and high-throughput sequencing technology were used to compare and analyze the differences of microbial communities in the biofilm under the influence of different concentrations of organic matter. In comparison with the reactor without any organic matter, the results indicated that the total nitrogen removal rate increases by 5.08% and 10.41% under the influent chemical oxygen demands (COD) of 30 and 60 mg L^{-1}, respectively. While the total nitrogen removal rate only increases by 4.71% when the COD concentration rises to 90 mg L^{-1}. In comparison with the reactor without any organic matter, the MPN method and high-throughput sequencing result showed that the denitrifying bacteria number was increased and the abundance of Planctomycetes and Proteobacteria was increased in the reactor under COD of 60 mg L^{-1}. Under the effect of 90 mg L^{-1} COD, the abundance of Proteobacteria continued to increase, while the abundance of Planctomycetes decreased sharply. At mentioned point, denitrification played a leading role. The experimental data showed that the denitrifying bacteria and anammox bacteria in the reactor achieved symbiosis and complementary advantages under certain organic concentration, but the growth of anammox bacteria was inhibited under the condition of high organic concentrations. This study can provide a reference value for anammox biofilm reactor to treat wastewater containing organic matter.

Keywords: Anammox; Denitrification; Microbial community; Nitrogen removal; Organic matter

1. Introduction

As a new and efficient denitrification technology, anammox has advantages of no additional organic carbon source, less sludge yield and higher nitrogen load compared with the traditional nitrification–denitrification technology [1–3]. However, the production of NO_3^-–N in the denitrification process will affect the further improvement of the denitrification rate [4,5]. Anammox bacteria use inorganic carbon sources for autotrophic metabolism. Nevertheless, many studies have shown that organic carbon sources have an impact on the denitrification efficiency of anammox activated sludge reactor. Zhang et al. [6], studied that when the chemical oxygen demand (COD)/N ratio exceeds 1.7 or COD concentration exceeds 100 mg L^{-1}, organic matter will seriously inhibit the performance of anammox reactor, and the proportion of anammox bacteria will decrease with the increase of COD concentration [7]. In the two-stage PN/A process, 75% of biodegradable COD can be removed during nitrification

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[8]. The remaining refractory COD is mainly composed of humic acid and fulvic acid, which can stimulate the growth of anammox bacteria at low concentrations [9].

The above studies focused on the anammox-activated sludge system, but the anammox mainly exist in the granules or biofilms and have the characteristics of aggregate growth [10]. Different from the anammox activated sludge system, the anammox bacteria from biofilm system attach to the filler to finish growth and enrichment. The fillers can effectively intercept the biomass and improve the biofilm microbial diversity, thus enhancing the stability of the reactor [11,12]. Nowadays, many studies on the removal of nutrients and organic matter from wastewater have been carried out [13–15], but there are few studies on the changes of microbial communities in anammox biofilm under the effects of organic matter. It is generally believed that in the process of denitrification, organic matter can be used as carbon source to improve the nitrogen removal efficiency, while in anammox, organic matter would inhibit the nitrogen removal efficiency. This study explores whether the concentration of organic matter can be controlled to reduce its adverse effects on anammox bacteria, and at the same time, the nitrogen removal effect of denitrifying bacteria can be used to make the two types of nitrogen removal microorganisms coexist and form coupling to improve the overall nitrogen removal effect of the biofilm system. The experiment examines the effects of different concentrations of organic matter on the anammox biofilm reactor with polyurethane porous carrier as filler to provide reference value for the biofilm reactor treatment of nitrogen-containing wastewater with certain organic content.

2. Materials and methods

2.1. Reactor configuration and operation

An up-flow anammox biofilm reactor with an effective volume of 6.28 L which adopts a peristaltic pump to continuously feed water with a daily inflow of 38 L and a hydraulic retention time (HRT) of 4 h was constructed, as shown in Fig. 1. The bottom of the reactor is laid with a 10 cm high pebble layer, and the middle of the reactor is filled with a 1 cm × 1 cm × 1 cm polyurethane porous carrier, accounting for 47% of the volume. At the top of the reactor is a three-phase separator. The reactor was cultured in a thermostatic chamber without light, and the water temperature was controlled at 30°C~32°C by an indoor temperature control system (Fig. 1).

2.2. Inoculate sludge and test water

Reactor inoculation sludge was collected from an A²/O (anaerobic–anoxic–oxic) process sewage treatment plant in Lanzhou, and the amount of sludge was 5 L. In this experiment, the synthetic wastewater prepared by standing tap water for 24 h was used as the inlet water in the reactor. Ammonium chloride (NH_4Cl) and sodium nitrite ($NaNO_2$) were used as a source of NH_4^+–N and NO_2^-–N, respectively.

Experimental drugs such as NH_4Cl and $NaNO_2$ were all analytical pure. The stoichiometry of ammonia nitrogen (NH_4^+–N) to nitrite nitrogen (NO_2^-–N) in water was 1:1.32, and the concentrations were 39.04~40.91 mg L^{-1} and 51.22~54.07 mg L^{-1}, respectively. The influent COD prepared by sucrose was 0, 29.10~31.50, 59.05~63.00, 88.15~93.20 mg L^{-1}. The influent pH range was 7.4~7.7. The addition amount of trace elements was 2 mL L^{-1} [16].

2.3. Test items and methods

Influent and effluent routine detection indexes include NH_4^+–N, NO_2^-–N, NO_3^-–N, total nitrogen (TN), and COD, these indexes were detected by Nessler's reagent spectrophotometry, N-(1-Naphthalene)diaminoethane, peroxide potassium sulfate-ultraviolet spectrophotometry respectively.

2.4. Most probably number method

The denitrifying bacteria in the reactor were counted by most probable number method. In this study, denitrifying bacteria in the bottom of anammox reactor with different COD concentrations (0, 60, and 90 mg L^{-1}) were tested by a series of gradient dilution culture and color detection to find out the corresponding number of possible bacteria according to the number of color tube. In the test, about 10 g of the packing with biofilm was taken and stirred in buffer solution or sterile water for 30 min, and the mixture of bacteria and water was diluted for culture.

2.5. Microbial community analysis

When the COD concentration was 0, 60, and 90 mg L^{-1}, the biofilms on the filler surface at 16 cm from bottom to top in the reactor were taken as samples, which were named ANB0-16, ANB60-16 and ANB90-16, respectively. The three samples were explored by Illumina MiSeq sequencing to determine the microbial community structure. Samples were sent to Majorbio Company (Shanghai, China). The universal primer pair 338 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′) was utilized for amplification and sequencing of the 16S rRNA gene

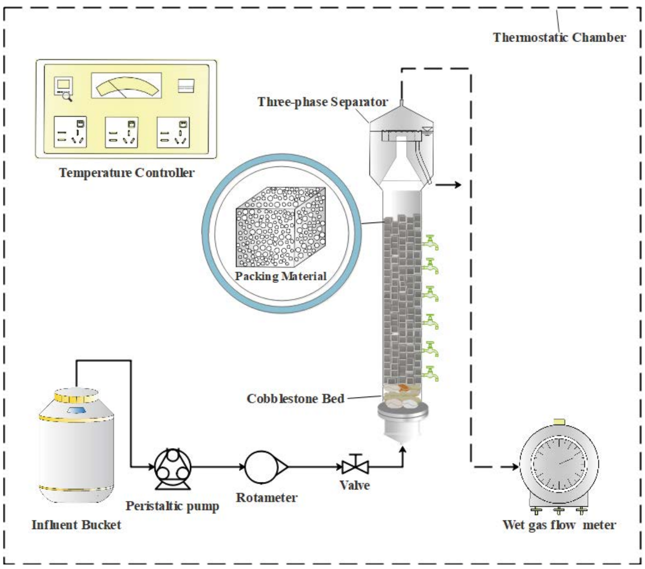

Fig. 1. Anammox biofilm reactor.

V3~V4 region. All data analyses were finished by I-Sanger Cloud Platform (www.i-sanger.com). Sequencing reads were clustered into operational taxonomic units (OTUs) at an identity threshold of 0.97. The functional genes were established using PICRUSt2 function prediction. 31,152 reads were obtained from each sample after subsampling and the coverage was greater than 99.6%, indicating that the diversity of the microbial community could be well [17].

2.6. Reactor start-up and operation

Nitrogen removal efficiency of anammox reactor in star-up period is shown in Fig. 2. The total nitrogen load of the reactor was 0.56 kg-N $(m^3\ d)^{-1}$. After 120 d of the start-up phase in the presence of organic matter in water, the removal rates of NH_4^+–N and NO_2^-–N reached 98.44% and 99.63%, respectively. A large number of anammox bacteria gathered and grew, and the biofilm showed brick red.

According to the two charts, after the 78th day, the concentration of NH_4^+–N and NO_2^-–N in the effluent of the reactor fluctuated slightly. It indicates that the gradual reduction of HRT will not significantly affect the stable operation of the reactor while promoting the enrichment of anammox bacteria, and there is no significant loss of anammox bacteria due to the shortened HRT. From the 104th day, the removal rates of NH_4^+–N and NO_2^-–N in the reactor decreased after the substrate concentration increased, but rose rapidly after a few days, indicating that the reactor could quickly adapt to the impact load caused by the substrate concentration change. After increasing the substrate concentration, the removal rates of NH_4^+–N and NO_2^-–N reached 98.5% and 99.21%, respectively, and the reactor achieved a good operation effect. At mentioned point, it is considered that the start-up of the anammox biofilm reactor was successful.

3. Results and analysis

After the successful start-up and stable operation of the anammox biofilm reactor, the experiment was divided into four stages. Each stage was run for 15 d, and the corresponding concentrations of organic matter were 0, 30, 60, and 90 mg L^{-1} to investigate the effect of organic matter on the reactor treatment and microorganism.

3.1. Influence of organic matter on nitrogen removal efficiency of reactor

Anammox process has the advantages of high nitrogen removal efficiency and energy saving. However, due to anammox bacteria are chemoautotrophic microorganisms, they are often easily affected by organic matter. In practical application, the anammox process is often used to treat wastewater with low C/N ratio [18]. The concentration of organic matter in the influent directly affects the number of heterotrophic bacteria in the anammox reactor, and then affects the living environment of the anammox bacteria.

At present, most of the research on anammox is on the laboratory scale, and the influent water of the reactor is mainly completed by synthetic water. In this experiment, sucrose is used as the external organic carbon source. The concentration of external organic matter is planned to be 0, 30, 60, and 90 mg L^{-1} (the measured value is shown in the subsequent experimental analysis). The HRT is 4 h, the temperature of the incubator where the reactor is located is controlled at about 36.0°C, and the effluent water temperature of the reactor is about 30.5°C. In the test process, the concentration changes of NH_4^+–N, NO_2^-–N, NO_3^-–N, and TN were analyzed to judge the denitrification efficiency of an anammox reactor. Parallel samples were set in the test process of each test index, and the average value was taken as the data each time.

As shown in Fig. 3a, COD at the first stage is 0 mg L^{-1}, and NH_4^+–N has a good removal rate of 99.62%. The COD of the second and third stages is relatively low, and the average removal rates of NH_4^+–N are 98.68% and 97.90%, respectively, indicating that a low concentration of organic matter has no significant effect on the nitrogen removal efficiency of anammox in the reactor. Stage 4 COD to 90 mg L^{-1}, NH_4^+–N removal rate continued to decline to 80.20%, and eventually back up to 83.25%. It shows that COD at this concentration has a negative effect on the removal of NH_4^+–N by anammox.

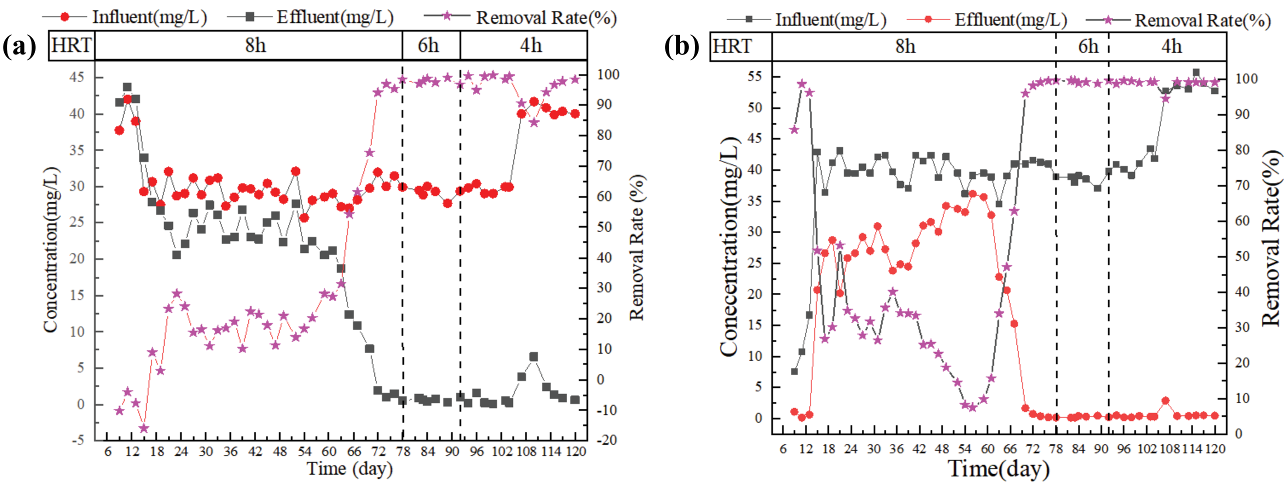

Fig. 2. Nitrogen removal efficiency of anammox reactor in start-up period: (a) NH_4^+–N and (b) NO_2^-–N.

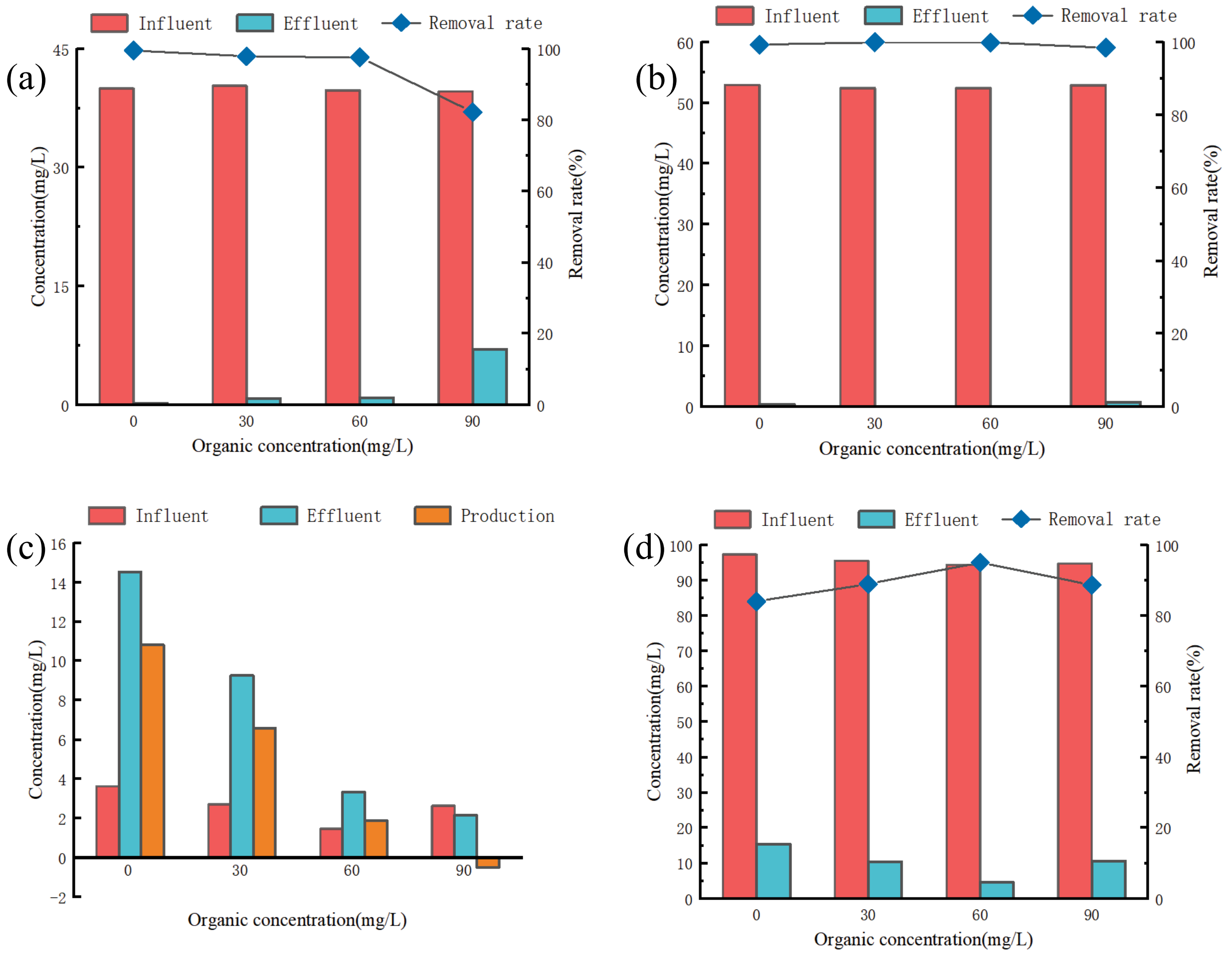

Fig. 3. Effect of organic matter on nitrogen removal efficiency. Influence on (a) NH_4^+–N, (b) NO_2^-–N, (c) NO_3^-–N, and (d) TN.

In Fig. 3b the NO_2^-–N removal rate in the four stages of operation is all higher than 98%, and organic matter has no obvious influence on the NO_2^-–N removal effect of the reactor.

In Fig. 3c the effluent NO_3^-–N concentration of the reactor at the first stage is 14.97 mg L^{-1}, and with the increase of COD, it gradually decreases. At the fourth stage, the average effluent concentration of NO_3^-–N was 2.61 mg L^{-1}. Thus, the effluent concentration of NO_3^-–N in the reactor was negatively correlated with the concentration of organic matter, and the increase of organic matter concentration was conducive to the removal of NO_3^-–N in the reactor. The NO_3^-–N concentration of the effluent in the fourth stage is far less than 8.56 mg L^{-1}, which is the theoretical value of NO_3^-–N concentration produced by anammox. It is speculated that the denitrification in the reactor is enhanced.

As shown in Figs. 3d and 4, the average removal rate of TN at the first stage was 83.94%, and the main nitrogen in the effluent was NO_3^-–N. With the increase of organic matter concentration, the TN removal rate of the second and third stage reactor increased continuously due to the decrease of effluent NO_3^-–N concentration, and reached the maximum of 94.35% in the third stage, which increased by 5.08% and 10.41%, respectively, compared with the first stage. The COD of the fourth stage is 90 mg L^{-1}, and the TN removal rate drops to 89.05%. The main nitrogen in the effluent is NH_4^+–N, and the TN removal rate decreases with the increase of NH_4^+–N concentration. It is speculated

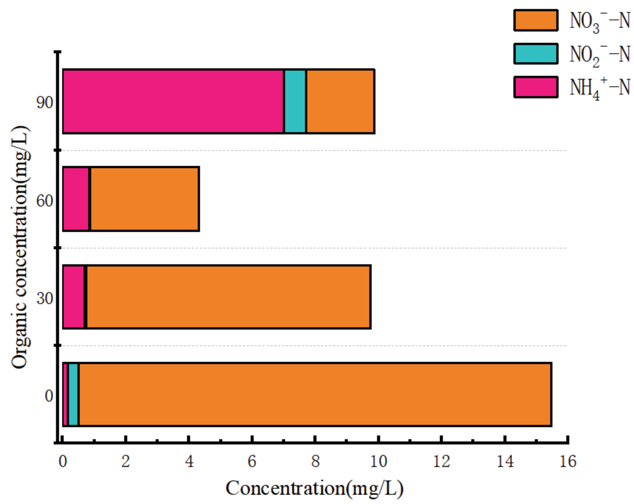

Fig. 4. Changes in nitrogen concentration in effluent pollution.

Table 1
Counting results of denitrifying bacteria at different organic concentrations

Bacteria	Concentration of organic matter (mg L^{-1})	Dilutability							Bacterial count (CFU cm^{-3})
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
Denitrifying bacteria	0	5^+	4^+	2^+	2^+	0^+	0^+		2.54×10^5
	60	5^+	5^+	4^+	4^+	2^+	0^+	0^+	1.69×10^7
	90	5^+	5^+	5^+	5^+	4^+	3^+	0^+	4.24×10^7

that under the condition of a high concentration of organic matter, the action of anammox bacteria is inhibited so that the removal effect of ammonia nitrogen is worse.

The results show that the organic matter with the appropriate concentration range can improve the TN removal efficiency of the reactor, and the TN removal efficiency will become worse if the organic matter concentration is too high.

3.2. *Influence of organic matter on the number of denitrifying bacteria*

As shown in Table 1, the number of denitrifying bacteria in the biofilm was calculated. It can be seen that the number of denitrifying bacteria is positively correlated with the concentration of organic matter. Compared with the condition without organic carbon source, the number of denitrifying bacteria was increased by two orders of magnitude by COD of 60 mg L^{-1}, indicating that organic matter could stimulate a large number of denitrifying bacteria. Hence, the appropriate concentration of organic matter can improve the NO_3^-–N removal effect of the reactor.

3.3. *Effect of organic matter on microbial community*

In this study, high throughput sequencing was used to analyze the microbial community in the biofilm at the concentration of three kinds of organic matter. The microbial diversity curves of the three samples are shown in Fig. 5. It can be seen that the curves of the three samples eventually tended to be flat, indicating that the amount of sequencing data was reasonable and the sequencing depth was sufficient [19].

The microbial diversity index of the three samples is shown in Table 2, and the library coverage rate of each sample is 1.00. The sequencing results can effectively analyze the microbial community in the sample biofilm. As can be seen from the table, with the gradual increase of COD concentration, ACE, Shannon, and Chao indexes all gradually increase, while Simpson index gradually decreases. ACE, Shannon and Chao indexes were positively correlated with the number of microbial species, while Simpson was negatively correlated with the diversity of microbial community [20]. Therefore, it can be believed that the addition of organic matter significantly increased the number of microbial species and enriched the community in the reactor.

As shown in Fig. 6, the main dominant bacteria in the three samples include Planctomycetes, Proteobacteria, Chloroflexi, and Acidobacteria. The Planctomycetes detected in the anaerobic reactor are the functional bacteria of anammox

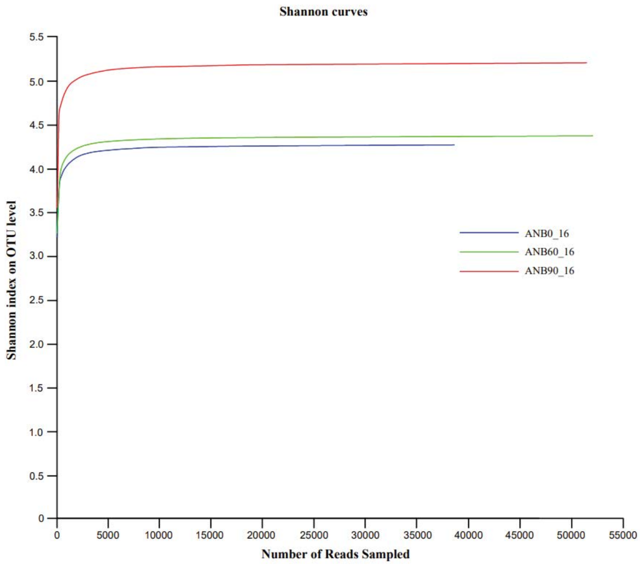

Fig. 5. Sample dilution curve.

Table 2
Biofilm microbial community diversity index table

Sample/Estimators	ACE	Chao	Simpson	Shannon
ANB0_16	917	912	0.0496	4.4541
ANB60_16	938	927	0.0460	4.6631
ANB90_16	1,070	1,062	0.0157	5.2059

[21]. Compared with 24.71% in ANB-0, the Planctomycetes abundance in ANB-60 increased to 31.78%, indicating that both of them were the most abundant phyla. However, in ANB-90, the abundance of Planctomycetes was significantly reduced to 5%.

Most of the Proteobacteria play an important role in microbial denitrification and the degradation of many organic compounds [22]. Their abundances in ANB-0, ANB-60, and ANB-90 were 16.16%, 21.93% and 25.93%, respectively. Compared with the non-organic stage, the abundance of the two kinds of nitrogen removal bacteria in the biofilm increased significantly under the COD of 60 mg L^{-1}, indicating that the coupling of anammox and denitrification improved the nitrogen removal efficiency of the reactor. When the concentration of organic matter further increased to 90 mg L^{-1}, the abundance of Proteobacteria further increased, but the abundance of Planctomycetes decreased sharply, resulting in a corresponding decrease in nitrogen removal efficiency. Chloroflexi bacteria are autotrophic microorganisms that can fix inorganic carbon [23]. Under anaerobic conditions, organic matter can be hydrolyzed by them and participate in the formation of biofilm [24]. It can be found that the abundance of Chloroflexi in ANB-0 and ANB-90 is higher than that in ANB-60. It can be inferred that the growth of Chloroflexi makes the abundance of the competing Planctomycetes significantly decrease due to the decrease of available inorganic carbon sources. Acidobacteria has acidophilic properties and the pH value in the reactor determines the abundance of Acidobacteria [25]. Since both denitrification and anammox produce alkalinity, the abundance of both denitrification bacteria determines the pH value of the reactor. The figure shows that in ANB-60, the sum of two kinds of bacteria abundance in three samples reached the highest 54.21%, while the abundance of Acidobacteria was only 4.25%. Under this condition of the organic matter concentration, the growth of Acidobacteria was inhibited, the abundance of Acidobacteria decreased obviously, and the competitive advantage of Planctomycetes and Proteobacteria in biofilm increased, which enhanced the nitrogen removal of the reactor.

As shown in Fig. 7, at the genus classification level, Brocadia and Jettenia were detected in all the three samples. The two genera are commonly found in ecosystems such as sewage plants and reactors. Jettenia is suitable for survival in low salinity environment [26]. Brocadia has a stronger affinity for NH_4^+ and NO_2^-, as well as a faster growth rate, making it easy to become the dominant bacteria genus. Among the three samples, the abundance of Brocadia was 20.38%, 28.58%, 2.12%, and the abundance of Jettenia was 1.38%, 1.24%, 0.26%, respectively. The results showed that the COD of 60 mg L^{-1} significantly promoted the abundance of Brocadia, but had little effect on the abundance of Jettenia. Under COD of 90 mg L^{-1}, Brocadia and Jettenia bacteria of the genus had strongly inhibited the abundance.

Thauera is a genus of Proteobacteria, which has the function of degrading organic matter and denitrification [27]. According to the figure, the abundance of *Thauera* in the three samples was 0.28%, 1.15%, and 0.44%, respectively. Under COD of 60 mg L^{-1}, the abundance of *Thauera* is higher than the other two samples, suggesting that *Thauera* may play a key role in maintaining the accumulation characteristics of high nitrite. It can be inferred that under the

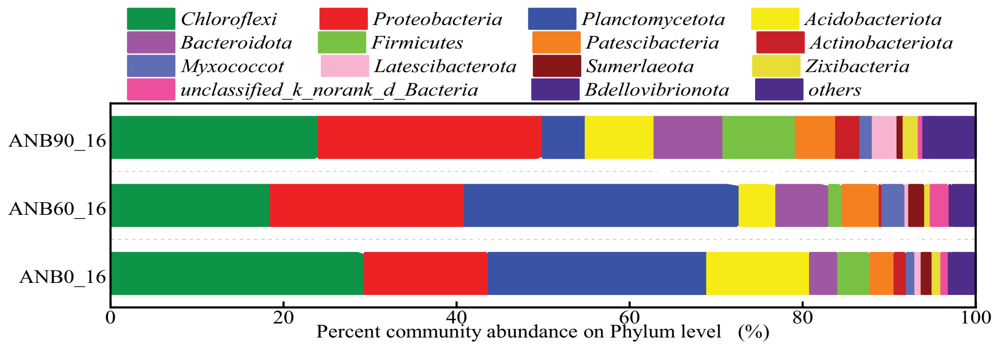

Fig. 6. Analysis of changes in the main phylum of microorganisms in biofilms.

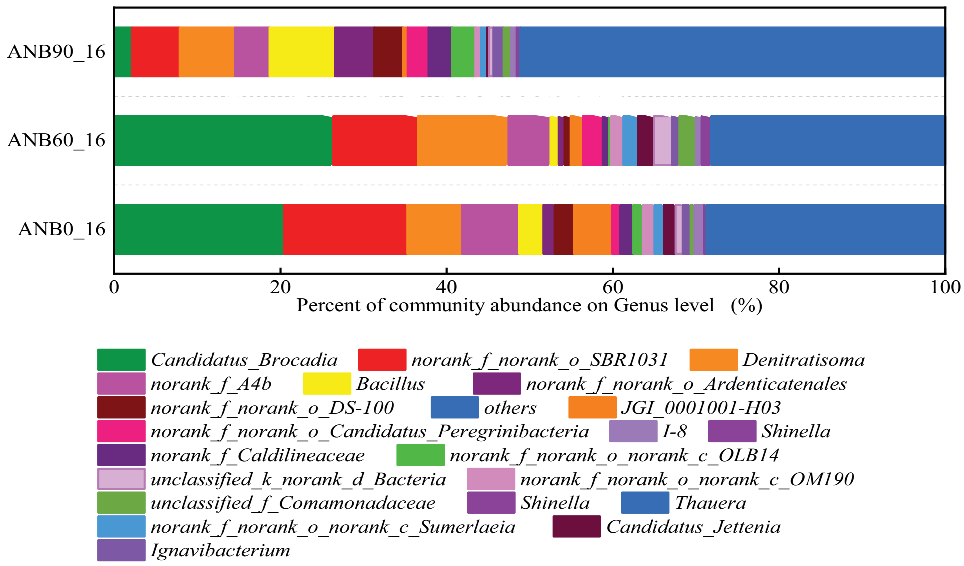

Fig. 7. Analysis of the main bacteria in the biofilm.

condition of insufficient concentration of organic matter, complete denitrifying bacteria cannot grow and develop sufficiently, thus increasing the abundance of some nitrate-reducing bacteria. Bacillus belongs to Firmicutes and is an organic vegetative bacterium capable of denitrification [28]. Its abundance in ANB-0, ANB-60, and ANB-90 was 2.9%, 2.75%, and 7.8%, respectively. Therefore, the increase of the concentration of organic matter can promote heterotrophic denitrifying bacteria in the biofilm of proliferation. *Ignavibacterium* is a genus of Chloroflexi, and its abundance in the three samples were 0.96%, 0.9% and 1.2%, respectively, which indicated that the presence of Chloroflexi had certain inhibitory effect on the genus Planctomycetes. It can also be seen from the figure that at 90 mg L^{-1} COD, the genus Paracoccus begins to appear, and its abundance is 1.4%, Paracoccus plays an important role in denitrification, and its presence indicates that denitrification has taken a dominant role under this concentration of the organism.

Therefore, it can be concluded that the appropriate concentration of organic matter can not only increase the abundance of Brocadia bacteria with the function of anammox, but also maintain a sufficient number of heterotrophic denitrification bacteria in the reactor, and the two kinds of denitrification bacteria coexist in the reactor. Thus, a highly efficient and stable coupled biofilm is formed to improve the nitrogen removal efficiency of the reactor.

3.4. Changes in microbial metabolic pathway level 1

As shown in Table 3, the statistical values of the abundance of metabolic pathways such as cell process, environmental information processing, genetic information processing, metabolism, and biological system of microorganisms in ANB-60 samples were significantly higher than those of ANB-0 and ANB-90 through the function prediction analysis of PicRust1. Under COD of 60 mg L^{-1}, the metabolic function of the microbial community in the biofilm is significantly enhanced, and the nitrogen removal function of the microbial community is enhanced. Thus, the nitrogen removal performance of the reactor was improved.

4. Discussion

As shown in Fig. 8, denitrifying bacteria can further transform NO_3^-–N and NO_2^-–N into N_2 to achieve nitrogen removal. Compared with ANB0_16, the abundance of Planctomycetes in sample ANB60_16 increased by 8.32% when influent organic matter increased from 30 mg L^{-1} to 60 mg L^{-1}, indicating that appropriate organic carbon source had no obvious influence on anammox bacteria. The NO_3^-–N produced by anammox bacteria in the reactor is utilized by denitrifying bacteria, and denitrifying bacteria can provide inorganic carbon source and NO_2^-–N for anammox bacteria [29]. Anammox and denitrifying bacteria show symbiotic relationship, they promote each other, and the two bacteria in the biofilm cooperate to remove nitrogen, so that the anammox and denitrification in the reactor are effectively coupled, thus improving the denitrification efficiency of the reactor. Excessive organic matter would promote the growth and proliferation of denitrifying bacteria in the biofilm reactor. When the organic matter was 90 mg L^{-1}, the abundance of Proteobacteria in the reactor increased significantly compared with 0 and 60 mg L^{-1}. However, the abundance of Planctomycetes in ANB90_16 decreased to 5.00% compared with 24.71% in ANB90_16, making denitrifying bacteria compete with anammox bacteria for common substrate NO_2^-–N and living space [30]. Under the inhibition of organic matter, the activity of anammox decreased, and the denitrification efficiency decreased because of the contradiction between anammox and denitrifying bacteria in the demand of organic carbon source.

5. Conclusion

- Compared with no organic matter, at 90 mg L^{-1} organic matter, the denitrifying bacteria in the biofilm competed with the anammox bacteria, which was not conducive to the growth of anammox bacteria, and the nitrogen removal performance of the reactor was poor. When the concentration of organic matter was 30 and

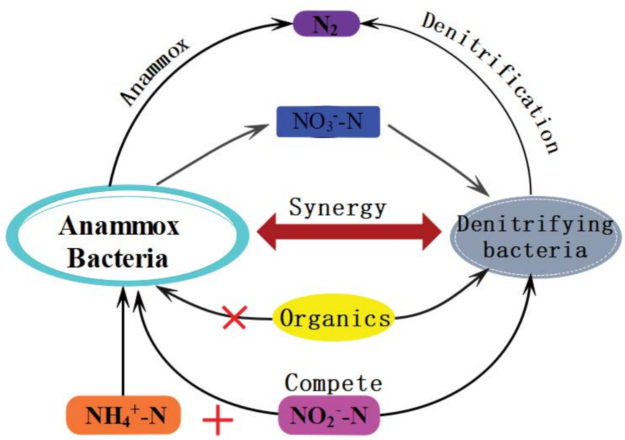

Fig. 8. Relationship between anammox bacteria and denitrifying bacteria.

Table 3
Biofilm microbial community metabolic pathway level 1 abundance table

Sample/ Estimators	Cellular processes	Environmental information processing	Genetic information processing	Metabolism	Organismal systems	Unclassified
ANB0_16	2,885,038	6,836,878	10,029,594	30,456,441	469,321	9,145,284
ANB60_16	5,289,324	11,323,587	17,369,472	52,774,302	826,498	16,162,358
ANB90_16	1,865,873	6,718,761	7,365,183	23,098,628	356,929	6,341,471

60 mg L^{-1}, respectively, denitrifying bacteria and denitrifying bacteria in the reactor coexisted and supplemented each other, making the reactor have a strong coupling nitrogen removal capacity.

- Relative to no organic matter, under COD of 60 mg L^{-1}, the abundance of Planctomycetes and Proteobacteria increased by 8.32% and 5.77%, respectively. The abundance of Cadia, Hauera, Enitratisoma and Comamonadaceae have all increased, indicating the realization of the synergistic nitrogen removal by denitrification and anammox. Under COD of 90 mg L^{-1}, the abundance of Planctomycetes decreased by 20.25%, Proteobacteria increased by 11.6%, and the abundance of Bacillus that can perform denitrification increased by 4.9%. In addition, a new Paracoccus genus with an abundance of 1.4% was generated. At this time, denitrification became dominant.

Therefore, it is not difficult to find that for the anammox biofilm reactor, a small amount of organic pollution will not inhibit the nitrogen removal efficiency of the reactor. On the contrary, it will be strengthened to a certain extent because of the coupling effect between anammox and denitrification.

Declarations

Conflict of interest I wish to declare that there is no conflict of interest.

Acknowledgments

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