Water Science & Technology

Water Science & Technology Vol 84 No 3, 725 doi: 10.2166/wst.2021.258

Effect of organic concentration on biological activity and nitrogen removal performance in an anammox biofilm system

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ABSTRACT

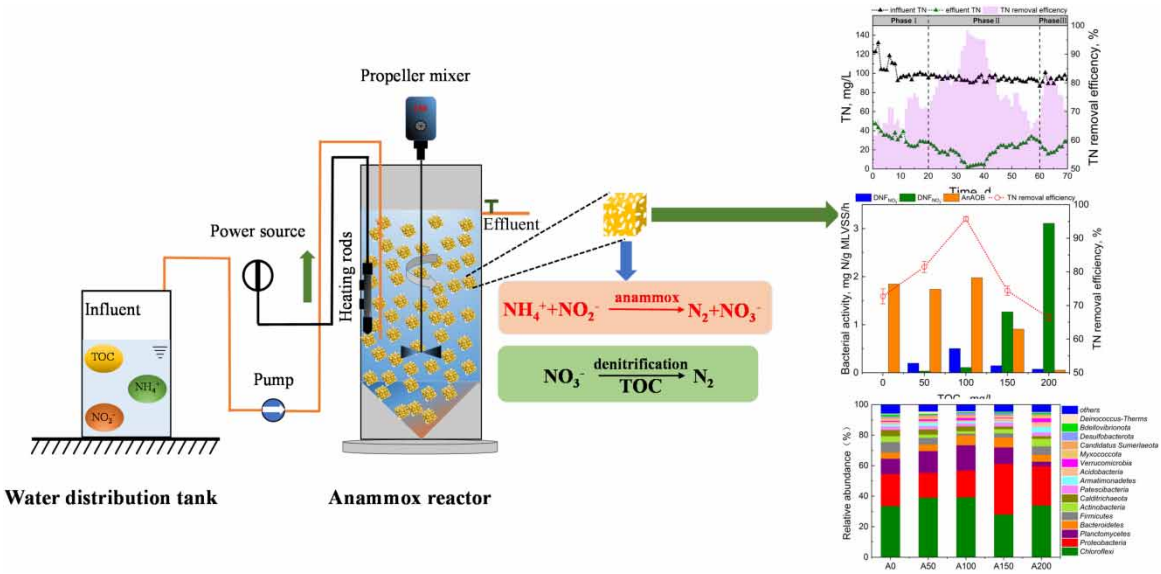
The effects of different concentrations of organic matter on the biological activity and nitrogen removal performance of the anaerobic ammonium oxidation (anammox) system was studied. The results showed that under the conditions of low influent total organic carbon (TOC $\leq$ 100 mg/L), the activity rate of anammox bacteria was basically unaffected, the anammox bacteria and denitrifying bacteria formed a good synergistic effect, and the maximum total nitrogen (TN) removal efficiency reached 95.77%. However, when the influent TOC concentration was up to 200 mg/L, the activity of anammox bacteria was seriously inhibited. At this time, denitrification becomes the main pathway of nitrogen removal, the effluent ammonia nitrogen content increases, and the TN removal efficiency decreases to 64.17%. High-throughput sequencing analysis showed that with the increase in organic matter concentration, the relative abundance of *Proteobacteria* and *Planctomycetes* changed significantly. In particular, the relative abundance proportion of *Proteobacteria* increased from 21.06% to 25.57%, the *Planctomycetes* dropped from 10.01% to 3.03% and the *Candidatus* Brocadia genus had the largest decrease. In conclusion, the concentration range of organic matter for collaborative denitrification was proposed in this study, which provided theoretical reference for the practical application of anammox biofilm process.

Key words: anammox system, biological activity, microbial community, moving bed biofilm reactor, nitrogen removal, organic concentration

HIGHLIGHTS

- A continuous flow anammox/denitrification moving bed biofilm reactor (MBBR) was operated.
- The optimum denitrification performance of the reactor was achieved at the influent organic matter concentration of 100 mg/L.
- The denitrification form of biofilm changed with the increase in organic matter.
- Predominant genus changed with the increase in organic matter concentration.

GRAPHICAL ABSTRACT



INTRODUCTION

The anaerobic ammonium oxidation (anammox) reaction is a biological process in which ammonium (NH_4^+) as electron donor is oxidized to nitrogen gas (N_2) by anammox bacteria using nitrite (NO_2^-) as electron acceptor under hypoxic conditions (Strous *et al.* 1999). Compared with the traditional nitrification and denitrification process, without the participation of organic matter, the anammox process can theoretically reduce nitrogen effluent by 89%, thus providing efficiency benefits in sewage treatment (Lackner *et al.* 2014).

In practice, it is difficult to realize large-scale enrichment of anammox bacteria in a short period due to its slow growth rate (about 0.072/days at 32 °C) and low yield coefficient (about 0.13 g dry weight/g NH_4^+ oxidized) (Wu *et al.* 2018). The influent fluctuation of organic matter, shown as the diversity of total organic carbon (TOC) concentration, usually inhibits the anammox bacteria activity, affecting the nitrogen removal performance in the anammox system (Wang *et al.* 2020a). Previous studies have shown that the tolerance capability of anammox bacteria to organic concentration also depends on the system's denitrification process (organic matter was consumed), while denitrification is caused by the competition and coexistence of anammox and denitrification (Anjali & Sabumon 2014; Jenni *et al.* 2014). At low TOC concentration, denitrifying bacteria use NO_3^-, which preliminary accumulated in the anammox process, to oxidize TOC in wastewater and provide nitrite for the next anammox reaction, which can improve nitrogen removal performance (Mulder *et al.* 2012). However, the surplus TOC in wastewater encourages the heterotrophic denitrifying bacteria to rapidly multiply and compete with anammox bacteria for nitrite, while the proportion of anammox bacteria decreases and its activity is decreased (Qin *et al.* 2017). What's more, under the conditions of higher TOC, the activity of anammox bacteria will be completely inhibited, and denitrification becomes the main nitrogen removal pathway, which results in a profitless approach in TN removal efficiency, indicating that high organic matter concentration might have a negative impact on anammox bacteria (Blackburne *et al.* 2008). Consequently, for reducing the competitive intension between anammox bacteria and denitrifying bacteria to improve the nitrogen removal efficiency, the conducive organic matter concentration range to the favorable coexistence of anammox bacteria and denitrifying bacteria requires further study. Whereas previous studies have mainly focused on the nitrogen removal effect of the anammox process, both the regular pattern of nitrogen transfer and microbial community structure change under different organic concentrations were not clear.

In this study, to maintain a high biological activity of anammox bacteria in the reactor, a moving bed biofilm reactor (MBBR) was used to enrich anammox bacteria. Under different influent TOC concentrations, the diversity of nitrogen removal performance, extracellular polymer substances (EPS), biological activity and microbial community structure on the biofilm were investigated. Then, through mass balance analysis, the regularity of nitrogen transfer was explored and

the synergistic nitrogen removal mechanism of anammox bacteria and denitrifying bacteria was further revealed. In the end, this study suggested the concentration range for organic matter for synergistic nitrogen removal, providing a theoretical reference for the practical application of anammox process.

MATERIALS AND METHODS

Experimental apparatus and operating conditions

A moving bed biofilm reactor (MBBR) was used as the anammox system in this study (Figure 1). The reactor was made of acrylic, with an inner diameter of 25 cm, a height of 70 cm and an effective volume of 21.6 L. MBBR was filled with 20% cubic polyurethane sponge as a microbial attachment carrier, with a density of 0.95 g/cm^3 and the specification of 10 × 10 × 10 mm. A stirrer was placed inside the MBBR and the stirring speed was 150 rpm/min and the reactor temperature was controlled at 32 ± 1 °C. The hydraulic retention time (HRT) of the reactor was maintained at 24 h, the whole MBBR was covered with black plastic film to avoid light and the outer layer was wrapped with tin foil for heat preservation.

Experiment with water and inoculated sludge

This experiment used artificially prepared simulated wastewater. The simulated wastewater was continuously added from the bottom of the reactor through a peristaltic pump (0.9 L/h). The entire operating cycle was 70 days, divided into three stages. TOC were 0, 50, 100, 150 and 200 mg/L, approximately, NH_4^+ and NO_2^- concentration were maintained at about 40 and 52 mg/L. The actual influent indexes of the reactor are shown in Table 1. Other components included $NaHCO_3$ 200 mg/L, KH_2PO_4 30 mg/L, $MgSO_4{\cdot}7H_2O$ 100 mg/L, $CaCl_2$ 15 mg/L, EDTA 5 mg/L, $FeSO_4{\cdot}7H_2O$ 5 mg/L, H_3BO_3 14 μg/L,

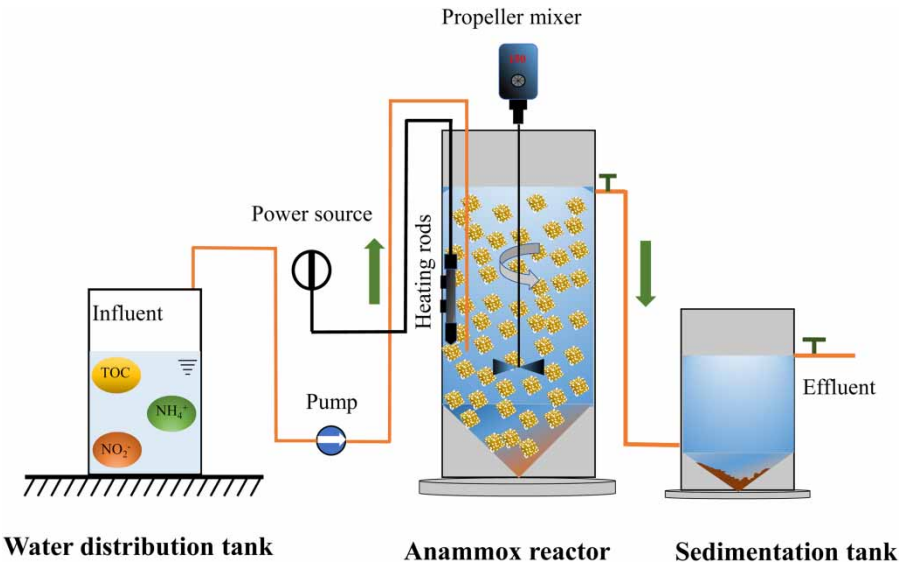


Figure 1 | Schematic diagram of the experimental device.

Table 1 | Influent pollutant concentration in anammox reactor

Phase	Time, d	NH_4^+, mg/L	NO_2^-, mg/L	TOC, mg/L
I	0–20	38.50–59.90	49.08–69.11	1.10–2.56
II	21–30	40.42–42.73	52.12–54.87	45.51–57.23
	31–40	39.25–41.96	50.65–54.94	97.5–112.82
	41–50	39.95–49.75	49.35–56.57	142.21–161.33
	51–60	38.65–42.05	50.11–52.95	192.76–209.69
III	61–70	39.36–43.85	49.55–58.45	1.02–2.88

$MnCl_2$•$4H_2O$ 990 μg/L, $CuSO_4$•$5H_2O$ 250 μg/L, $CoCl_2$•$6H_2O$ 240 μg/L, $ZnSO_4$•$7H_2O$ 430 μg/L, $NiCl_2$•$6H_2O$ 190 μg/L, $NaMoO_4$•$2H_2O$ 220 μg/L, $CaCl_2$•$2H_2O$ 440 μg/L (Ma *et al.* 2017).

The inoculated sludge was used as laboratory-grown nitrifying sludge and anammox sludge, which were put into the reactor at the ratio of 2:1 and the initial sludge mixed liquid suspended solids (MLSS) concentration was 2,986 mg/L.

All samples were filtered through a 0.45 μm pore size filter before analysis. Water quality indexes such as TOC, NH_4^+, NO_2^- and NO_3^- concentrations were measured by standard methods (Eaton *et al.* 1966), pH and temperature (T) were measured by a lightning multi-parameter analyzer and the MLSS and MLVSS were also measured by the standard methods.

Biological activity analysis

The activities of anammox bacteria, nitrite denitrifying bacteria and nitrate denitrifying bacteria on the polyurethane sponge biofilm in the reactor were measured on the 20th, 30th, 40th, 50th and 60th days, respectively. Polyurethane sponge and simulated wastewater (Table 2) were taken from the reactor and put into an 80 ml serum bottle, which was added to make the working volume to 80 ml. The serum bottles were blown off with nitrogen for 15 min and sealed with rubber and aluminum caps to ensure the anaerobic conditions. Then, the TOC concentration was added at different stages according to demand (about 0, 50, 100, 150, 200 mg/L), wrapped in a black plastic cloth to avoid light and placed at a constant temperature shaker at 32 °C (150 r/min) for incubation. After that, the concentrations of NH_4^+, NO_2^- and NO_3^- were measured by sampling every 1 h.

EPS extraction and analysis

EPS were extracted from the polyurethane sponge biofilm at different stages of the reactor using a modified heat extraction method (Du *et al.* 2019) and then protein (PN) and polysaccharide (PS) in the soluble type EPS (S-EPS), loose-bound EPS (LB-EPS) and tightly-bound EPS (TB-EPS) were determined. What's more, the total EPS (T-EPS) of samples were also calculated. Proteins were determined using the Lowry method with bovine serum albumin as the standard. PS was determined using the anthrone method with glucose as the standard (Hou *et al.* 2015).

Microbial community analysis

At the 20th, 30th, 40th, 50th and 60th days, samples of biofilm were collected, named A0, A50, A100, A150 and A200, then sent to Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China) for Illumina MiSeq analysis. Purity and concentration of the extracted DNA were detected by agarose gel electrophoresis, using common bacterial primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Xu *et al.* 2016).

Nitrogen transfer analysis

The nitrogen loading rate (NLR) and nitrogen removal rate (NRR) of the reactor were studied according to Equations (1) and (2):

$$NLR = TN_{inf} \times Q \times H/(1{,}000 \times V) \quad (1)$$

$$NRR = (TN_{inf} - TN_{eff}) \times Q \times H/(1{,}000 \times V) \quad (2)$$

where TN_{inf} and TN_{eff} indicate the TN concentration of influent and effluent respectively, mg/L; Q is the inflow rate, m^3/d; H is the HRT, d; V represents the working volume, 21.6 L, respectively.

In this study, ammonium, nitrite and glucose were added to the reactor. Theoretically, without the presence of oxygen, anammox bacteria used CO_2 as a carbon source and nitrite nitrogen to oxidize ammonia nitrogen to N_2, as shown in

Table 2 | Determination of biological activity

	Activity determination		
Compound, mg/L	**Anammox activity**	**Denitrification activity for nitrite nitrogen**	**Denitrification activity for nitrate nitrogen**
NH_4^+	40	0	0
NO_2^-	52	52	0
NO_3^-	0	0	52

Equation (3):

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (3)$$

At the same time, the experimental wastewater was not deoxidized and nitrification occurred, which was combined with chemical Equations (4) and (5):

$$NH_4^+ + 1.37O_2 + 0.02HCO_3^- + 0.08CO_2 \rightarrow 0.98NO_2^- + 0.02C_5H_7NO_2 + 0.97H_2O + 1.96H^+ \quad (4)$$

$$NO_2^- + 0.01NH_4^+ + 0.45O_2 + 0.01HCO_3^- + 0.01H_2O + 0.04CO_2 \rightarrow NO_3^- + 0.01C_5H_7NO_2 \quad (5)$$

Based on the above reaction, the following equation can be established:

$$R_{AMX} + R_{AOB} + 0.01R_{NOB} = \Delta NH_4^+ \quad (6)$$

$$1.32R_{AMX} - 0.98R_{AOB} + R_{NOB} = \Delta NO_2^- \quad (7)$$

$$0.26R_{AMX} + R_{NOB} = \Delta NO_3^- \quad (8)$$

where R_{AMX} and R_{AOB} respectively represent the content of ammonia nitrogen consumed by anammox and partial nitrification during the denitrification process of the reactor, R_{NOB} represents the content of nitrite consumed in the nitrosation process, ΔNH_4^+, ΔNO_2^-, ΔNO_3^- respectively represent the difference in the concentration of water in and out of the reactor without the addition of organic matter.

At the stage of discussing the influence of organic matter, the nitrogen removal performance of the reactor is affected by both anammox bacteria and denitrifying bacteria. To evaluate the contribution ratio of anammox and denitrification to nitrogen removal, a nitrogen-based mass balance was established. Theoretically, 1 mg/L NO_3^- and 1 mg/L NO_2^- consume 2.86 mg/L and 1.71 mg/L TOC, respectively. This study was carried out under a low nitrogen load. Considering the consumption of biological assimilation NH_4^+ during denitrification (Deng *et al.* 2020), the following equations can be established:

$$\Delta NH_4^+ = \left(1 - \frac{1.32i_{NBM}}{1.71Y_H} + \frac{0.26i_{NBM}}{2.86Y_H}\right) NH_{4AMX}^+ + \frac{i_{NBM}}{2.86Y_H}\Delta NO_3^- + \frac{i_{NBM}}{1.71Y_H}\Delta NO_2^- \quad (9)$$

$$\Delta NO_2^- = 1.32NH_{4AMX}^+ + NO_{2DNF}^- \quad (10)$$

$$\Delta NO_3^- = 0.26NH_{4AMX}^+ - NO_{3DNF}^- \quad (11)$$

where NH_{4AMX}^+, NO_{2DNF}^- and NO_{3DNF}^- represent the content of ammonia nitrogen consumed in the process of anammox, the content of nitrite and nitrate removed by denitrification, respectively. Parameter 1.32 and 0.26 are the stoichiometric values of nitrite consumption and nitrate production in the anammox process, respectively (Strous *et al.* 1998). Where ΔNH_4^+, ΔNO_2^-, ΔNO_3^- represent the differences of influent and effluent nitrogen concentrations. Assuming that the biomass nitrogen content (i_{NBM}) is 0.087 g N/g TOC, the yield coefficient of heterotrophic microorganisms (Y_H) is 0.625 g TOC/g TOC (Henze *et al.* 1999).

RESULTS AND DISCUSSION

Effects of organic matter concentration on nitrogen removal performance

The whole reaction was divided into three stages. The first stage was the start-up stage of the anammox reactor (0–20 d). The influent NH_4^+ and NO_2^- were about 38.50–59.90 mg/L and 49.08–69.11 mg/L. After 20 days of operation, the effluent of the reactor was stable and the effluent NH_4^+, NO_2^- and NO_3^- were about 1.37 mg/L (Figure 2(a)), 6.62 mg/L (Figure 2(b)) and 19.52 mg/L (Figure 2(c)). The TN removal efficiency was 70.57% (Figure 2(d)). What's more, the ratio of $\Delta NO_2^-/\Delta NH_4^+$ was 1.19, close to 1.32 (Figure 2(e)), indicating that the reactor start-up was successful.

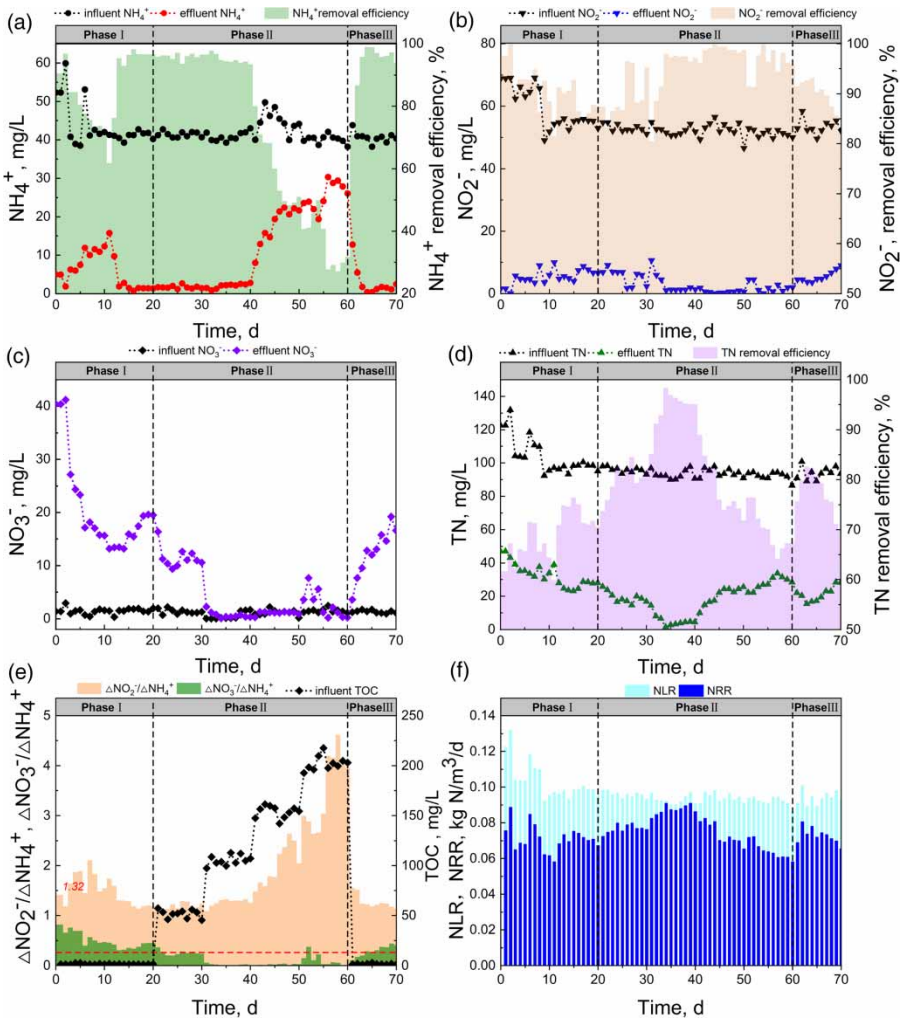


Figure 2 | Nitrogen removal performance of the anammox reactor. (a) The NH_4^+ concentration and NH_4^+ removal efficiency of the influent and effluent. (b) The influent and effluent NO_2^- concentration and NO_2^- removal efficiency. (c) The concentration of NO_3^- in influent and effluent. (d) The concentration of TN in influent and effluent and TN removal efficiency. (e) Influent and effluent TOC concentration, $\Delta NO_2^-/\Delta NH_4^+$, $\Delta NO_3^-/\Delta NH_4^+$. (f) Nitrogen loading rate (NLR), Nitrogen removal rate (NRR).

The second stage (21–60 d) was introduced to investigate the organic matter concentration impact. The nitrogen removal rate (NRR) was maintained at 0.09 kg N/(m^3·d). When the influent TOC concentration was 50 mg/L, the effluent NO_3^- dropped from 19.52 mg/L to 10 mg/L (Figure 2(c)) with the effluent NH_4^+ and NO_2^- both maintained at about 1.5 mg/L. As the influent TOC concentration was increased to 100 mg/L, the effluent NH_4^+, NO_2^- and TN were 1.5 mg/L, 1.43 mg/L and 4.4 mg/L. Under these conditions, the TN removal efficiency increased from 70.57% to 95.77%, indicating that the nitrogen removal performance of the reactor could be significantly improved by setting the appropriate concentration of organic matter. However, when the influent TOC was up to 150 mg/L, the effluent NH_4^+ rapidly increased from 2 mg/L to 20 mg/L and the TN removal efficiency rapidly dropped to 74%. At this point, $\Delta NO_2^-/\Delta NH_4^+$ was over 2.0, indicating that part of NO_2^- was removed by denitrification. Moreover, when the influent TOC concentration was 200 mg/L, the effluent NH_4^+ reached 26 mg/L, the NH_4^+ removal efficiency dropped to 30% and the removal efficiency of TN was lower than 64.17%, which seems much more deteriorated. But the effluent NO_2^- and NO_3^- content were 2 mg/L and 0.23 mg/L, which was close to complete removal, and compared with the $\Delta NO_2^-/\Delta NH_4^+$ was close to 4.0, indicating that denitrification was the main nitrogen removal process in the reactor at this stage.

The third stage was the activity recovery stage (61–70 d), to remove the effect from organic matter on the denitrification performance and study the impact load resistance in the reactor. It was found that on the third day, the effluent NH_4^+ rapidly

and gradually decreased to 2 mg/L, the effluent NO_3^- and TN content were maintained at about 16.64 mg/L and 28.14 mg/L respectively, the TN removal efficiency increased to 70% and the effluent $\Delta NO_2^-/\Delta NH_4^+$ was close to 1.1. The denitrification performance of this anammox reactor quickly recovered and indicated that the reactor showed good impact load resistance.

Effects of organic matter concentration on biological activity in a reactor

Figure 3 shows the changes in the activity of anammox bacteria and denitrifying bacteria under different organic conditions. As the figure shows that under the condition of low influent organic matter (TOC $\leq$ 100 mg/L), the activity rate of anammox bacteria (AnAOB) was basically unaffected, remained stable at 1.847 mg N/g MLVSS/h, while nitrate denitrifying bacteria ($DNF_{NO_3^-}$) could use NO_3^- accumulated in the process of anammox to oxidize organic matter. Its activity was rapidly increased to 0.504 mg N/g MLVSS/h and the removal efficiency of TN in the reactor increased to 95.77 $\pm$ 0.68%. However, as the concentration of organic matter continued to increase, nitrite denitrifying bacteria ($DNF_{NO_2^-}$) began to proliferate in large numbers and competed with anammox bacteria for electron acceptor nitrite. Anammox bacteria were in the 'hunger' state due to the lack of necessary growth substrate, and their activity gradually decreased. when the influent TOC concentration increased to 150 mg/L, the anammox bacteria activity was significantly inhibited, decreased to 0.91 mg N/g MLVSS/h. When the influent TOC concentration was 200 mg/L, the anammox activity decreased to 0.053 mg N/g MLVSS/h, which was close to 0, indicating that its activity was seriously inhibited. The figure also shows that when TOC concentration exceeded 200 mg/L, the activity of nitrite denitrifying bacteria ($DNF_{NO_2^-}$) rapidly increased from 0.11 mg N/g MLVSS/h to 3.11 mg N/g MLVSS/h, which caused the nitrogen removal pathway to gradually change from anammox to nitrite denitrification. Therefore, for maintaining the activity of anammox bacteria and promoting the synergistic nitrogen removal of anammox bacteria and denitrifying bacteria, the concentration of influent organic matter should be maintained at 0–100 mg/L.

EPS composition and characteristics

EPS is an important part of the biofilm system which helps to maintain the stability of the microbial community (Sheng *et al.* 2010). Therefore, the changes in the EPS composition of the biofilm attached at the polyurethane sponge under different organic concentrations were studied.

Figure 4(a) shows in the start-up phase, due to the less biofilm on the polyurethane sponge, the actual T-EPS content measured was relatively low, only 24.23 mg/g VSS. With the gradual increase of the organic matter concentration, the denitrifying bacteria multiplied and microorganisms stored more EPS to promote the attachment of biofilm and the T-EPS content increased to 52 mg/g VSS. In the layered analysis of the carrier biofilm EPS (Figure 4(b)), it was found that the TB-EPS content on the carrier biofilm was greater than LB-EPS and S-EPS at different organic concentrations and with the increase of the organic concentration, the content of TB-EPS in the biofilm reached 78%. Studies have shown that TB-EPS is beneficial to the

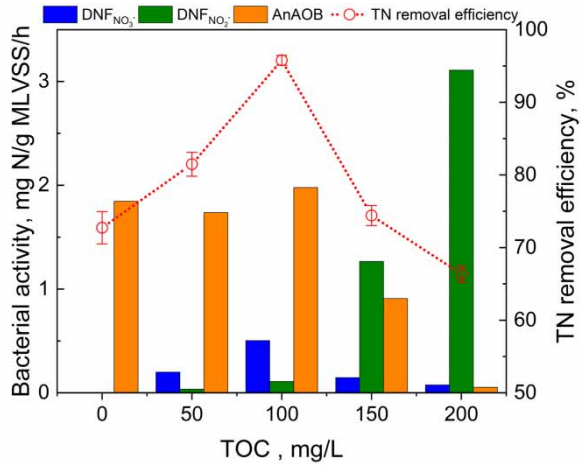


Figure 3 | The activity of nitrite denitrifying bacteria ($DNF_{NO_2^-}$), nitrate denitrifying bacteria ($DNF_{NO_3^-}$), anammox bacteria (AnAOB) and TN removal efficiency under different concentrations of organic matter.

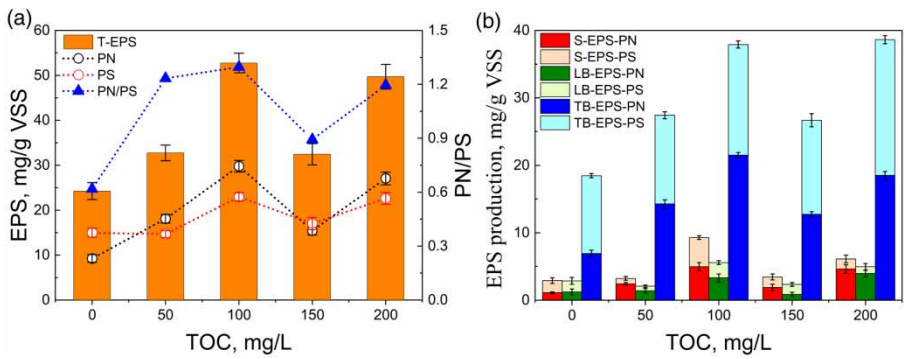


Figure 4 | (a) T-EPS, PN, PS content on the biofilm and PN/PS in T-EPS under different organic concentrations; (b) EPS composition under different organic concentrations.

enrichment of anammox bacteria (Gu *et al.* 2017). In contrast, excessive LB-EPS will weaken cell adhesion, and deteriorate the structure of anammox biofilm (Ali *et al.* 2018; Wang *et al.* 2020b).

In the analysis of the EPS composition, the PN/PS in the EPS on the biofilm under different organic concentration conditions were found at Figure 4(a). With increase in organic concentration, PN/PS gradually increased from 0.6 to 1.29. Studies showed that PN was a hydrophobic substance, while PS was a hydrophilic substance. Increasing the ratio of PN/PS might improve the performance of the biofilm (Yu *et al.* 2012). When TOC concentration was 150 mg/L, the activity of anammox bacteria was inhibited. PN/PS gradually decreased to 0.90, which was not conducive to microbial attachment. In addition, the content of T-EPS decreased rapidly. With the further increase in TOC concentration, heterotrophic bacteria multiplied and the content of T-EPS rapidly increased to 49.69 mg/g VSS, indicating that the increase in the higher influent TOC concentration ($\geq$200 mg/L) was beneficial to the production of EPS on the biofilm, which was consistent with the results of previous studies (Miqueleto *et al.* 2010).

Effect of organic matter concentration on microbial community structure

The 16S rRNA gene high-throughput sequencing was used to analyze the microbial community composition of the reactor biofilm under different organic concentrations. It can be seen from Table 3 that the sequence numbers of samples A0, A50, A100, A150 and A200 ranged respectively from 56,325 to 73,830 and 3,087,728 valid sequences were obtained. The coverage index of the five samples was above 99%, suggesting that the sequencing depths for all samples were sufficient to cover the whole microbial diversity. The Chao1 index and ACE index showed that the bacterial abundance on the biofilm first decreased and then increased with the addition of organic matter concentration. The Shannon index shows that the diversity of the microbial community on the biofilm first decreased and then gradually increased with the addition of organic matter. The reason for this phenomenon was that under low organic matter concentration, autotrophic bacteria could not adapt to the environmental change and the amount of decrease was greater than the amount of increase in heterotrophic bacteria and the diversity and abundance of the microbial community gradually decreased. With excessive increase in organic matter concentration, heterotrophic bacteria began to multiply coupled with the bacterial community and diversity on the biofilm gradually increased.

Table 3 | Richness and diversity analysis of the microbial community

Sample	Sequences	Shannon	ACE	Chao1	Coverage
A0	61,001	5.218772	1,532.701	1,491.706	0.993073
A50	69,895	5.073956	1,530.674	1,556.110	0.992108
A100	56,325	5.109480	1,418.474	1,418.139	0.993121
A150	73,830	5.283771	1,584.370	1,607.262	0.991915
A200	57,677	5.515892	1,577.192	1,560.800	0.994352

At the phylum level, the microbial community showed significant changes with the increase in organic matter concentration (Figure 5). *Chloroflexi*, *Proteobacteria*, *Planctomycetes* and *Bacteroidetes* were the main predominant flora at the phylum level, indicating that the predominant flora species on the biofilm did not change after the addition of organic matter, only the relative abundance changed, which was consistent with the results of previous studies on the microbial community in anammox reactors (Chu *et al.* 2015). The nitrogen removal function bacteria of the reactor were *Proteobacteria* and *Planctomycetes*. *Planctomycetes* related to the anammox bacteria were detected in all five biofilm samples. Therefore, the change in the relative abundance of *Planctomycetes* could well reflect the survival status of anammox bacteria in the reactor. When the influent TOC concentration was 100 mg/L, its relative abundance increased from 10.01% to 16.58%. With the addition of organic matter, heterotrophic bacteria multiplied and consumed dissolved oxygen to promote the reproduction of anammox bacteria. At the same time, the abundance of *Proteobacteria* dropped from 21.06% to 17.61%. With further increase in the influent organic matter concentration, when the influent TOC concentration was maintained at 200 mg/L, the relative abundance ratios of *Proteobacteria* and *Planctomycetes* changed significantly. Denitrifying bacteria can multiply, and compete with anammox bacteria for nitrite. The *Planctomycetes* phylum was inhibited under the influence of higher organic concentrations and its relative abundance sharply decreased to 3.03%, *Proteobacteria* rapidly proliferated with the help of large amounts of organic matter and its relative abundance increased to 25.57%.

To further explore the microbial flora on the biofilm at different concentrations of organic matter, Figure 6 shows the relative abundance distribution of species at the taxonomic level of the genus on the biofilm at different concentrations of organic matter. It can be seen that *Candidatus* Brocadia and *SM1A02* accounted for the large proportion of *Planctomycetes* phylum (Liu *et al.* 2017). In this study, *Candidatus* Brocadia used NO_2^- as the energy source and CO_2 as the carbon source. It was the typical anammox strain to be enriched and identified (Kuenen & Jetten 2001), it was well enriched in the reaction and its relative abundance reached 5.69%. When the influent TOC concentration was 100 mg/L, its relative abundance increased to 7.62%. However, with the influent organic matter concentration of 200 mg/L, its relative abundance sharply decreased to 0.22%. In addition, the relative abundance of *SM1A02* bacteria maintained at 0.46–1.07% and the percentage change was small, indicating that the concentration of organic matter had little effect on the abundance of *SMIA02* bacteria.

Among *Proteobacteria*, *Denitratisoma*, *Limnobacter*, *Hydrogenophaga* and *Gemmobacter* account are dominant. Among them, the number of *Denitratisoma* and *Limnobacter* decreased with increase in organic matter concentration. When the TOC concentration was 200 mg/L, the relative abundance decreased from 11.28% and 0.68% to 3.58% and 0.53%, respectively. When the influent TOC concentration was 100 mg/L, the relative abundance of *Hydrogenophaga* and *Gemmobacter* reached the maximum, which were, respectively, 2.23% and 0.3%. *Hydrogenophaga* belonged to *β-Proteobacteria* and is a representative of autotrophic denitrifies. Studies have shown that the fermentation of organic matter by *Chloroflexi* would produce hydrogen for nitrate reduction by *Hydrogenophaga* (Speth *et al.* 2016). The increase in organic matter concentration led to the death of autotrophic bacteria, which led to fermentation enhancement and more hydrogen generation, which was used as electron donor and nitrate as electron acceptor to convert the two into nitrogen, thus improving the TN removal efficiency (Zhang *et al.* 2009). The fermentation of organic matter therefore, increased the appearance of Hydrogenophaga.

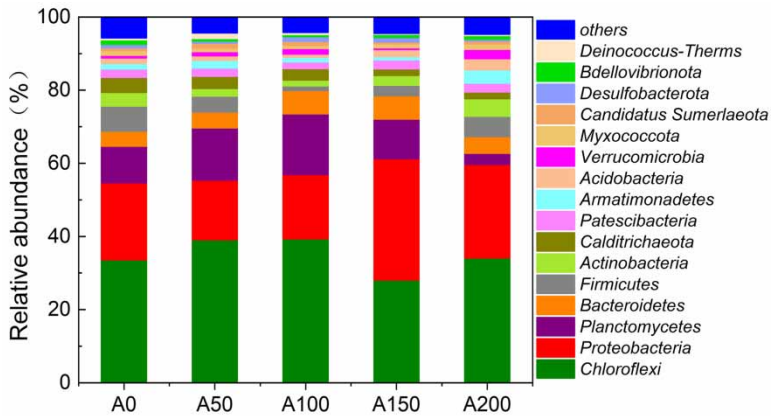


Figure 5 | The relative abundance of microbial communities at the phylum level.

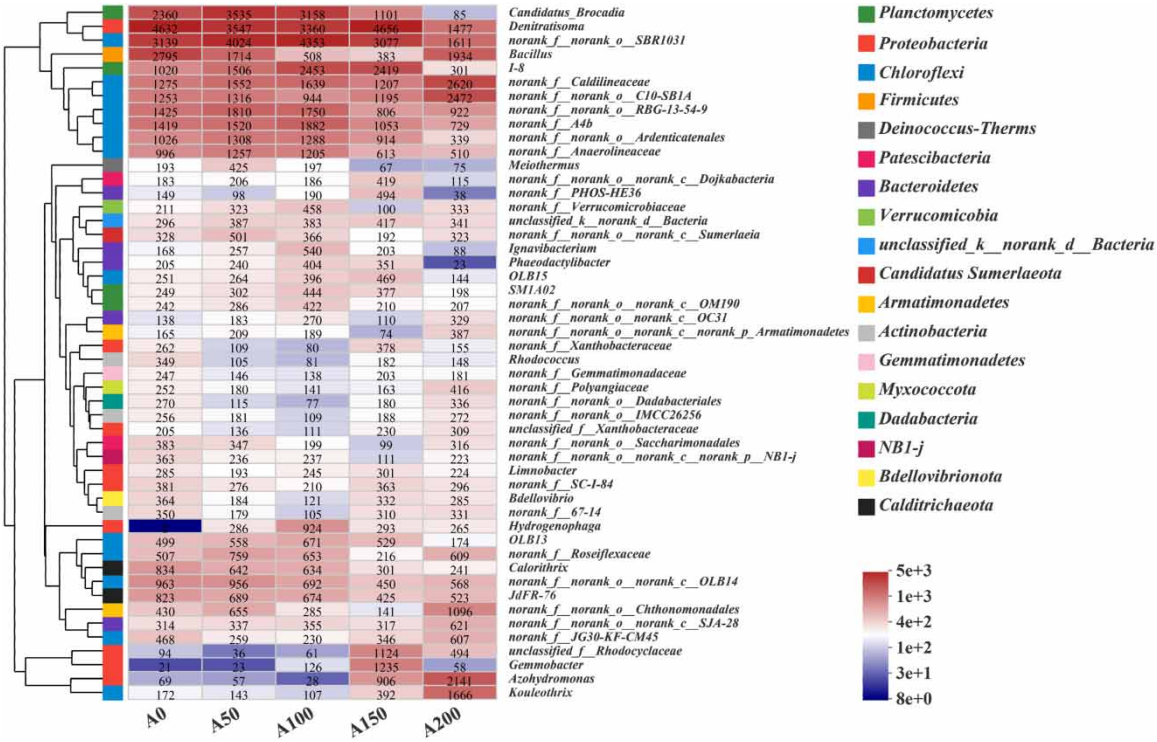


Figure 6 | Community heatmap at the genus level.

Migration and transformation of nitrogen in different concentrations of organic matter

The production of nitrate and the TN removal efficiency under different organic concentrations in the reactor further indicated the coexistence of anammox bacteria and denitrifying bacteria in the reactor. Using stoichiometric equations and experimental data, the transformation rule of nitrogen-containing compounds at different concentrations of organic matter was evaluated. Figure 7 shows the regulation of nitrogen conversion in the reactor under different organic matter addition conditions.

When the influent water did not contain organic matter, the denitrification of the reactor was mainly based on anammox. At the same time, as the influent water contained little dissolved oxygen, about 6 mg/L NH_4^+ was converted into NO_2^- through AOB (Figure 7(a)) and about 9 mg/L NO_2^- was transformed into NO_3^- through NOB (Figure 7(b)). As TOC concentration was increased from 0 mg/L to 100 mg/L, the nitrate nitrogen denitrification ($DNF_{NO_3^-}$) consumption in the reactor increased to 9.34 mg/L (Figure 7(d)). At this time, the contribution ratios of anammox and denitrification for nitrogen removal were 86.78% and 13.22%, respectively. When the TOC concentration was increased from 100 mg/L to 200 mg/L, the nitrogen removal pathway of the reactor was gradually transformed into denitrification. The denitrification contribution ratio of denitrification gradually increased to 99.59%, while the nitrogen removal contribution ratio of anammox dropped to 0.41%. The consumption of NH_4^+ and NO_2^- was mainly due to the denitrification of nitrite nitrogen ($DNF_{NO_2^-}$). This was mainly due to the fact that the growth rate of anammox bacteria was lower than that of denitrifying bacteria. The increase in the concentration of organic matter led to the proliferation of denitrifying bacteria and gradually became the dominant strain. Therefore, controlling the concentration of organic matter in the influent of the anammox reactor is the key to ensure the efficient denitrification of the anammox reactor.

CONCLUSION

In this study, biological activity and nitrogen removal performance of anammox biofilm system under difference organic concentration have been investigated. When the influent organic concentration was about 100 mg/L, the TN removal efficiency could reach 95.77%, the denitrification performance of the reactor was favorable, anammox bacteria and denitrifying bacteria formed a good synergy and the nitrogen removal contribution rate of anammox and heterotrophic denitrification respectively

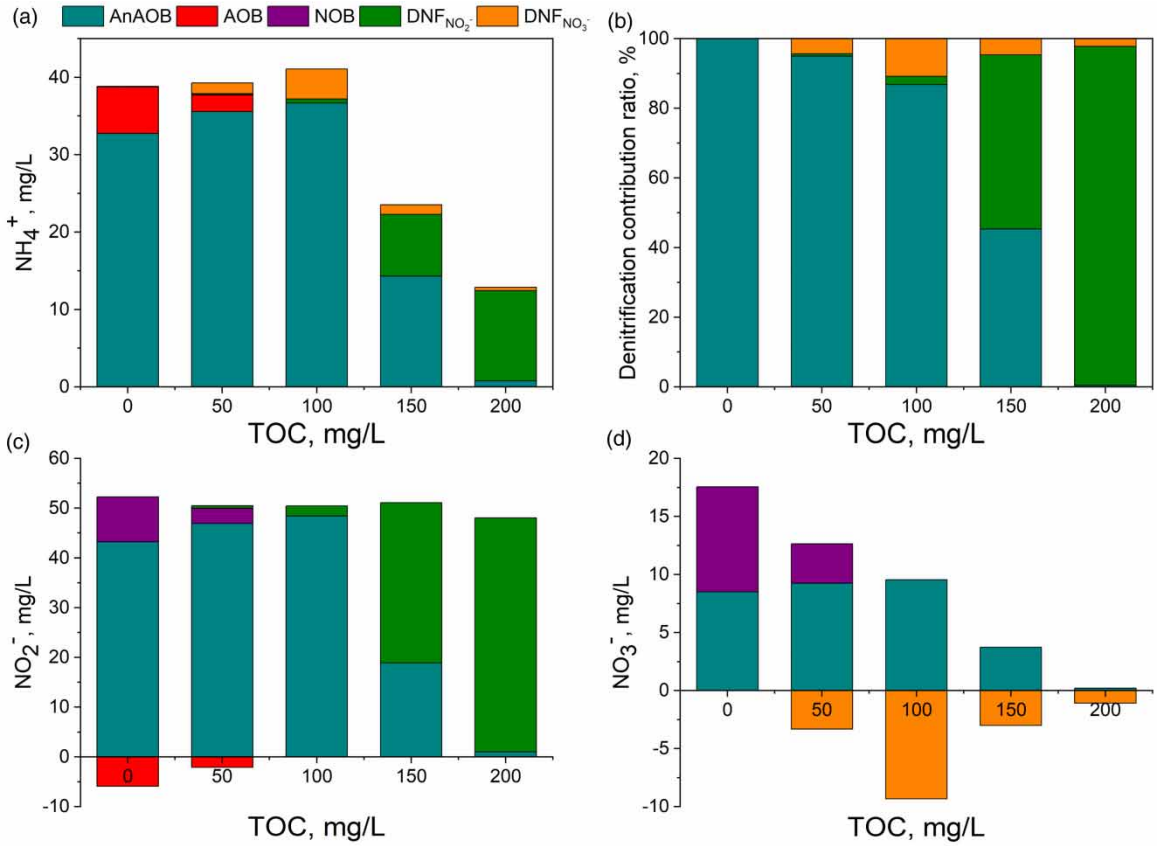


Figure 7 | Nitrogen transfer under different organic conditions (a) NH_4^+; (b) denitrification contribution ratio; (c) NO_2^-; (d) NO_3^-.

reached 86.78% and 13.22%. The further analysis of the microbial community structure showed that with the increase in the organic concentration, the relative abundance of the *Proteobacteria* increased from 21.06% to 25.57%, of which *Denitratisoma* was the genus that changed the most, the relative abundance of *Planctomycetes* phylum decreased from 10.01% to 3.03%. This study recommended a proper influent organic concentration for sustainable synergistic effect between the denitrification bacteria and anammox bacteria, which can be used for practical engineering application or enlighten further anammox process research.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support from the Research and Development Program of Guangxi, China (Grant No. AB19110001) and the Science and Technology Base and Talent Project of Guangxi, China (Grant No. AD17195092).

DECLARATION OF COMPETING INTEREST

The authors declared that they have no conflicts of interest to this work.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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First received 1 April 2021; accepted in revised form 20 June 2021. Available online 1 July 2021