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Removal of tetracycline in nitrification membrane bioreactors with different ammonia loading rates: Performance, metabolic pathway, and key contributors☆

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ABSTRACT

Membrane bioreactors (MBRs) have been widely applied for the treatment of wastewater that contains high concentrations of both ammonium and antibiotics. Nonetheless, information about tetracycline (TC) removal in nitrification MBRs with high ammonium loading rates (ALRs) is still very limited. Herein, the fate of TC at four different concentrations of 1, 5, 20, and 50 mg/L in three parallel lab-scale nitrification MBRs with different ALRs (named AN50, AN500, and AN1000) were investigated in this study. Excellent nitrification performance and high TC removal efficiency (90.46%) were achieved in AN1000 at influent TC concentration of 50 mg/L. Higher ALRs promoted the removal of TC at lower influent TC concentration (≤5 mg/L), while no significant difference was observed in TC removal efficiencies among different ALRs MBRs at higher influent TC concentration (≥20 mg/L), implying that the heterotrophic degradation could be strengthened after long-term exposure to high concentration of TC. Batch tests demonstrated that adsorption and biodegradation were the primary TC removal routes by nitrification sludge, of which both autotrophic ammonia oxidizers and heterotrophic microorganisms played an important role in the biodegradation of TC. FT-IR spectroscopy confirmed that amide groups on the sludge biomass contributed to the adsorption of TC. Mass balance analyses indicated that biodegradation (63.4–88.6% for AN50, 74.5–88.4% for AN500 and 74.4–91.4% for AN1000) was the major mechanism responsible for the removal of TC in nitrification MBRs, and its contribution increased with influent TC concentration, while only 1.1%–15.0% of TC removal was due to biosorption. TC was progressively degraded to small molecules and the presence of TC had no notable effect on membrane permeability. These jointly confirmed TC could be effectively removed via initial adsorption and subsequent biodegradation, while biodegradation was the primary mechanism in this study.

1. Introduction

Tetracyclines (TCs) are one of the most widely used broad-spectrum antibiotics in the livestock and aquaculture industries (Huang et al., 2020). A recent study showed that the average concentrations of TCs in soil and surface water in China were significantly higher than those of quinolones, macrolides, and sulfonamides (Lyu et al., 2020). A large amount of wastewater rich in NH4+-N and TC is generated in the pharmaceutical industry, and the traditional treatment methods have limited removal effect on TC (Zhang et al., 2006). In addition, animal farm wastewater was also characterized by high NH_4^+-N and TC concentrations (Zhou et al., 2013), which poses a significant risk of antibiotics resistance gene transmission (Chen et al., 2022). Therefore, it is of great importance to develop an efficient, stable, and economical

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process to control both NH_4^+-N and antibiotic pollution in response to the urgent need for ammonium-rich antibiotic wastewater treatment.

Biological treatment processes are expected to be an eco-friendly and cost-effective technique for handling antibiotic wastewater (Fischer & Majewsky, 2014), and it has been demonstrated that biodegradation and adsorption are two of the most important removal mechanisms of antibiotics during biological wastewater treatment processes (Yu et al., 2018). An extended sludge retention time (SRT) and a high sludge concentration is generally thought to be beneficial for the removal of both NH_4^+-N and antibiotics (Kumwimba & Meng, 2019). Membrane bioreactors (MBRs) can achieve complete sludge retention for attaining high sludge concentrations and long SRTs by effective biomass-effluent separation with membrane modules, providing sufficient time for the growth of slow-growing microorganisms like ammonia oxidizing bacteria (AOB) (Shen et al., 2014) and contributing to the development of specialized microbial species capable of decomposing compounds (such as antibiotics) of lower biodegradability (Shao et al., 2019; Shi et al., 2021). The combination of these factors suggests that MBRs may be more advantageous in terms of high NH_4^+-N antibiotic wastewater treatment.

In addition, a large number of studies have been reported that AOB were capable of improving the degradation of a wide range of toxic or refractory organic pollutants (Joss et al., 2006; Park et al., 2017). For example, Park et al. (2017) performed batch tests using activated sludge from a MBR and demonstrated AOB make a huge contribution to the removal of a variety of micropollutants. Much evidence confirmed the contributions of AOB to the biodegradation of TC, and suggested that ammonia monooxygenase (AMO), a key enzyme of AOB catalyzing the first step of ammonia oxidation to nitrite, played a pivotal role in the co-metabolism of TC (Shi et al., 2011; Wang et al., 2021; Yang et al., 2023). In our previous studies, we found that the abundance of AOB and the ammonia oxidation activity of sludge in MBR increased with the elevation of NH_4^+-N loading rates (ALRs) within a certain range (Wang et al., 2016; Xu et al., 2022). Thus, the highly activated nitrifying sludge in MBR with high ALRs is expected to play a more significant role in enhancing TC removal. Although MBRs have been widely adopted to treat antibiotic pharmaceutical wastewater (Hou et al., 2016; Xiao et al., 2019), and been confirmed to be able to efficiently remove NH_4^+-N and TC simultaneously (Sheng et al., 2018), the influent NH_4^+-N concentration in most studies was generally lower than that of 300 mg/L (Sheng et al., 2018), information about TC removal in MBRs with high ALRs is still very limited. Meanwhile, the presence of high TC concentrations in wastewater may affect the nitrification process. Katipoglu-Yazan et al. (2015) found that 50 mg/L of TC caused the nitrifying bacteria to be phased out, eventually leading to complete inhibition of the nitrification capacity of the sludge. Thus, the effects of high concentrations of TC on the performance of MBR with high ALRs should also be studied.

In this study, three parallel lab-scale nitrification MBRs with different ALRs were operated (named AN50, AN500, and AN1000) with the increasing TC dosage (1 mg/L-50 mg/L). The TC removal efficiencies, removal routes and metabolic pathway in MBRs were determined. The influences of TC, in turn, on the nitrification performances of MBRs were also evaluated. This study demonstrated the potential of MBR in the treatment of wastewater with high concentrations of TC and ammonia nitrogen.

2. Materials and methods

2.1. Experiment setup and operating conditions

Three sets of lab-scale MBRs were operated, all with an effective volume of 2 L and a hydraulic retention time of 14 h. The membrane modules were made of PVDF hollow fiber membrane with a pore size of 0.05 μm and a membrane flux of 7.5 L/(m^2·h). The influent and effluent of the MBRs were realized by peristaltic pumps. Pressure sensors were installed between the membrane modules and effluent pumps to record the variation in transmembrane pressure. Aeration was performed with blower pumps connected to the aeration head to maintain dissolved oxygen at 3–4 mg/L, which was measured daily using a portable dissolved oxygen meter. The reactors were operated at 25 ± 5 °C and the pH of the mixture was maintained at 7–8. The influent water was prepared as in our previous study except that no organic carbon source (glucose) was injected (Xu et al., 2022). The three MBRs, named AN50, AN500, and AN1000 (AN stands for ammonia nitrogen), operated stably for a long period at influent NH_4^+-N concentrations of 50, 500, and 1000 mg/L, respectively. The remaining operating conditions for the three MBRs with different NH_4^+-N loads were maintained constant, and the experimentally required concentration of TC was added during the preparation of influent water. In this study, the influent TC concentrations were 1, 5, 20, and 50 mg/L in ascending order from low to high. Each stage lasted at least 40 days to ensure that the reactors reached a stable state. No additional sludge was discharged during the operation except for taking it for experimental use. When the transmembrane pressure was greater than 15 kPa, membrane cleaning was performed following Jang et al. (2013).

2.2. Analytical methods

Standard methods were used to determine MLSS, MLVSS, NO_2^--N, and NO_3^--N (APHA et al., 2012). To exclude the interference of TC, an improved salicylic acid method was used instead of Nessler's reagent method to determine NH_4^+-N (Ma et al., 2019). The adsorption assay and FT-IR spectroscopy were performed according to the methods of Chen et al. (2012) and Feng et al. (2018), respectively. All reagents used in this study were purchased from Shanghai Titan Technology Co., Ltd., and the purity was above analytical grade.

2.3. High-Performance Liquid Chromatography and UPLC-QTOF-MS

TC was detected by High-Performance Liquid Chromatography (HPLC) using a C18 reversed-phase column with the following mobile phase ratio: methanol: 0.08% orthophosphoric acid aqueous solution: acetonitrile = 20%: 60%: 20% at a flow rate of 1 mL/min and a detection wavelength of 355 nm. The metabolic intermediates of TC in MBR were detected by UPLC-QTOF-MS according to the method of Xie et al. (2018). Briefly, separation was performed on a C18 reverse-phase column, and the injection volume was 20 μL. Mobile phase was consisted by 80% H_2O containing 0.2% formic acid and 20% acetonitrile with a flow rate of 0.2 mL/min. The wavelength of single online detection was set as 355 nm. The mass spectrometer analysis was operated in positive ionization mode over the range of m/z 100–600 with an electrospray ionization (ESI) source.

2.4. Batch experiments

Prior to the injection of TC into the influent of the MBRs, batch experiments were performed to examine the ammonia oxidation rate of nitrifying sludge at different initial TC concentrations by taking sludge from the reactor, and the experimental method was referred to Luo et al. (2019). AN50 sludge was used to measure the ammonia oxidation rate. Batch tests were also conducted for the different fates of TC in nitrifying sludge (including sludge adsorption, heterotrophic metabolism, and nitrifying cometabolism). The basic conditions of the reaction system and the pre-treatment process of the sludge were the same. To avoid the inhibition of sludge activity by the initial concentration of NH_4^+-N or COD, peristaltic pumps were used to supply the system with the appropriate load of NH_4^+-N or COD. The pump flow rate was set to 0.2 mL/min to avoid excessive volume changes in the reaction system. A total of seven sets of experiments were conducted under the following conditions: (1) control group, pumped with pure water; (2) pumped with NH_4Cl and $NaHCO_3$ solution so that the NH_4^+-N load was 0.1 mg NH_4^+-N/(gMLSS·min); (3) pumped with NH_4Cl and $NaHCO_3$ solution so

that the NH_4^+-N load was 0.2 mg NH_4^+-N/(gMLSS·min); (4) pumped with pure water. The sludge mixture was pre-loaded with 0.1% ProClin™ 950 bio-inactivator; (5) pumped with pure water. The sludge mixture was pre-loaded with 20 mg/L allylthiourea (ATU); (6) pumped with glucose solution so that the COD load was 0.1 mg COD/(gMLSS·min). The sludge mixture was pre-loaded with 20 mg/L ATU; (7) without sludge injection, the NH_4Cl and $NaHCO_3$ solution used in the group (3) was pumped. Since the reaction volume varied with the pumped liquid, the TC concentrations were calculated according to the volume of the sludge mixture at the sampling time. AN1000 sludge was used to perform TC removal experiments.

2.5. Extraction and quantification of extracellular polymers

The extraction and quantification of extracellular polymers (EPS) from sludge was performed in parallel with the determination of sludge concentration as follows: The sludge mixture was shaken well and two identical portions of 25 mL each were taken, one of which was used to measure the sludge concentration and the measured MLSS was recorded in g/L. The EPS of another sludge was extracted according to the method of Meng et al. (2012) and the protein and polysaccharide concentrations were measured in mg/L, and the EPS content per unit mass of activated sludge in mg/gMLSS was obtained by dividing the result by the MLSS concentration measured above.

2.6. Statistical analysis

The statistical method of two-factor repeated ANOVA was used to analyze some of the data in this study, and the statistical analysis was done by the software SPSS 26, $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Tetracycline removal, removal routes and metabolic pathway

3.1.1. Tetracycline removal

The other operating conditions of the three MBRs with different NH_4^+-N loads were maintained constant, and the TC concentration in the influent was gradually increased in stages, in the order of 1, 5, 20, and 50 mg/L. The TC concentration in the effluent during operation is recorded in Fig. 1(A), and the removal rate statistics are shown in Fig. 1 (B). At an influent TC concentration of 1 mg/L, the removal rate of AN50 was close to 90% in the first week and then decreased to about 80%, which may be related to the saturation of AN50 sludge on TC after a week. The average TC removal in both AN500 and AN1000 at this stage was above 90%, and such high efficiencies were likely driven by the higher ammonia loads. After the influent TC was raised to 5 mg/L, the removal rates of AN50, AN500, and AN1000 fluctuated and decreased to some extent, with averages of 70.89%, 85.91%, and 82.54%, respectively. AN500 and AN1000 were still signally higher than AN50, but AN1000 was slightly lower than AN500, presumably because the saturation adsorption capacity of AN1000 sludge for TC was lower than that of AN500.

The influent TC was further increased to 20 and even 50 mg/L. Although the removal rate of the three MBRs remained relatively high, a certain degree of TC accumulation was observed due to the leap in influent concentration. It can be found that the removal rates of TC by the three MBRs tend to be close in these two stages. A two-factor repeated ANOVA was performed on the TC removal rates of the three MBRs at these two stages using the software SPSS 26. The two factors, i.e., influent NH_4^+-N and TC concentrations were taken at three levels of 50, 500, and 1000 mg/L for NH_4^+-N and two levels of 20 and 50 mg/L for TC. The results obtained from the ANOVA were as follows: p of NH_4^+-N = $0.181 > 0.05$, p of TC = $0.000 < 0.05$, p of NH_4^+-N*TC = $0.080 > 0.05$. This statistical result indicated that there was no statistical difference in the removal rate of TC by MBRs with three different NH_4^+-N loads for influent TC concentrations of 20 and 50 mg/L.

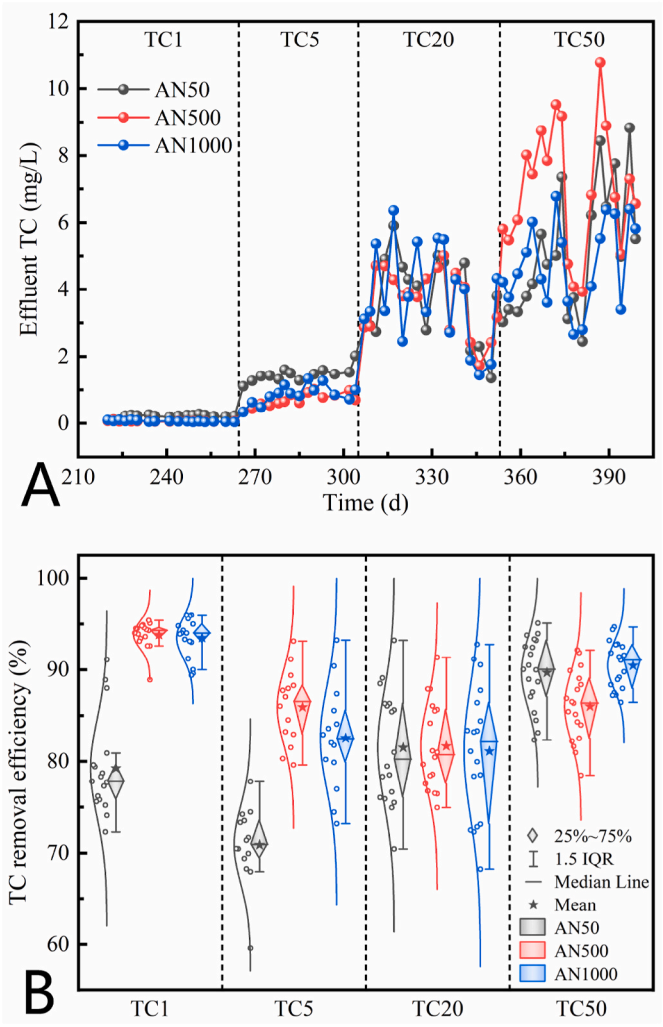

Fig. 1. Record and statistics of effluent from the MBRs.

The reason for this result may be that the selective effect of high concentrations of TC on the heterotrophic metabolism functional flora led to the growing heterotrophic degradation of TC in nitrifying sludge, and the percentage of contribution of heterotrophic metabolism to the removal of TC was greatly increased, which in turn leveled the gap in the ability of the three MBRs with different NH_4^+-N loads to remove TC by ammonia oxidation cometabolism. Batch tests for the efficiency of heterotrophic metabolism were performed by adding ATU to inhibit AOB by taking sludge from three reactors at the end of MBR operation. It was found that AN50 sludge had the highest heterotrophic removal efficiency for the same sludge concentration, while AN1000 was significantly lower than AN50 and AN500, which may be because the relative abundance of heterotrophs in high ammonia-loaded sludge was lower than that in low ammonia-loaded sludge.

3.1.2. Contributions of biodegradation and biosorption to tetracycline removal

To investigate the contribution of biosorption and biodegradation to the removal of TC, unit sludge adsorption amounts at the end of each stage was examined (Fig. 2(A)). The adsorption of TC per unit mass of sludge increased with influent TC concentrations, in accordance with the equilibrium thermodynamics. The unit sludge adsorption capacity of AN50 was greater than that of AN500, and AN1000 was the smallest. Cheng et al. (2008) pointed out that due to the negative charge on the

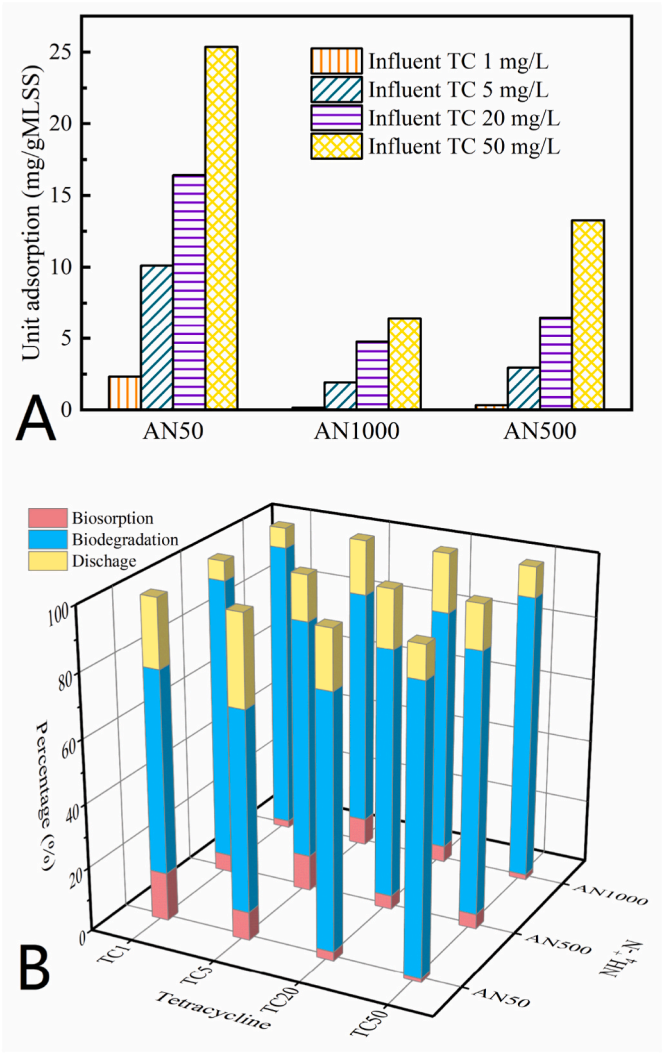

Fig. 2. Distribution of tetracycline removal: (A) Unit adsorption amounts in the end of each stage; (B) Contributions of biodegradation and biosorption to tetracycline removal.

sludge surface, cations such as H^+, Na^+, and Ca^{2+} present in an atmosphere with low pH or high ionic strength will compete with the contaminants for the adsorption sites on the sludge surface thus causing a decrease in adsorption. To provide the alkalinity required for nitrification, $NaHCO_3$ in the influent was elevated proportionally with NH_4^+-N, so the difference in ionic strength was responsible for the different adsorption per unit sludge mass in the three MBRs.

The contribution of biosorption and biodegradation to the removal of TC at each stage was calculated according to the mass balance equation (Liu et al., 2018):

$$TC_{in} = TC_{sor} + TC_{deg} + TC_{out} \quad (1)$$

$$TC_{sor} = \left(C_{s,end} - C_{s,beg}\right) \times M + C_{s,end} \times \Delta M \quad (2)$$

$$TC_{in} = Q \times \int C_{w,in} dT \quad (3)$$

$$TC_{out} = Q \times \int C_{w,out} dT \quad (4)$$

where TC_{in} is influent TC, TC_{sor} is the amount of TC biosorbed onto sludge, TC_{deg} is the amount of TC biodegraded and TC_{out} is the amount of TC in effluent. $C_{s,beg}$ and $C_{s,end}$ are the amounts of TC biosorbed by per unit biomass at the beginning and the end of each stage, respectively. M is the amount of biomass at the end of each stage and ΔM is the biomass grown at each stage. $C_{w,in}$ and $C_{w,out}$ are the TC concentrations in the influent and effluent, respectively. T is the running time, Q is the flow rate of the MBRs, which in this experiment was 3.43 L/d. The integral calculations were done with the software Origin 2021 based on the TC concentration profiles of the influent and effluent.

As shown in Fig. 2(B), most of the TC could be removed by biodegradation in long-term tests, with a small percentage of biosorption. It can be found that the percentage of biosorption decreased with increasing influent concentration, while that of biodegradation increased accordingly, especially for AN50. This evidence also reaffirmed that the ability of sludge to biodegrade TC can be domesticated. Early studies concluded that TC was usually difficult to be biodegraded (Kim et al., 2005), but as research progressed, scholars believed that longer SRT and a gradual acclimation strategy could effectively enhance the biodegradation of TC (Liu et al., 2018), which is consistent with what was observed in this study. It was also confirmed from the gene expression level that some rare microbial taxa that play a crucial role in the biodegradation of antibiotics are enriched under long SRT (Vuono et al., 2016).

The results of batch experiments for the removal of TC by nitrifying sludge are shown in Fig. 3. It is easy to find that the aqueous solution of TC was stable when the sludge was absent. While after sludge dosing, the TC concentration in the aqueous phase decreased rapidly in the first 15–30 min, which was mainly caused by sludge adsorption. Sheng et al. (2018) also believed that sludge adsorption was the main reason for the removal of TC from the aqueous phase. It can be seen from the inactivation group that the adsorption equilibrium was reached after about 5 h. The best removal of TC was observed in the two groups with NH_4^+-N injection, and the removal rate was faster in the group with higher NH_4^+-N load, indicating that the enhancement of NH_4^+-N load in a certain range improved the removal of TC, which may be degraded through the ammonia oxidation cometabolic pathway (Wang et al., 2021; Yang et al., 2023). The only sludge group was close to the group that was dosed with AMO inhibitor ATU, suggesting that the AMO may not be activated when the substrate (NH_4^+-N) was lacking so that TC can not be effectively removed. This feature also coincided with the cometabolic pathway, further validating the possibility that TC can be biodegraded by cometabolism, an inference that was consistent with Shi et al. (2011). However, Sheng et al. (2018) found that nitrosifying sludge was the least effective in removing TC at an initial NH_4^+-N concentration of 200 mg/L. It was speculated that the reason might be related to the high initial

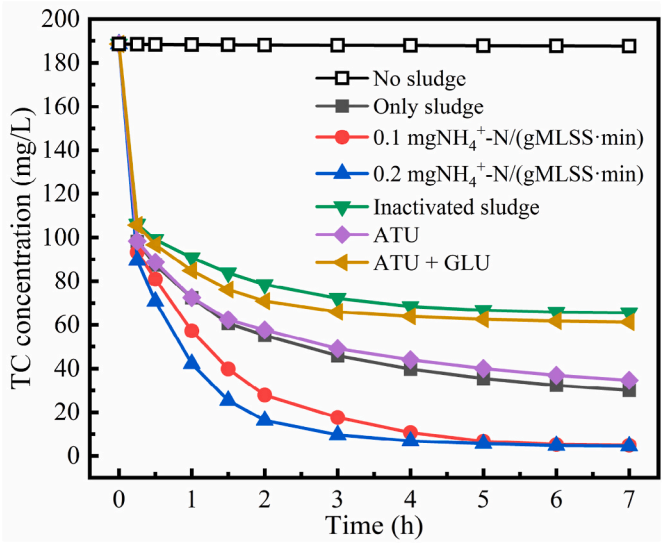

Fig. 3. Removal curves of tetracycline by nitrifying sludge under different conditions.

NH_4^+-N concentration, which produced free ammonia that inhibited the ammonia oxidation activity (Xu et al., 2022).

The removal effect of both the only sludge and ATU groups was better than that of the inactivated group, suggesting that the metabolism of heterotrophic bacteria may also contribute to the degradation of TC, as verified by glucose (GLU) injection. The removal of TC in the group with glucose was close to that of the inactivated group and inferior to the others. This was because the glucose was an easily metabolizable substance that was preferentially utilized by heterotrophs thus affecting the heterotrophic metabolism of TC. Readily metabolizable organics may cause competitive inhibition of heterotrophic metabolism of refractory organics, for example, Donoso-Bravo et al. (2009) also found that an anaerobic sequencing batch reactor with phenol as the sole carbon source was better for phenol removal than other reactors with additional glucose dosing.

3.1.3. Transformation pathways and products

TC degradation intermediates were detected by UPLC-QTOF-MS. Based on the obtained mass spectral information, the presumed conversion pathway of TC in MBR is shown in Fig. 4. A total of 6 possible intermediates were detected. The first reacting group of TC was the dimethylamino on the A-ring, and the conversion was by two reaction pathways, demethylation, and methyl oxidation, forming products TP415 and TP475, respectively. Demethylation of the dimethylamino on the A-ring was found in several studies to be the prevalent first step in the biodegradation pathways of TC (Shao et al., 2019; Tan et al., 2021), consistent with that observed in the present study. This reaction is of great importance in the bacterial biodegradation of TC because the bacterial inhibitory effect of TC is completely lost when the methyl group of dimethylamino is replaced or eliminated (Fuoco, 2012). The dehydration of the tertiary alcoholic hydroxyl group on the C-ring to form a C=C bond also occurred during the formation of TP415. Further, the hydroxyl and amide groups on the A-ring of TP415 were removed to form TP340, a process similar to that previously found by Tan et al. (2021). The A-ring of TP340 was then opened to further hydrolyze to form TP227.

Similar to the findings of Zhao et al. (2020) the benzene ring of TP227 was opened and the side ring was removed to form TP167, which in turn was stripped of a hydroxyl group to form TP151. The mass spectra (Fig. S1) showed that the relative peak height of TP227 was notablely higher than the other products, indicating a possible accumulation of TP227. This phenomenon implies that the biotransformation of TC occurs more by the removal or oxidation of side chain groups. The ring structure of organic compounds usually has high bond energy, and it is not easy to degrade by microbial metabolism for ring opening (Chen et al., 2018). In the presence of bacteria, TC can be biodegraded via demethylation, dehydroxylation, deacylation, deamination, ring-opening hydrolysis, etc., and usually, the antibacterial activity of these transformation products was much lower than that of the TC itself (Shao et al., 2019). By comparison, in some advanced oxidation processes, such as ozone or electrochemical oxidation of TC, intermediates were formed that had higher biological toxicity than the parent (Wu et al., 2012; Wang et al., 2018). From this perspective, the superiority of the biodegradation method for the treatment of TC wastewater was also further illustrated.

3.2. Effects of tetracycline on MBR performance

3.2.1. Effects of TC on nitrification performance

The ammonia oxidation rates under the influence of initial TC concentrations of 0, 1, 10, 50, 100, and 200 mg/L were tested, respectively. An additional control group was set up with the dosage of 20 mg/L ATU

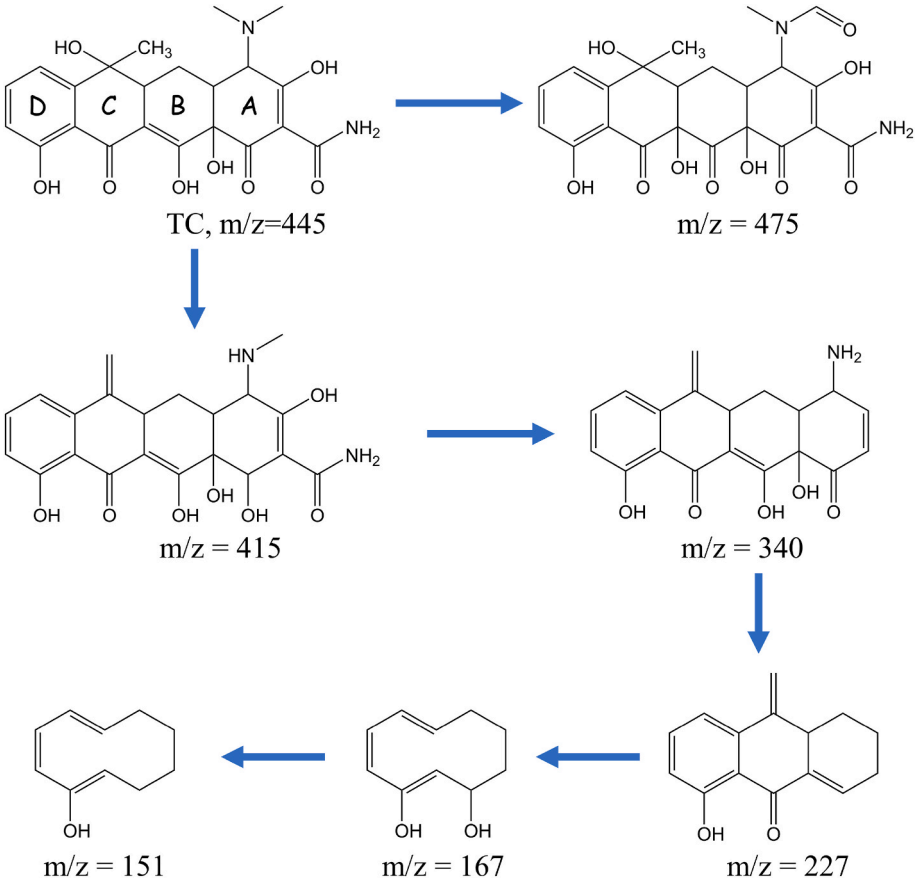

Fig. 4. Intermediate products and transformation pathway of tetracycline in MBR.

as an ammonia oxidation inhibitor. As shown in Fig. 5(A), the ammonia oxidation function of sludge was completely lost under the inhibition of ATU. This control group also ruled out the possibile loss of NH_4^+-N through other ways, proved that the ammonia oxidation of sludge completely contributed to the loss of NH_4^+-N in the reaction system. The presence of TC at different concentrations exhibited different effects on ammonia oxidation. For example, when the concentration of TC was less than 10 mg/L, the ammonia oxidation rate (2.01–2.03 mg/(gMLSS·h)) was slightly higher than that of the blank group (1.74 mg/(gMLSS·h)), indicating that a low concentration of TC may improve the nitrification activity of sludge. It was hypothesized that the phenomenon was related to the hormesis effect of antibiotics, i.e., bacteria exhibit a hormetic response to increase their resilience when exposed to antibiotic sub inhibitory concentrations (Iavicoli et al., 2021). When the concentration of TC was higher than 50 mg/L, the ammonia oxidation rate of sludge was decreased, but still maintained at 1.60 mg/(gMLSS·h) even if it reached 200 mg/L. This result proved that nitrifying sludge had strong tolerance to TC in short-term exposure, which also reflected the potential of nitrifying sludge in the treatment of TC-containing wastewater.

As for the continuous flow tests, it can be seen that the nitrification performance of the three reactors was not affected at all during the whole operation, as the ammonia (Fig. 5(C)) and nitrite (Fig. 5(D)) in the effluent always tended to be close to zero. Occasional transient elevation of nitrite was due to the failure of the aeration system, which can be quickly recovered after troubleshooting. This is a large gap from some previous studies. Katipoglu-Yazan et al. (2015) found that direct injection of 50 mg/L of TC in a sequencing batch reactor decreased the abundance of AOB and the expression level of the *amo*A gene to converge to zero within one month, and eventually the nitrification function of the system was completely inhibited. Conversely, it has also been reported that progressively increasing the TC concentration in the influent of a partial nitrification reactor, eventually reaching even 150 mg/L did not affect the ammonia oxidation performance (Xu et al., 2019). Combined with the above studies, it was hypothesized that the gradual transition from low to high concentrations would allow the nitrifying sludge to gradually adapt and thus maintain nitrification activity under the stress of high TC contents.

3.2.2. *Biomass and EPS at different ammonia and tetracycline loads*

Sludge concentration is an important factor affecting the treatment efficiency of the reactor. High biomass can make the reactor more resistant to shock loading and more advantageous in treating toxic organic matter such as antibiotics (Li & Zhang, 2010). The variation of sludge concentration in the three MBRs is shown in Fig. S2(A). The sludge concentration increased significantly for AN500 and AN1000, with MLSS exceeding 18000 mg/L and MLVSS/MLSS ratio above 80% for AN500 at the end of the operation, and exceeding 22000 mg/L and 90% for AN1000, respectively. The sludge concentration in AN50 was stable between 4000 and 5000 mg/L due to low NH_4^+-N load and scarcity of growth substrate, but the ratio of MLVSS was also increasing gradually with influent TC concentration, indicating good cell yield. The above phenomenon implied that TC was likely to act as an organic carbon source to promote the growth and reproduction of heterotrophs. The ability to maintain high sludge concentrations is one of the advantages of MBRs compared to other types of high-load reactors that may suffer from sludge loss due to increased aeration (Chai et al., 2015). Although the unit sludge adsorption capacity of AN500 and AN1000 was low, the very high sludge concentration made sure for the total adsorption of TC.

The components of EPS include proteins, polysaccharides, and other biomolecules, which can account for 50%–80% of the total biomass and play an important role in promoting microbial aggregation and resisting adverse external conditions (Shi et al., 2017). The detection of EPS content is shown in Fig. S2(B), and it is easy to see that the higher the NH_4^+-N load the higher the EPS content of the sludge. Many studies have also found that activated sludge under high salinity, ammonia, or antibiotic stress produces more EPS with a higher content of protein fractions (Meng et al., 2012; Shen et al., 2014). The EPS content of the sludge in the three MBRs did not change significantly in the pre-operational period, suggesting that low TC concentration did not cause toxic or inhibitory effects on the sludge (Zhao et al., 2023). In the two stages of 20 and 50 mg/L, it can even be observed that there were varying degrees of decline; and the protein/polysaccharide ratios of AN500 and AN1000 sludge showed an overall decreasing trend except for a significant increase in the early stage when the TC jumped to 20

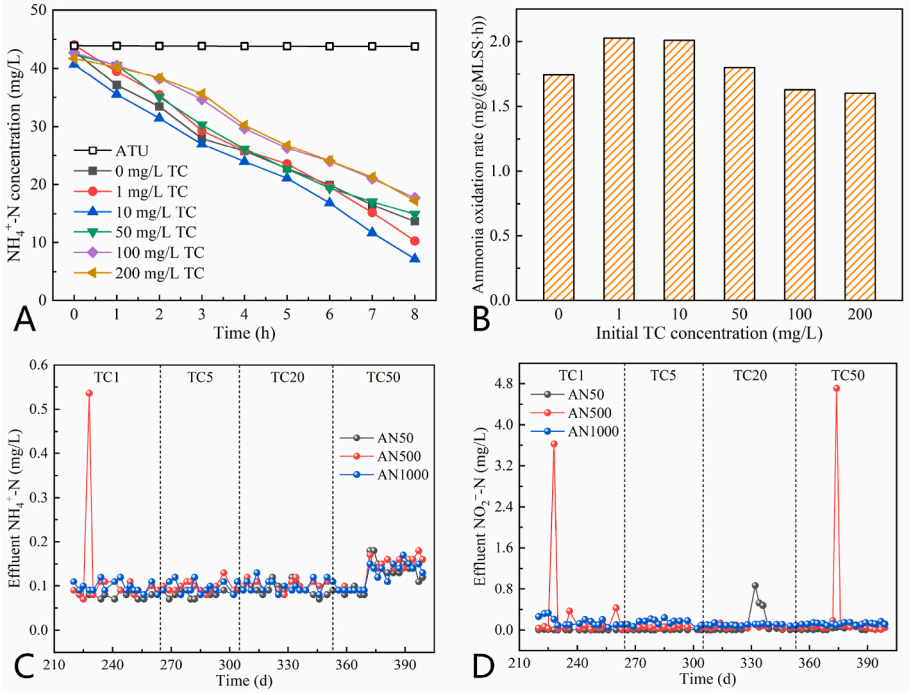

Fig. 5. Effects of TC on nitrification performance: (A) Ammonia oxidation curves; (B) Ammonia oxidation rates calculated from the curves; (C) NH_4^+-N concentrations in the effluent of the MBRs; (D) NO_2^--N concentrations in the effluent of the MBRs.

mg/L. This seems to demonstrate that even high concentrations of TC did not have a significant effect on the sludge; on the contrary, the sludge adapted well to the incoming TC, and even the stressing effect of high NH_4^+-N was diminished by the later stages of the operation.

Protein-like substances in EPS play roles in promoting cell aggregation, adsorbing nutrients, bridging enzymes and substrates, and building cell barriers, which are key factors affecting sludge flocculation and adsorption (More et al., 2014), explaining that more protein-like EPS are produced when sludge is stressed by environmental factors. In addition, proteins are more likely to be adsorbed by membrane pores than polysaccharides or humic acids, thus causing more membrane fouling (Li et al., 2015). Daily monitoring of the transmembrane pressure (TMP) (Fig. S3) showed that the TMP of AN50 hardly increased, indicating that the membrane fouling problem of MBR can be effectively controlled at a low NH_4^+-N load. However, the membrane fouling rate of AN1000 was much higher than that of both AN500 and AN50, requiring frequent cleaning to restore the membrane flux, making the TMP cyclical in general. No significant changes in membrane fouling were observed for all three MBRs at different concentrations of influent TC, implying that NH_4^+-N was still the main factor influencing the membrane fouling rate and TC had little to do with it. This also coincided with the trend of EPS variation.

Considering that sludge adsorption is an important pathway for antibiotic removal, the sludge before and after the adsorption of TC was characterized by FT-IR spectroscopy as shown in Fig. 6(A). As seen the following absorption peaks: 3419 cm^{-1} and 3415 cm^{-1} may be the O–H stretching vibration of the hydroxyl group or N–H stretching vibration of amide, 1650 cm^{-1} and 1643 cm^{-1} may be the C═O stretching vibration of amide or ketone, 1549 cm^{-1} and 1558 cm^{-1} may be the N–H bending vibration or N–H stretching vibration of secondary amine, 1454 cm^{-1} may be the backbone vibration of the benzene ring, 1232 cm^{-1} may be the C–H bending vibration of a benzene ring or C–C stretching vibration of ketone, 1032 cm^{-1} may be the C–O stretching vibration of alcohol or phenol or N–H bending vibration of a primary amine. The C═O and N–H absorption peaks of amide showed more obvious shifts or intensity changes after the adsorption of TC, indicating that the amide bond may be the main site for the adsorption of TC. And the newly appeared peaks 1454 cm^{-1} and 1232 cm^{-1} after adsorption might be from TC and its intermediate products.

The amide I region at wave numbers from 1600 to 1700 cm^{-1} was further analyzed to obtain information on the secondary structure of proteins on the sludge surface. The subpeaks after split peak fitting are shown in Fig. 6(B). The subpeaks at different wave numbers corresponded to different secondary structures, and the relative contents of various secondary structures were calculated based on the percentage of the subpeak area to the total area (Feng et al., 2018), and the results were tallied in Table S1. The α-helix is the key structure in the protein secondary structure to promote protein flocculation (Yuan et al., 2011), while the antiparallel β-sheet and random coil will play the opposite role (Badireddy et al., 2010). The percentage of α-helix of the sludge without TC adsorption was 21.82%, and the sum of the antiparallel β-sheet and the random coil was 27.25%; after TC adsorption, it was 21.33% and 27.60%, respectively. The α-helix/(antiparallel β-sheet + random coil) ratio of the sludge without TC adsorption was 0.80, while the value was 0.77 after adsorption, which was reduced, indicating that the flocculation of proteins on the sludge surface may be reduced to some extent after TC adsorption, thus affecting the cohesiveness and sedimentation of the sludge. However, the magnitude of the observed changes here was too small to draw a convincing conclusion. Overall the relative percentages of protein secondary structures changed little before and after adsorption, leading to the belief that TC had no significant effect on them.

4. Conclusions

The removal efficiency of TC by MBRs with different ALRs and the interaction between TC and nitrification sludge were investigated. The results confirmed that three MBRs with different ALRs (AN50, AN500, and AN1000) could achieve efficient simultaneous removal of ammonia and TC when the influent TC concentration increased in a gradient of 1, 5, 20, 50 mg/L. AN500 and AN1000 exhibited higher removal rates than AN50 at low concentrations (≤5 mg/L). In contrast, when raised to 20 and even 50 mg/L, there was no statistical difference in the removal of TC by the three MBRs (all mean values were above 80%), which the batch tests verified was due to enhanced heterotrophic degradation. The removal of TC from nitrification sludge by biosorption and biodegradation was also confirmed by batch tests, where both AOB-mediated co-metabolism and heterotrophs-mediated heterotrophic metabolism played an important role in biodegradation. As for sludge adsorption, FT-IR spectra showed that the amide bond was the main adsorption site,

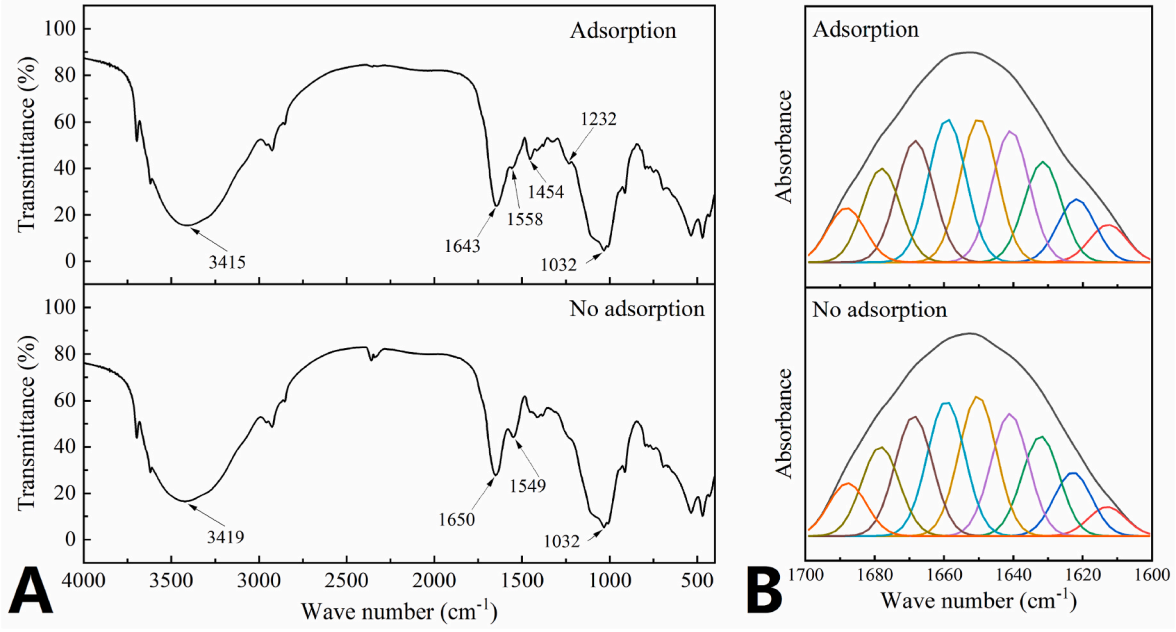

Fig. 6. FT-IR spectrum of sludge: (A) Full-band scanning spectrum; (B) The curves fitted to the amide I region corresponding protein secondary structures.

but TC had no significant effect on the protein secondary structure. The mass balance revealed that most of the TC (63.4–88.6% for AN50, 74.5–88.4% for AN500 and 74.4–91.4% for AN1000) was degraded and only a small fraction was adsorbed in the sludge. TC can be gradually biodegraded to small organic molecules by demethylation, dehydroxylation, deacylamino, deamino, ring-opening hydrolysis, etc. This study demonstrated the great potential of nitrifying MBRs for the treatment of TC-containing wastewater, and in addition implied the importance of screening efforts for heterotrophic degrading functional bacteria.

Credit authors statement

Huaihao Xu: Methodology, Data curation, analysis, preparing original draft; Yuepeng Deng: Methodology, analysis; Mingji Li: Analysis; Kaoming Zhang: Analysis, Data curation; Jie Zou: Data curation; Yunhua Yang: Reviewing and editing; Peng Shi: Reviewing and editing; Yiping Feng: Reviewing and editing; Chun Hu: Resources, reviewing and editing; Zhu Wang: Resources, analysis, Methodology, reviewing and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121922.

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