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Influence of C/N ratio on MBBR denitrification for advanced nitrogen removal of wastewater treatment plant effluent

Quan Yuan[a,b], Hai-yan Wang[a, b,*], Zhao-sheng Chu[a,d], Qian-yu Hang[a,b], Kai Liu[a,b,c], Chun-mei Li[a, b]

[a]*State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, No. 8 Da Yang Fang, Anwai, Chaoyang District, Beijing 100012, China, Tel. +86 10 84915322, Fax +86 10 84915322, email: yuanquan1990@hotmail.com (Q. Yuan), wanghy@craes.org.cn (H.-Y. Wang), chuzs@craes.org.cn (Z.-S. Chu), 397204795@qq.com (QY. Hang), 470012574@qq.com (K. Liu), 554389675@qq.com (C.-M. Li)*

[b]*Research Center for Water Pollution Control Technology, Chinese Research Academy of Environmental Sciences, No. 8 Da Yang Fang, Anwai, Chaoyang District, Beijing 100012, China*

[c]*Henan Polytechnic University, Jiaozuo 454150, China*

[d]*Research Center of Lake Eco-Environments, Chinese Research Academy of Environmental Sciences, No. 8 Da Yang Fang, Anwai, Chaoyang District, Beijing 100012, China;*

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ABSTRACT

High total nitrogen (TN) content is an urgent issue for the reuse of wastewater treatment plant (WWTP) effluent, and moving bed biofilm reactor (MBBR) is preferable for advanced denitrification of WWTP effluent. The influence of COD/NO_3^--N (hereinafter referred to as C/N) ratio on nitrate removal was studied. The results showed that the denitrification rate was influenced by influent NO_3^--N concentration, and the maximum value (13.9 ± 2.7 mg NO_3^--N·(L·d)$^{-1}$) was achieved for 4.6 C/N ratio; dissimilatory nitrate reduction to ammonium (DNRA) might be occurred under higher C/N ratios (8.0 and 8.4), and reduced the TN removal rate because of NH_4^+-N accumulation. Dissolved organic matter (DOM) concentration of effluent was lower than that of influent, except for 6.7 C/N ratio. 8.0 C/N ratio had the lowest community structure diversity and evenness of denitrification nosZ gene, and the narG and nosZ gene content of 4.6 C/N ratio was the highest. Overall, 4.6 C/N ratio was the optimum choice for nitrogen removal of WWTP effluent by denitrification MBBR.

Keywords: C/N ratio; Denitrification genes; Denitrification MBBR; qPCR; WWTP effluent

1. Introduction

Municipal wastewater treatment plant (WWTP) effluent may be used as an alternative water source due to water shortage. Israel, a semi-arid country, has investigated wastewater recycling technology since 1970s, and now 94% of sewage is collected while 91% is treated, which is among the highest recycling rates in the world [1]. Urban sewage contains only about 1% of pollutants, which has higher economic and environmental benefits compared with long-distance water desalination and other projects [2]. But the higher total nitrogen (TN) content becomes the main limitation for wastewater recycle. The TN and ammonia limitation of Class I (A) of *Discharge Standard of Pollutants for Municipal Wastewater Treatment Plant of China* (GB18918-2002) are 15 mg/L and 5 mg/L respectively. The organic nitrogen content of WWTP effluent is so low that can be neglected, and the remaining nitrogen is mainly in nitrate form. The COD of WWTP effluent is mostly refractory organics, and external carbon source should be added as electron donor for denitrification. Common used carbon sources are methanol [3], ethanol [4], sodium acetate [5],

*Corresponding author.

and glyceride [6] etc. Innovative moving bed biofilm reactor (MBBR) technology for post-denitrification is considered as an established technology for wastewater treatment. It is believed that the MBBR technology have the potential of small footprint to fit the limited space [3]. MBBR was used as post-denitrification for two WWTPs in Sweden Malmö City, Sjölunda and Klagshamn, where methanol and ethanol were used as external carbon source respectively, and the carbon dosage was adjusted through the NOx-N online monitoring [5].

As nutrient and energy source for bacterial metabolism, carbon is essential for the biological wastewater denitrification process [7]. For non-aromatic carbon, the minimum COD/NO_3^--N (hereinafter referred to as C/N) ratio for complete denitrification increases with increase of its molecular weight [8]. K.A. Bill et al [6] added methanol as carbon source for denitrification in MBBR, and found that the maximum denitrification rate was 0.6 g N m^{-2} d^{-1} under the condition of 5.7 $COD_{applied}/NO_3^-$-N and 30 min HRT. For bio-filter treatment of WWTP secondary effluent, the TN removal rate increased with the increase of sodium acetate dosage, and when the sodium acetate dosage increased to 50 mg/L, i.e., 11.2 C/N ratio, the effluent TN was lower than 1.5 mg/L, and TN removal rate reached 88% or even higher [9]. The above-mentioned studies indicate that the C/N ratio requirement is different for different treatment processes, water quality and carbon type, and appropriate C/N ratio is the basic factor for maximum denitrification rate.

Increasing attention is paying to the negative impact of dissolved organic matter (DOM) from WWTP effluents on water body quality. DOM is generated from natural organic matter, soluble microbial products, and synthetic organic chemical constituents [10] etc. Some components of DOM are mostly linked to biotoxicity and disinfection by-products (DBPs) formation. Therefore, a comprehensive analysis of DOM is needed urgently. As a rapid reagentless technique that requires little sample preparation, fluorescence spectroscopy is commonly used in the study of DOM. Fluorescence measurements can be used as tracer for quantitative and qualitative changes occurring in the DOM pool as a whole [11].

Denitrification is a biochemical process involving the stepwise reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to produce the gaseous NO, N_2O, and N_2 under anoxic conditions, which is mediated by physiologically diverse groups of microorganisms. The denitrification bacteria, namely denitrifiers, belong to more than 50 genera [12].

Molecular identification based on 16S rRNA genes does not necessarily correspond to metabolic function [13]. Fortunately, genes that encode key enzymes (nitrate-, nitrite-, nitric oxide-, and nitrous oxide reductases) in nitrate reduction processes have been established as molecular markers [14]. These functional genes include nitrate reductase genes (narG and napA), nitrite reductase genes (nirS and nirK), nitric oxide reductase gene (norB) and nitrous oxide reductase gene (nosZ). In this study, the response of narG and nosZ genes to different C/N ratio in denitrification MBBR were investigated by terminal restriction fragment length polymorphism (T-RFLP) and quantitative polymerase chain reaction (qPCR) molecular approaches.

Due to its low price, easy preparation and non-byproducts, methanol is widely used as external carbon source for WWTP. External carbon source dosage will increase the WWTP operation cost, which requires the highest organic matter utilization efficiency, i.e., minimize the external carbon source dosage for certain TN removal efficiency, thus to reduce operation costs. High TN content is an urgent problem for the reuse of WWTP effluent. MBBR is feasible for advanced denitrification of WWTP effluent, and appropriate C/N ratio is the key factor for denitrification process. However, there is little study about the influence of C/N ratio on actual WWTP effluent denitrification, as well as the full-scale C/N ratio optimization. In this study, denitrification MBBR with methanol external carbon source was used for advanced nitrogen removal of WWTP effluent, and the C/N ratio influence on denitrification efficiency in full-scale was investigated.

2. Materials and methods

2.1. Equipment and carriers

As shown in Fig. 1, plexiglass column with 120 mm inner diameter, 500 mm height, 6.0 L volume and 5.7 L effective volume was used as MBBR denitrification reactor, and polyethylene particles with 25 mm diameter, 10 mm height, 0.96~0.98 g/L density and 620 m^2/m^3 specific surface area were used as carriers.

2.2. Experiment and operation conditions

The experiment was carried out and optimized for four stages, i.e., under the conditions of 4.6, 6.7, 8.0 and 8.4 C/N ratio and the period of each stage were shown in Table 1. Secondary sedimentation tank effluent of a Beijing WWTP was used as MBBR influent. The influent C/N ratio and water quality under stable operation conditions were shown in Table 1. To maintain the C/N ratio, 12.4, 32.6, 6.1 and 7.2 mg·L^{-1} methanol were added in stage I, II, III and IV respectively. The carriers were sampled for microbial characteristics analysis at stable operation period.

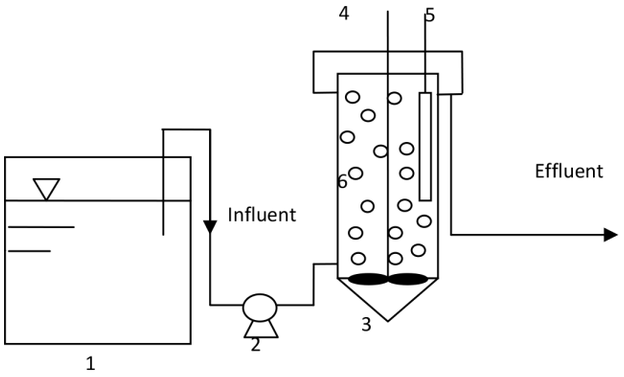

Fig. 1. Flow chart of MBBR denitrification process.

Table 1
The influent C/N ratio and water quality under stable operation conditions

Experimental stage	Carbon source	C/N ratio (w/w)	Methanol added (mg/L)	Operation time (d)	COD (mg/L)	TN (mg/L)	NO_3^--N (mg/L)
I	WWTP	4.6	12.4	26	48.1 ± 13.1	11.5 ± 4.4	10.5 ± 4.6
II	effluent	6.7	32.6	20	78.9 ± 16.5	14.9 ± 2.4	11.7 ± 3.3
III	and	8.0	6.1	24	33.5 ± 8.1	13.3 ± 5.4	4.2 ± 1.3
IV	Methanol	8.4	7.2	25	33.5 ± 7.4	11.6 ± 5.1	4.0 ± 1.1

The MBBR reactor was operated with 30% filling rate (volume ratio of carriers to reactor), 30 rpm agitation speed to keep carriers suspended and 25 ± 1ºC temperature. Peristaltic pump (BT100-1L, Baoding Lange constant pump Ltd.) was used for continuously operation.

The MBBR reactor was inoculated with activated sludge taken from the anoxic part of Carrousel Oxidation Ditch 3000 Process of a Beijing WWTP, and the sludge was of 7000 mg/L MLSS, 3549 mg/L MLVSS, 0.51 MLVSS/MLSS ratio, 66% SV and 94 mL/g SVI.

2.3. Analytical methods

2.3.1. Water Quality Index Detection

TN, NO_2^--N, NO_3^--N and DOM were analyzed after the water samples filtered by 0.45 μm membrane. NH_4^+-N was measured by Nessler's reagent spectrophotometry method. TN was determined using the TN unit of TOC-V_{CPH} TOC analyzer. TP was examined by persulfate oxidation - molybdate spectrophotometry method. COD was analyzed by rapid digestion method. NO_2^--N and NO_3^--N were measured by ion chromatography (DIONEX ICS-1000, Dionex, USA). pH was determined by glass electrode method. The samples were taken every two days, and then pH, DO, temperature, COD, NH_4^+-N, NO_2^--N, NO_3^--N and TN were analyzed. The chemicals used were analytical grade reagents (Sinopharm Chemical Reagent Beijing Co., Ltd.).

2.3.2. Three-Dimensional Fluorescence Spectroscopy

DOM was analyzed by fluorescence protractor (Hitachi F-7000 fluorescence spectrophotometer, Japan), and the excitation and emission slit width was 5 nm. The scanning scope of excitation wavelength (Ex) and emission wavelength (Em) was ranged from 200 nm to 500 nm with 12000 nm/min scanning speed.

Fluorescence index $f_{450/500}$, which means the intensity ratio of fluorescence emission spectrum at 450 nm and 500 nm with 370 nm Ex, can be used to characterize the source of DOM humus [15].

Some studies suggest that the intensity ratio of protein-like fluorescence and visible region fulvic acid-like fluorescence r(S, M) can reflect the water contamination generally [16]. r(S, M) is calculated according to Eqn. (1):

$$r(S, M) = I_S / I_M \tag{1}$$

where I_S and I_M are the protein-like and the visible region fulvic acid-like fluorescence intensity respectively.

The intensity ratio of UV region and visible region of fulvic acid-like fluorescence r(A, M), which is influenced by organic molecule size, solution pH value, etc., is related with organic structure and maturity [17]. r(A, M) is calculated according to Eqn. (2):

$$r(A, M) = I_A / I_M \tag{2}$$

where I_A and I_M are the UV region and the visible region fulvic acid-like fluorescence intensity respectively.

2.3.3. DNA extraction and T-RFLP

Some sludge was collected for DNA extraction in stable period. DNA was extracted by UltraClean DNA extraction kit (Mobio Laboratories, Carlsbad, USA). Amplification reactions of T-RFLP were performed with 50 μL reaction mixture, which contained 25μL 2×Taq mix, 1 μL primer (10 μmol/L) for each type, 1.0 μL template DNA, and 13 μL ddH_2O. The primers were nosZ-F (5′-CGYTGTTCMTCG-ACAGCCAG-3′; 5′- end labeled with carboxy fluorescine) and nosZ1622R (5′-CGSACCTTSTTGCCSTYGCG-3) [18]. The PCR amplification conditions were as follows: 1 cycle at 94°C for 5 min; 35 cycles at 95°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1.5 min, final extension at 72°C for 10 min [13]. Hha I was used to digest PCR products that purified by QIA quick PCR purification kit (Qiagen Inc., Germany) at 37°C for 3h. Shannon-Weiner index and Bray-Curtis similarity were used to evaluate the diversity of bacteria species and the similarity of different samples. The Shannon–Wiener index is based on the concept of evenness or equitability, which can be calculated by following Eqn. (3) [19]:

$$H = -\sum_{i=1}^{s} Pi \ln(Pi) \tag{3}$$

where P is the proportional abundance of each species and ln is the natural logarithm; Bray-Curtis similarity index is calculated as Eqn. (4) [20]:

$$D_{1,2} = \Sigma qi \tag{4}$$

where $D_{1,2}$ is the similarity between sample 1 and 2; qi is the less one of the two relative abundances of species i.

Evenness index (E) is calculated as Eqn. (5):

$$E = H / Hmax = H / lnS \tag{5}$$

where S is the number of species.

2.3.4. qPCR

Amplification reactions of qPCR were performed with 20 µL reaction mixture, which contained 10.0 µL SYBR Master Mix (ROCHE), 0.4 µL primer for each type, 1.0 µL template DNA, and 8.2 µL ddH_2O. The fractions were PCR amplified using primers NarG-F (5′-TCGCCSATYCCGGCSATGTC-3′) and narG-R (5′-GAGTTGTACCAGTCRGCSGAYTCSG-3′), nosZ-F (5′-CGCRACGGCAASAAGGTSMSSGT-3′) and nosZ-R (5′-CAKRTGCAKSGCRTGGCAGAA-3′) [18]. The thermo cycling steps of the real-time PCR for denitrification genes amplification were as follows: 95°C for 120 s, 40 cycles at 95°C for 15 s, 53°C for 20 s, and 72°C for 20 s. The specificity of each PCR assay was confirmed by both melting curve analysis and agarose gel electrophoresis methods. All the measurements were carried out in triplicate.

Peak Scanner Software v 1.0, PRIMER 5.0, ABI-7500 System SDS Software V1.4 and Origin 8.0 were used for information statistics, data analysis and plotting.

3. Results and discussion

3.1. Influence of C/N Ratio on Nitrogen Removal

3.1.1. Influence of C/N Ratio on NO_3^--N Removal

Fig. 2 illustrates the NO_3^--N concentration, NO_3^--N removal efficiency and denitrification rate under the conditions of 4.6, 6.7, 8.0 and 8.4 C/N ratios. It can be seen from Fig. 2 that under the conditions of 4.6, 6.7, 8.0 and 8.4 C/N ratios, 10.5 ± 4.6, 11.7 ± 3.3, 4.2 ± 1.3 and 4.0 ± 1.1 mg/L influent NO_3^--N concentrations, the effluent NO_3^--N concentrations were 3.5 ± 4.2, 5.9 ± 3.1, 0.7 ± 0.3 and 0.6 ± 0.3 mg/L, and the removal rate were 75.2 ± 24.3%, 69.4 ± 18.4%, 81.7 ± 8.1% and 84.2 ± 5.6% respectively. The removal rate changed with the variation of C/N ratio. However, when the C/N ratio increased from 8.0 to 8.4, the increase of NO_3^--N removal efficiency was not obvious. Denitrification rates were 13.9 ± 2.7, 11.6 ± 2.1, 6.9 ± 2.5 and 6.7 ± 1.8 mg NO_3^--N·(L·d)$^{-1}$, which were closely related with influent NO_3^--N concentration, and the higher the influent concentration the higher the denitrification rate. These denitrification rates mentioned above were much lower than 96 mg NO_3^--N·(L·d)$^{-1}$, which was for the MBBR with methanol as carbon source for synthetic wastewater treatment at 30 min HRT [6]. Because of the long HRT of 12 h and the relatively low NO_3^--N concentration in WWTP effluent, the denitrification rate showed great difference. The main purpose of this study was to figure out the suitable C/N ratio for WWTP effluent denitrification, so stable HRT of 12 h was used for different C/N ratios.

The study of Foglar et al. [21] showed that the minimum CH_3–OH/N (w/w) ratio for complete denitrification was 2.5, and the maximum ratio was 3.5, that is to say, the C/N ratio ranged from 3.8 to 5.3. Her et al. [8] concluded that the appropriate TOC/N ratio was 0.9~10.0 when using methanol as carbon source, and the higher the C/N ratio the faster the decrease of NO_3^--N and NO_2^--N. Wang et al. [7] suggested that suitable C/N ratio ranged from 2.7 to 7.1, and the denitrification rate did not increase when the C/N ratio was higher than 7.1, which meant that 7.1 C/N ratio could satisfy the complete denitrification requirements. Generally speaking, the denitrification rate increases with the increase of C/N ratio until the maximum denitrification rate appears. For the maximum denitrification rate, all of the biological reaction enzyme active sites are occupied by matrix molecules, the increase of substrate concentration will not influence the denitrification reaction any more [22], and further external carbon dosage is not needed. Under the conditions of 4.6, 6.7, 8.0 and 8.4 C/N ratio in this study, the TN removal rate increased with the increase of C/N ratio, and the minimum C/N ratio of 4.6 was higher than reported 3.8 for complete denitrification [21], which may result from the difference of carbon source. In the study of Foglar et al, the influent was simulated wastewater with methanol as carbon source [21], and the influent of this study was the secondary sedimentation tank effluent with methanol addition as carbon source (Table 1), and part of the COD, which was from the secondary sedimentation tank effluent, was more complex and difficult for degradation.

3.1.2. Influence of C/N Ratio on the removal of Nitrogen in other forms

As shown in Table 2, the NH_4^+-N removal rate varied greatly with the increase of C/N ratio, NH_4^+-N accumulation occurred when C/N ratio was higher than 8.0, and the higher the C/N ratio the greater the NH_4^+-N variation. The NO_2^--N

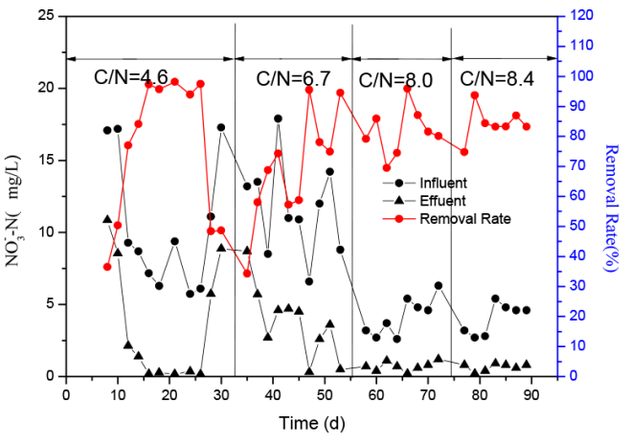

Fig. 2. NO_3^--N removal efficiency at different C/N ratios.

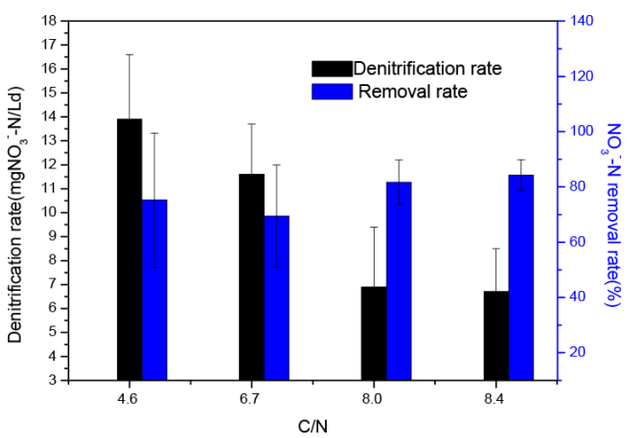

Table 2
Nitrogen Removal Rate at Different C/N Ratios

C/N	NH_4^+-N (mg/L)			NO_2^--N (mg/L)			TN (mg/L)		
Ratio	Influent	Effluent	Removal rate	Influent	Effluent	Removal rate	Influent	Effluent	Removal rate
4.6	1.0 ± 0.6	1.0 ± 0.6	10.8 ± 38.1	1.1 ± 0.9	0.9 ± 0.7	2.2 ± 33.5	11.4 ± 4.4	4.5 ± 2.9	61.9 ± 16.8
6.7	1.0 ± 1.1	0.5 ± 0.0	14.2 ± 67.5	1.7 ± 0.5	1.5 ± 0.2	6.5 ± 27.3	14.9 ± 2.4	5.7 ± 1.2	61.6 ± 7.5
8.0	6.6 ± 5.4	7.9 ± 4.1	–11.0 ± 24.9	2.3 ± 0.7	1.8 ± 0.3	8.9 ± 47.6	13.3 ± 5.4	8.8 ± 4.4	34.4 ± 15.1
8.4	5.5 ± 3.1	6.1 ± 2.9	–37.4 ± 85.0	2.2 ± 1.0	1.7 ± 0.2	8.3 ± 36.8	10.9 ± 5.3	6.6 ± 2.6	37.0 ± 10.0

removal rate varied slightly with the change of C/N ratio. Because of NH_4^+-N variation, the TN removal rates at 8.0 and 8.4 C/N ratios were significantly lower than those at 4.6 and 6.7 C/N ratios. This may be caused by the reaction of dissimilatory nitrate reduction to ammonium (DNRA), a way for NO_3^- dissimilatory reduction caused mostly by facultative anaerobic bacteria such as *Aeromonas Enterobacteria* [23] etc. The ammonium and nitrite production was due to the bacterial cell synthesis or metabolite production. Studies suggested that DNRA reaction was likely to be occurred when the easy degradation carbon and NO_3^--N ratio were high [24].

The TN removal rate changed with the increase of C/N ratio, which slowed down when C/N ratio increased from 8.0 to 8.4. DNRA reaction maybe occurred under high C/N ratio conditions, which led to NH_4^+-N accumulation and lower TN removal rate. Therefore, the C/N ratio should be controlled lower than 6.7. Bernat et al. [25] suggested that the CH_3OH/NO_3^--N stoichiometry ratio for denitrification was 2.47, i.e. 3.7 C/N ratio. Wong and Lee [26] indicated that the theoretical COD of butyrate required for complete denitrification of 1 mg NO_3^--N was 2.86 mg COD, while that for the DNRA of 1 mg NO_3^--N was 4.57 mg COD. The difference of COD consumption for 1 mg NO_3^--N reduction resulted from different nitrate reduction pathway and carbon sources.

3.2. Influence of C/N Ratio on organic pollutant removal

3.2.1. Influence of C/N ratio on COD removal

Under the conditions of 4.6, 6.7, 8.0 and 8.4 C/N ratio, when influent COD were 48.1 ± 13.1, 78.9 ± 16.5, 33.5 ± 8.1 and 33.5 ± 7.4 mg/L, the effluent COD were 26.6 ± 5.2, 35.3 ± 8.9, 22.1 ± 8.8 and 26.8 ± 5.9 mg/L respectively. As shown in Fig. 3, The Class IV limitation of *Environmental Quality Standards for Surface Water of China* (GB 3838-2002) for COD was 30 mg/L. However, when the C/N ratio was 6.7, the effluent COD concentration exceeded the limit value. DNRA reaction might be occurred at 8.0 and 8.4 C/N ratios. As we know, 8 mol electrons from carbon source were consumed when 1 mol NO_3^- as electron acceptor was reduced, but only 5 mol electrons would be consumed for 1 mol NO_3^- reduction during denitrification process [27], which meant that DRNA consume more carbon, and thus lead to lower effluent COD for 8.0 and 8.4 C/N ratios.

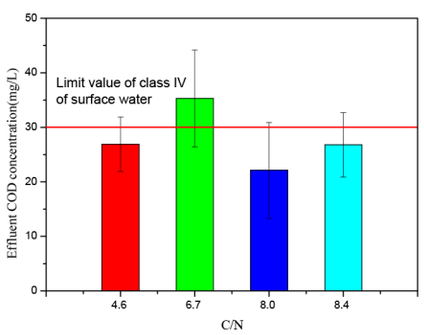

Fig. 3. Influent and effluent COD at different C/N ratios.

3.2.2. Influence of C/N Ratio on DOM removal

The fluorescence in different spectral regions is related with different organic functional groups in DOM. Fig. 4 shows the DOM fluorescence spectra of the MBBR influent and effluent.

It can be seen from Fig. 4 that four major fluorescence peaks were included in the influent and effluent three-dimensional fluorescence spectra: Peak T (Ex/Em: 280/345-365) and Peak S (Ex/Em: 235-245/345-380) represented the protein-like substances, Peak M (Ex/Em: 305-315/395-400) represented the visible fulvic acid-like substance, and Peak A (Ex/Em: 240-245/385-410) represented the UV fulvic acid-like substance. Under the conditions of 4.6, 8.0 and 8.4 C/N ratio, the total fluorescence intensity of effluent decreased 2.6%, 3.9% and 2.0%, Peak M and Peak T decreased 3.0% and 8.9%, 2.1% and 3.9%, 0.6% and 6.1%, Peak A increased 1.4% and 0.6% except for 8.0 C/N ratio, Peak S decreased 2.8% and 1.4% except for 4.6 C/N ratio. The total fluorescence intensity of effluent increased 5.2% for 6.7 C/N ratio, Peak A, M and S increased to some extent while Peak T decreased 12.1%.

Peak S was related to simple aromatic proteins such as tyrosine, Peak T reflected tryptophan-like material, and was related with biological activity, and it also had the strongest correlation with BOD_5 [28]. Peak S was relatively high when C/N ratio was low (4.6 and 6.7), which represented the increase of tyrosine-like substances. Peak T decreased under high C/N ratio conditions (8.0 and 8.4), which might result from the microbial degradation of tryptophan-like material by DNRA reaction or ammonification, thus led to the increase of NH_4^+-N. Peak M and Peak A belonged to humic substances, which reflected the fulvic and humic acids with exogenous inputs, and the fluorescence peaks were related with the hydroxyl and carboxyl structure of such substances.

Table 3 shows EEMs parameters of influents and effluents at different C/N ratios. The fluorescence ratio at different wavelength has been used to reflect the difference of

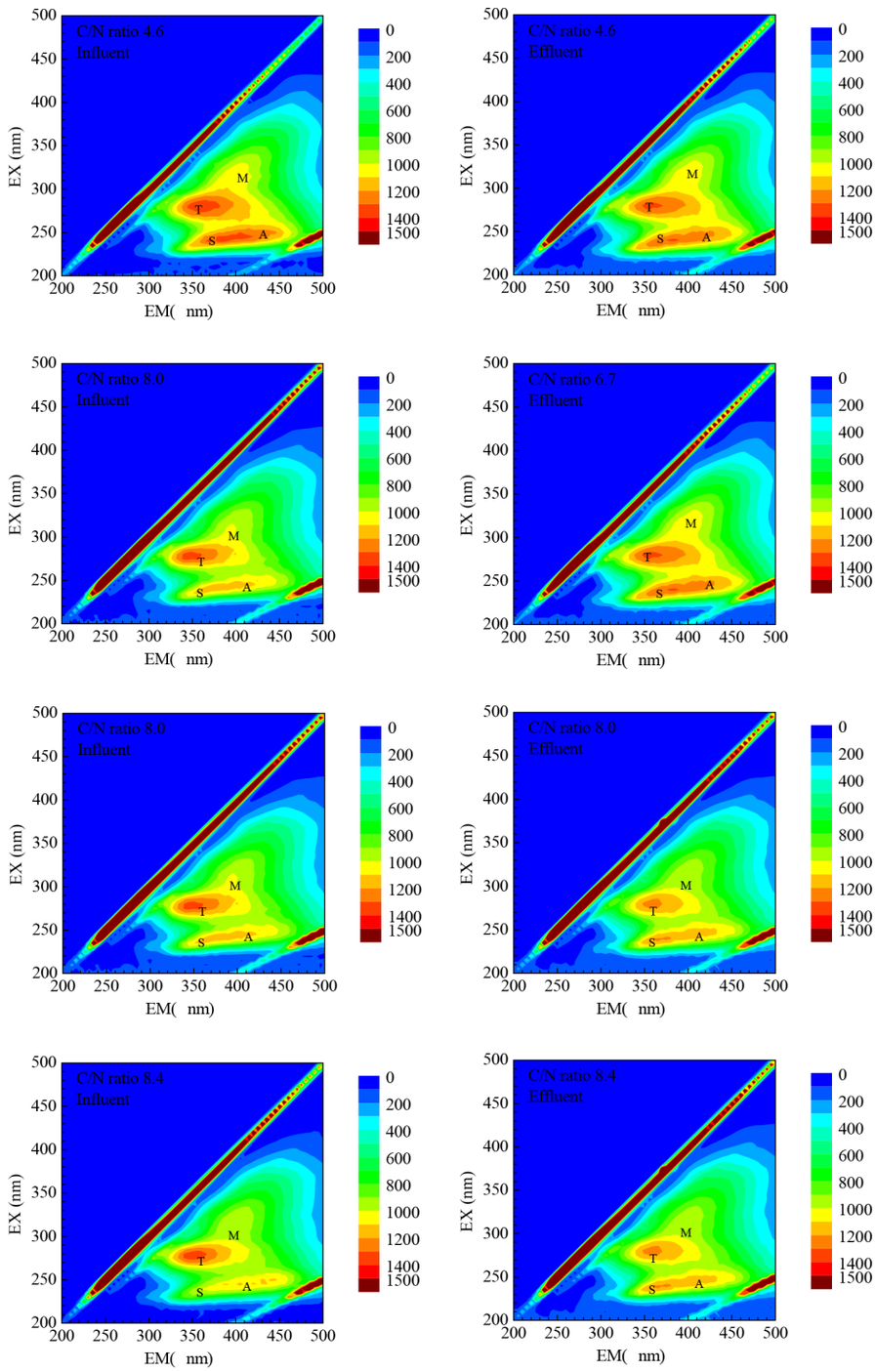

Fig. 4. Influent and effluent three-dimensional fluorescence spectra at different C/N ratios.

DOM. It was reported that the fluorescence index $f_{450/500}$ was correlated to the source of fulvic acid fluorescence [29]. The microbially derived fulvic acids had higher fluorescence $f_{450/500}$ index than terrestrially derived fulvics [16]. The lowest $f_{450/500}$ value was found in freshwater samples, in which terrestrial fulvic acid dominated the DOM pool. The highest $f_{450/500}$ was found in wastewater samples, in which terrestrial DOM was quite limited, and the fulvic acid was most likely microbially derived [11]. All the influent and effluent $f_{450/500}$ of this study were higher than 1.9, which indicated that humus was mostly of biological origin. Since $f_{450/500}$ had negative correlation with fulvic acid aromatic substances, higher $f_{450/500}$ of this study meant that the humic substances contain less aromatic, i.e. less benzene ring structure materials.

Table 3
EEMs parameters of influents and effluents at different C/N ratios

C/N ratio	Influent / effluent	$f_{450/500}$	*r* (S, M)	*r* (A, M)
4.6	Influent	2.05	1.17	1.15
	Effluent	2.07	1.22	1.20
6.7	Influent	2.12	1.05	1.13
	Effluent	2.07	1.40	1.16
8.0	Influent	2.09	1.22	1.25
	Effluent	2.07	1.21	1.19
8.4	Influent	2.07	1.13	1.22
	Effluent	2.07	1.12	1.23

It is generally considered that the *r*(S, M) of polluted river DOM is higher than 1.5 [16]. The *r*(S, M) in this study ranged from 1.05 to 1.40, which indicated that the effluent contamination degree was not high. The maximum effluent *r*(S, M) was 1.4 for 6.7 C/N ratio, which meant higher contamination level than other C/N ratio conditions, and these results were consistent with the COD removal rates discussed above.

Constant *r*(A, M) value represents the type of fulvic acid fluorophores. The *r*(A, M) in this study varied for different samples, which suggested that there were more than one type of fulvic acid fluorophores in the influent and effluent DOM under different C/N conditions [16].

3.3. Influence of C/N Ratio on diversity and abundance of denitrification genes

3.3.1. nosZ-based T-RFLP

Fig. 5 shows the differences of denitrification bacteria community under different C/N ratio based on nosZ genes analysis. The fragments of 62, 116, 167, 169 and 259 bp were predominant when C/N ratio was 4.6, and the relative abundance of 116 and 259 bp were 45.8% and 12.2%, respectively. When C/N ratio was 6.7, the fragments of 51, 91, 96, 127, 167 and 220 bp were predominant, which accounted for 5.6%, 10.6%, 7.8%, 9.4%, 26.4% and 13.0%, respectively.

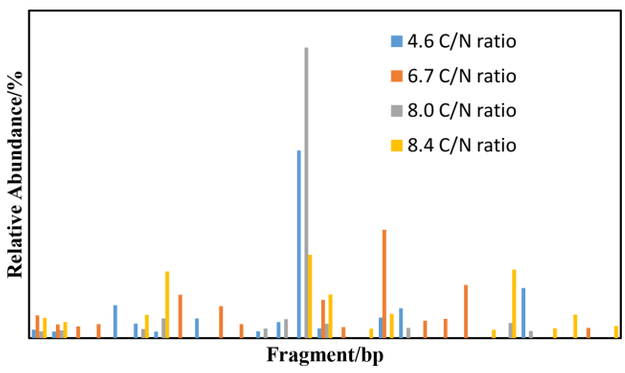

Fig. 5. nosZ community structure at different C/N ratios.

The predominant fragments were 51, 53, 73, 80, 111, 115, 116, 127, 169, 232 and 259 bp when C/N ratio was 8.0, and 116 bp fragment accounted for 71.0%. Under 8.4 C/N ratio, the predominant fragments were 51, 53, 73, 80, 116, 127, 155, 167, 228, 232, 277, 305 and 364 bp, and the relative abundance of 80, 116, 127, 167, 232, 305 bp were 16.3%, 20.4%, 10.7%, 16.8%, 5.9% and 5.7%.

Microbial diversity index H (Shannon-Weiner index) were 1.919, 2.351, 1.251 and 2.288 for 4.6, 6.7, 8.0 and 8.4 C/N ratio respectively, which meant that the community structure diversity of nosZ gene under 8.0 C/N ratio was the lowest. Microbial evenness for different C/N ratio were 0.7482, 0.8907, 0.5216 and 0.8919, which showed similar variation trend with diversity index H. Bray-Curtis similarity index, which represented the similarity of microbial community between different C/N ratio conditions, varied between 15.2% and 50.0%, except for 4.6 and 8.0 C/N ratio, i.e. 70.6%. Diversity index H and evenness E were rather high under 4.6, 6.7 and 8.4 C/N ratio, which indicated more stable operation of the system under these C/N ratio conditions.

3.3.2. narG- and nosZ-based qPCR

SYBR green-based qPCR assays were used to determine the copy numbers of narG and nosZ genes in the carrier biofilm under different C/N ratios. The results showed that when C/N ratio were 4.6, 6.7, 8.0 and 8.4, the narG and nosZ gene number of the biofilms were 1.81×10^9 and 1.29×10^8, 1.0×10^8 and 1.74×10^7, 3.38×10^8 and 1.55×10^6, 5.22×10^7 and 1.31×10^6 copies/g-SS, from which it can be concluded that the abundance of nitrate-reducing genes was greater than that of nitrous oxide reducing genes. The content of narG and nosZ genes under 4.6 C/N ratio was the highest, which was consistent with the nitrogen and organic pollutant removal efficiency anaylzed in section 3.1 and 3.2.

Using SYBR green-based qPCR method, Kyongmi Chon et al. [30] studied the density and abundance of narG and nosZ in sediment soil samples taken from wastewater effluent-fed, and found that the functional genes ranged from 1.0×10^6 to 1.0×10^9 copies/g of soil. Biological processes involved in the nitrate removal in bioretention system characterized by low infiltration rates and long drainage times were studied, and the denitrification nosZ gene varied from 10^5 to 10^8 copies/gram [31]. The gene abundance in these literatures was similar with thatin our study.

3.3.3. Correlation analysis

The abundance of denitrification genes is influenced by environmental factors greatly [14]. The influent concentration and removal efficiency of NO_3^--N were remarkably negative correlated ($r = -0.496$, $P < 0.05$) in this study. There were significantly positive correlation between influent NO_3^--N and the quantity of nosZ gene ($r = 0.515$, $P < 0.05$). NO_3^--N content is one of the major factors that impact NO_3^--N removal efficiency and denitrification genes.

4. Conclusions

As a post denitrification process, MBBR is very preferable for the NO_3^--N removal of WWTP effluent, and C/N ratio is of great importance to guarantee the NO_3^--N removal and effluent COD concentration at the same time. The NO_3^--N

removal rate increased with the increase of C/N ratio. Maximum denitrification efficiency of 13.9 ± 2.7 mg NO_3^--N·(L·d)$^{-1}$ was achieved under 4.6 C/N ratio due to the influence of influent NO_3^--N concentration. DNRA reaction may occur under high C/N ratio conditions (8.0 and 8.4), which led to NH_4^+-N accumulation and the decrease of TN removal efficiency. The effluent COD concentration was lower than 30 mg/L except for 6.7 C/N ratio. All the MBBR influent and effluent contained DOM as fulvic acid-like and protein-like substance etc., and the fluorescence intensity of effluent was lower than that of influent except for 6.7 C/N ratio. When the C/N ratio was 4.6, the community structure diversity and evenness of nosZ gene, as well as the abundance of narG and nosZ genes were the highest, which was consistent with the nitrogen and organic pollutant removal efficiency. As a whole, 4.6 was the optimum C/N ratio for nitrogen removal of WWTP effluent by denitrification MBBR.

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References

[1] A. Tal, Rethinking the sustainability of Israel's irrigation practices in the Drylands. Wat. Res., 90 (2016) 387–394.
[2] X. Jian, Status and development trend of municipal wastewater reuse technology. Environ. Prot., 8 (1996) 15–17.
[3] B. Stinson, M. Peric, D. Neupane, M. Laquidara, E. Locke, S. Murthy, W. Bailey, S. Kharkar, N. Passarelli, R. Derminassian, J. Carr, M. Sultan, G. Shih, J. Barnard, G. Daigger, D. Parker, C. Randall, T. Wilson, Design and operating considerations for a post denitrification mbbr to achieve limit of technology effluent NOx < 1 mg/l and effluent TP<0.18 mg/l. Nutri. Remov., (2009) 1225–1254.
[4] C.S. Srinandan, G. D'Souza, N. Srivastava, B.B. Nayak, A.S Nerurkar, Carbon sources influence the nitrate removal activity, community structure and biofilm architecture. Biores. Technol., 117 (2012) 292–299.
[5] M. Mases, I. Dimitrova, U. Nyberg, C. Gruvberger, B .Andersson, Experiences from MBBR post-denitrification process in long-term operation at two WWTPs. Pro. Wat. Environ. Feder., 14 (2010) 458–471.
[6] K.A Bill, C.B Bott, S.N Murthy, Evaluation of alternative electron donors for denitrifying moving bed biofilm reactors (MBBRs). Wat. Sci. Technol., 60 (2009) 2647–2657.
[7] S. Wang, F. Yin, H. Hou, Biological denitrification with methanol as external carbon source. J. BJ Univer. Technol., 35 (2009) 1521–1526.
[8] J-J. Her, J-S. Huang, Influences of carbon source and C/N ratio on nitrate/nitrite denitrification and carbon breakthrough. Biores. Technol., 54 (1995) 45–51.
[9] M. Yuan, Q. Zhou, D. Yang, Acetate sodiumas external carbon source for nitrogen removal in anoxic biological fiter. Technol. Water Treat., 34 (2008) 23–25.
[10] H. Shon, S. Vigneswaran, S. Snyder, Effluent organic matter (EfOM) in wastewater: constituent, effects, and treatment. Crit Rev. Environ. Sci. Technol., 36 (2006) 327–374.
[11] C.A. Stedmon, S. Markager, Resolving the variability in dissolved organic matter fluorescence in a temperate estuary and its catchment using PARAFAC analysis. Limnol. Oceanogr., 50 (2005) 686–697.
[12] W.G. Zumft, Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev., 61 (1997) 533–616.
[13] D.J. Scala, L.J. Kerkhof, Nitrous oxide reductase (nosZ) gene specific PCR primers for detection of denitrifiers and three nosZ genes from marine sediments. Fems. Microbiol. Lett., 162 (1998) 61–68.
[14] Z.F. Zhou, Y.M. Zheng, J.P. Shen, L.M. Zhang, J.Z. He, Response of denitrification genes nirS, nirK, and nosZ to irrigation water quality in a Chinese agricultural soil. Environ. Sci. Pollut. Res., 18 (2011) 1644–1652.
[15] M. Tedettia, P. Cuetb, C. Guiguea, M. Goutxa, Characterization of dissolved organic matter in a coral reef ecosystem subjected to anthropogenic pressures (La Réunion Island, Indian Ocean) using multi-dimensional fluorescence spectroscopy. Sci. Total Environ., 409 (2011) 2198–2210.
[16] C. Yang, M. Wang, R. Ma, Excitation-emission matrix fluorescence spectra characteristics of DOM in a subsurface constructed wetland for advanced treatment of municipal sewage plant effluent. Spectrosc. Spect. Anal., 32 (2012) 708–713.
[17] N Patel-Sorrentino, S. Mounier, J.Y. Benaim, Excitation-emission flourescence matrix to study pH influence on organic matter flourescence in the Amazon basin rivers. Wat. Res., 36 (2002) 2571–2581.
[18] A. Vilar-Sanz, S. Puig, A. Garcia-Lledo, R. Trias, M.D. Balaguer, J. Colprim, L. Baneras, Denitrifying bacterial communities affect current production and nitrous oxide accumulation in a microbial fuel cell. PloS One, 8 (2013) 1–11.
[19] I.F. Spellerberg, Shannon–Wiener Index, in Encyclopedia of Ecology, Edited by Fath SEJD. Academic Press, Oxford, 3249–3252 (2008).
[20] M.G. Kelly, Use of similarity measures for quality control of benthic diatom samples. Wat. Res., 35 (2001) 2784–2788.
[21] L. Foglar, F. Briški, Wastewater denitrification process—the influence of methanol and kinetic analysis. Process Biochem., 39 (2003) 95–103.
[22] H.J. Hamlin, J.T. Michaels, C.M. Beaulaton, W.F. Graham, W. Dutt, P. Steinbach, Comparing denitrification rates and carbon sources in commercial scale upflow denitrification biological filters in aquaculture. Aquacult. Eng., 38 (2008) 79–92.
[23] S. Yin, J. Lu, Microbiological process of dissimilatory nitrate reduction to ammonium. Microbiology China, 24 (1997) 170–173.
[24] B. Kelso, R.V. Smith, R.J. Laughlin, S.D. Lennox, Dissimilatory nitrate reduction in anaerobic sediments leading to river nitrite accumulation. Appl. Environ. Microbiol., 63 (1997) 4679–4685.
[25] J.C. Akunna, C. Bizeau, R. Moletta, Denitrification in anaerobic digesters-possibilities and influence of wastewater COD/N-NOx ratio. Environ. Technol., 13 (1992) 825–836.
[26] B.T. Wong, D.J. Lee, Denitrifying sulfide removal and carbon methanogenesis in a mesophilic, methanogenic culture. Biores. Technol., 102 (2011) 6673–6679.
[27] W. Lu, W. Shi, Potential of dissimilatory nitrate reduction to ammonium in paddy soils in the lake region as affected by irrigation with tail water from pig rearing. Acta. Pedologica. Sinica., 49 (2012) 1120–1127.
[28] E. Parlanti, K. Wörz, L. Geoffroy, M. Lamotte, Dissolved organic matter fluorescence spectroscopy as a tool to estimate biological activity in a coastal zone submitted to anthropogenic inputs. Org. Geochem., 31 (2000) 1765–1781.
[29] D.M. McKnight, E.W. Boyer, P. Westerhoff, Spectrofluorometric characterization of dissolved organic matter for indication of precursor organic material and aromaticity. Limnol. Oceanogr., 46 (2001) 38–48.
[30] K. Chon, J-S. Chang, E. Lee, J. Lee, J. Ryu, J. Cho, Abundance of denitrifying genes coding for nitrate (narG), nitrite (nirS), and nitrous oxide (nosZ) reductases in estuarine versus wastewater effluent-fed constructed wetlands. Ecol. Eng., 37 (2011) 64–69.
[31] X. Chen, E. Peltier, B.S. Sturm, C.B. Young, Nitrogen removal and nitrifying and denitrifying bacteria quantification in a stormwater bioretention system. Wat. Res., 47 (2013) 1691–1700.