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Achieving stable partial nitritation for low strength wastewater at low temperature in a continuous moving bed biofilm reactor under high DO concentration through ratio control

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ABSTRACT

To promote the application of nitritation in actual domestic wastewater treatment, stable partial nitritation (PN) in a moving bed biofilm reactor treating low strength wastewater at low temperature (15°C, 12°C and 8°C) was successfully achieved under high dissolved oxygen (DO) concentration through ratio control which was the ratio of DO and total ammonia nitrogen (TAN). The mechanism of achieving nitritation through ratio control and the effect of organic carbon contained in wastewater on achieving stable PN at temperature of 8°C were investigated. Results indicated that at temperature of 12°C stable nitritation could be achieved through controlling ratio not exceeding 0.16 exerting effective repression on NOB populations which would be washed out slowly. Organic carbon is largely beneficial to achievement of stable PN at temperature of 8°C, resulting in looser oxygen-limited conditions (DO/TAN: 0.2) than that (DO/TAN: 0.16) at temperature of 12°C. But not the more organic carbon the better nitritation performance, an equal number of COD (C/N = 1) should be adequate. In practical applications, to achieve stable PN for actual mainstream under high DO concentration at temperature not lower than 8°C, TAN-dominated ratio control should be a suitable control strategy.

Keywords: Partial nitritation; Low temperature; Low strength wastewater; Ratio control; Wash-out of NOB; Organic carbon

1. Introduction

Nowadays, wastewater emission limits are becoming stricter, making biodegradable carbon less suitable for nitrogen removal via the nitrification–denitrification process [1]. Biological nitrogen removal by partial nitritation and anaerobic ammonium oxidation (PNA) has been proposed as a sustainable method for wastewater treatment [2,3]. Two-stage system can be thought as an appealing solution for possible applications of PNA in wastewater treatment [4], and the unit of nitritation can be transformed between the activated sludge, granular and biofilm reactors, where low concentrations of DO are required. As a whole, nitritation with granular and biofilm reactors have less effect on the subsequent anammox process than that with activated sludge, for instance, no sludge settling is needed [5,6]. An important component of biofilm treatment technologies is the substratum that is used for the attachment and growth of the biofilm. Zekker et al. [2] have pointed that in biofilm reactor both anammox and AOB populations increased

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in abundance during reactor operation when the temperature was decreased from 26°C to 20°C, while the relative abundance of NOBs remained below 10% [7]. In anammox process, ammonia and nitrite were simultaneously transformed to nitrogen gas by anaerobic ammonia oxidizing bacteria, with a small amount production of nitrate [8]. PNA installations were already successfully operated worldwide in side-stream treatment to reduce aeration energy for N-removal [2,3]. Now the research focus has moved to possible applications of PNA in mainstream treatment. But the nitrate produced from nitritation significantly affected the stable operation in the present autotrophic N-removal [9,10]. So the nitritation is the key process to achieve stable PNA, and then effective repression on NOB is the key factor to ensure the stable operation. For the domestic sewage with low strength, the repression on NOB is certainly an inevitable and major challenge in nitritation process, especially at low temperature [10–12]. Step-wise temperature decreasing cultivation has been confirmed as a suitable and effective strategy to perform N-removal sufficiently in moving bed biofilm reactor (MBBR) [13].

Recently, denitrification and NO was used to inhibit NOB to achieve nitritation [7,14], also an oxygen-limiting ratio control (DO/TAN) strategy was successfully applied to obtain nitritation under stable operating conditions [15,16]. Obviously, nitritation can be affected by temperature, to the best of our knowledge, a stable biofilm nitritation reactor at temperatures of 8°C and treating low strength ammonium wastewaters has rarely been reported [5,12]. Through simulation, Perez et al. [17] has pointed that oxygen limitation is very important for stable nitritation in biofilm reactor. Heterotrophic layer forms on the top of the nitrifying biofilm with the presence of organic carbon, which limits the nitrifiers' oxygen supply [18,19]. Therefore, when applying oxygen-limiting ratio control to achieve nitritation in biofilm reactor, the effects of the presence of organic carbon should not be ignored.

Noteworthy, the action of the control strategy based on the DO/TAN realized nitritation through wash out NOB but allowed nitritation to be achieved without the need to completely wash out the NOB from the reactor [15]. Making the wash-out mechanism of NOB during the process of nitrification to nitritation clear is beneficial to further broaden the possible application of nitritation achieved through ratio control. For two-stage PNA system, partial nitritation (almost 57% ammonia conversion to nitrite) is preferred due to the meet of subsequent anammox process. Even for low-strength municipal wastewater, the effluent TAN concentration of partial nitritation (PN) is largely higher than that of full nitritation which might lead to stricter ratio control for full nitritation and more feasibility of ratio control for PN [20].

Hence, we show that the results obtained with an MBBR in which ratio control was used to obtain PN of a low-strength ammonium concentration (about 60 mg N/L) wastewater. In the study, the main goals were to (i) explore the nitritation performance of MBBR through ratio control at different temperatures (15°C, 12°C and 8°C); (ii) demonstrate the effective repression on NOB through ratio control and make clear how were the NOB populations washed out from reactor during achieving nitritation; (iii) investigate the effect of organic carbon on the application of ratio control to achieve nitritation in MBBR and recommend a TAN-dominated ratio control strategy to achieve stable PN at low temperature. The results would promote the application of PNA in practice.

2. Materials and methods

2.1. Reactor set-up and inoculum

A cubic reactor (approximately 180 × 120 × 260 mm) with working volume of about 4.4 L was used; the detailed diagram was described in Fig. 1. The reactor was placed in a temperature control cabinet (model: DC-0789, Shanghai Haozhuang Instrument Co. Ltd., China) in which water temperature could be controlled as expected (15°C, 12°C and 8°C). Compressed air was supplied through an air diffuser placed at the bottom of the reactor. The DO concentration in the bulk liquid was measured by means of an online electrode. Aeration rate was manually controlled to maintain the DO concentration at different set-point values. Hydraulic retention time (HRT) was adjusted through influent pump (model: Longer BT300-2J, Halma, England) to obtain different setting TAN concentration in effluent. No pH adjustment was carried out in the reactor and in effluent it was about 7.3–7.4. Pall rings were used as biomass carriers. The volume of the carriers was about 30% of the working volume of the reactor. Initial inoculated pall rings were taken from a sequencing batch reactor which has a good nitritation performance and operated at temperature of 15°C.

2.2. Wastewater and operational conditions

The MBBR was fed with a synthetic wastewater, which contained, in average, 2.5 mg P/L, added as KH_2PO_4; 60 mg NH_4^+-N/L, added as NH_4Cl. $NaHCO_3$ was added for alkalinity, which was about 720 mg/L. No COD was contained between 0 and 190. From day 191, CH_3COONa was added for COD of which the concentrations in days 191–240 and 241–310 were approximately 60 and 120 mg/L, respectively. The pH of influent was about 7.7–7.8. The synthetic wastewater also contained 1 mL of trace elements solution per litre of influent [21].

The MBBR was run with continuous influent for 310 d, which could be divided into eight experimental periods. Main parameters applied in the different experimental periods are shown in Table 1.

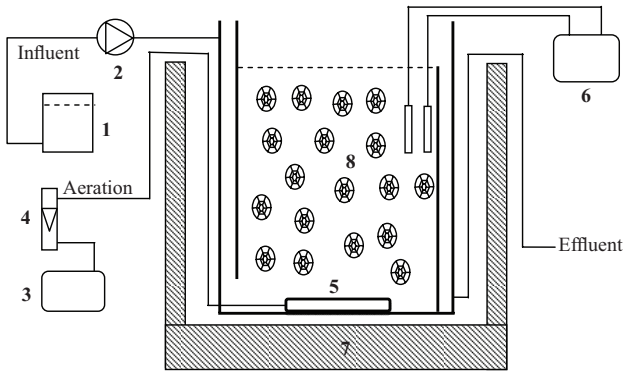

Fig. 1. Schematic diagram of the biofilm reactor showing the peripheral instrumentation: (1) influent tank; (2) influent pump; (3) air pump; (4) air flow meter; (5) air diffusor; (6) WTW (DO and pH probes); (7) temperature control basin; (8) pall ring.

Table 1
Main parameters applied in the different experimental periods

Period	Temperature (°C)	[DO] (mg of O_2/L)	[TAN] (mg of N/L)	*R* [DO]/[TAN]	COD (mg of N/L)	Duration (d)
A	15±1	4±0.2	25±1	0.16	0	20
B	12±1	4±0.2	25±1	0.16	0	20
C	12±1	4±0.2	10±1	0.4	0	40
D	12±1	4±0.2	25±1	0.16	0	90
E	8±1	4±0.2	25±1	0.16	0	20
F	8±1	5±0.2	25±1	0.2	60	50
G	8±1	5±0.2	18±1	0.28	120	30
H	8±1	5±0.2	25±1	0.2	120	40

2.3. Analytical methods

All the samples were filtered with a 0.45 μm filter before analyzing. NH_4^+-N, NO_2^--N, NO_3^--N, TSS, VSS and alkalinity were measured according to the standard methods [22]. Biofilms were, respectively, detached from pall rings by ultrasound (45 kHz, 120 W, 2–3 min) and subsequently centrifuged (10,000 g, 15 min) for VSS measurement. DO, pH, and temperature were monitored on-line by a WTW Multi 3420i meter (WTW Company, Germany). Aeration pump (45 KW, 50 L/min), flow meter (120 mL/min).

The free ammonia (FA) and free nitrous acid (FNA) concentrations were calculated by Eqs. (S1) and (S2). TAN and total nitrite nitrogen (TNN) concentrations were obtained through calculation where TAN = NH_4^+-N + FA-N and TNN = NO_2^--N + FNA-N.

The nitrite accumulation ratio (NAR, %) was calculated as Eq. (1):

$$\mathrm{NAR} = \frac{\mathrm{C(TNN)}}{\mathrm{C(TNN)} + \mathrm{C}(NO_3^- - \mathrm{N})} \quad (1)$$

TNN: concentration of TNN in the reactor. NO_3^--N: concentration of NO_3^--N in the reactor. Ammonium oxidation rate (AOR) and nitrite oxidation rate (NOR) were measured in batch tests which were performed in the two same 2 L beakers regularly and conducted synchronously with the main continuous MBBR in this study. For AOR measurement, influent and operation conditions were the same with that under continuous operation. AOR was defined by the ammonium average removal rate at a certain time. Similarly, for NOR measurement, influent contained no NH_4^+-N replaced by 60 mg/L NO_2^--N, added as $NaNO_2$. Also, operation conditions of NOR measurement were the same with that under continuous operation. NOR was defined by the nitrite average removal rate at a certain time.

The actual specific oxygen uptake rate (SOUR) was measured by static experiment: equal proportions of pall rings and liquid were quickly taken from the reactor to 500 mL sealed serum bottle, in which DO concentration decreased gradually and the value could be detected by a WTW Multi 3420i meter (WTW Company, Germany). Through linear fitting, the average oxygen uptake rate (OUR) could be obtained. Then the total biofilm mass of pall rings in serum bottle was measured, afterwards SOUR = OUR/MLVSS.

2.4. Microbial sampling and assignment

For quantitative analysis of the microbial population, the biofilm samples were, respectively, detached from pall rings by manual operation with the help of swap. And then according to the methods [23], the DNA was extracted from each sample. The extracted DNA was used for high-throughput pyrosequencing and phylogenetic assignment (Sangon, China).

Spatial distribution of bacterial populations (total bacteria, AOB and NOB) were identified by FISH-CLSM techniques. Biofilms were collected, washed, and fixed in 4% paraformaldehyde solution. The process in situ hybridization was carried out according to the study of Amann et al. [24]. Oligonucleotide probes used were listed in Table S1. All fluorescence signals were recorded with a CLSM (FV1200, Olympus, Japan), and the images were analyzed by the software of FV10-ASW. Biofilm thickness was obtained directly through CLSM.

Likewise, spatial distribution of live and dead bacteria was identified by fluorescence staining (FS)-CLSM techniques. Hoechst 33342 and propidium iodide (PI) were used to label live and dead bacteria, respectively. First, fresh biofilms were washed three times with 1xPBS and Hoechst 33342 was added to dye 30 min in a 200 rpm constant temperature shaker; second, the biofilms were washed three times by 1xPBS and PI was added to dye 30 min in the same shaker; and the biofilms were cleaned in the same way of the last step of FISH. Finally, a CLSM (FV1200, Olympus, Japan) was used to record the signals and the images were also analyzed by the software of FV10-ASW.

A heat extraction method was used to extract extracellular polymeric substance (EPS) samples and the detailed procedure of EPS extraction was described in a previous study [25]. 3D-EEM spectra of EPS samples were measured using a luminescence spectrometer (F-7000, Hitachi, Japan). The detailed spectra were set as emission wavelength (Em) from 240 to 600 nm at 2.0 nm increments by varying the excitation wavelength (Ex) from 200 to 400 nm at 5.0 nm increments.

3. Result and discussion

3.1. Performance of MBBR operation

After the inoculation from SBBR to MBBR in this study, nitrogen removal rate and nitritation performance rapidly recovered to similar level with that of SBBR resulting from the similar synthetic wastewater and temperature before and

after inoculation. The variation tendency of TNN, nitrate, and NAR in period A-H can be seen in Fig. 2.

As Fig. 2 shows, at temperatures of 15°C and 12°C nitritation both remained stable in periods A and B (days 1–40) where *R* was 0.16. In period C (days 41–80) where *R* was controlled at about 0.4 and temperature was also 12°C, NAR decreased gradually from nearly 100% to nearly 0% resulting in complete nitritation failing to complete nitrification and remained stable. When *R* reduced to 0.16 again in period D (days 81–170) where temperature was also controlled at 12°C, NAR increased gradually to nearly 100% and stable nitritation was achieved once more. It can be seen that in this study stable PN (effluent NH_4^+: NO_2^- = 1.24–1.33) at temperatures of 15°C and 12°C was achieved successfully through ratio control. Something different was that both with stable nitritation AOR at temperature of 12°C was only about 74% of that at temperature of 15°C. Remarkably, during stable nitritation process the DO concentration was controlled at 4 mg/L which had little effect on nitritation rate. When temperature decreased to 8°C in period E (days 171–190), nitritation failed at *R* = 0.16.

To achieve PN at temperature of 8°C through ratio control under high DO concentration, COD was added to the influent in periods F-H (days 191–310) to investigate the effect of organic carbon on the application of ratio control to achieve nitritation at low temperature. In period F (days 191–240) since an equal number of COD (C/N = 1) was added, several days later nitritation began to recover and complete PN was achieved even at *R* = 0.2 under DO = 5 mg/L. The positive effect of organic carbon on the application of ratio control to achieve nitritation under high DO concentration, especially at low temperature was clearly demonstrated in this study. In period G and H (days 241–310), double number of COD (C/N = 2) was added, meanwhile *R* was increased to 0.28 in period G (days 241–270) but stable nitritation was not remained. Once *R* reduced to 0.2 in period H (days 271–310) nitritation recovered and remained stable. It was observed that not the more organic carbon the better performance of ratio control to achieve stable nitritation at low temperature under high DO concentration but an equal number of COD (C/N = 1) should be adequate to achieve stable PN at temperature of 8°C.

3.2. Effective repression on NOB through ratio control

During the process of achieving complete nitritation in days 81–120, less and less nitrite was oxidized to nitrate due to the shares of NOB populations in total bacteria decreased or the limitation of activity of NOB populations. To distinguish the reasons, the shares of AOB and NOB populations in total bacteria were estimated and listed in Table 2. Meanwhile, AOR and NOR were also measured and listed in Table 2 as well. AOR/AOB and NOR/NOB were introduced to indirectly represent activity of AOB and NOB, respectively.

As can be seen from Table 2, in the 80–102 day of operation, the NOB population has only eluted less than 1%, and on the 102nd day, NAR has reached 50%. During this stage (days 81–102), suppression on activity of NOB populations (NOR/NOB decreased from 35.40 to 19.33) might be the main contributor to the increase of NAR. Even at day 120 complete nitritation was achieved, the share of NOB populations in total

Table 2
Shares of AOB and NOB in total bacteria with regard to AOR and NOR

Day	AOB (%)	NOB (%)	AOR (mg/(L·h))	NOR (mg/(L·h))	AOR/AOB	NOR/NOB
81	0.55	0.43	13.54	15.22	24.62	35.40
102	0.56	0.39	13.63	7.54	24.34	19.33
120	0.72	0.18	15.71	0.45	21.82	2.50
170	0.83	0.01	16.05	0	19.34	0

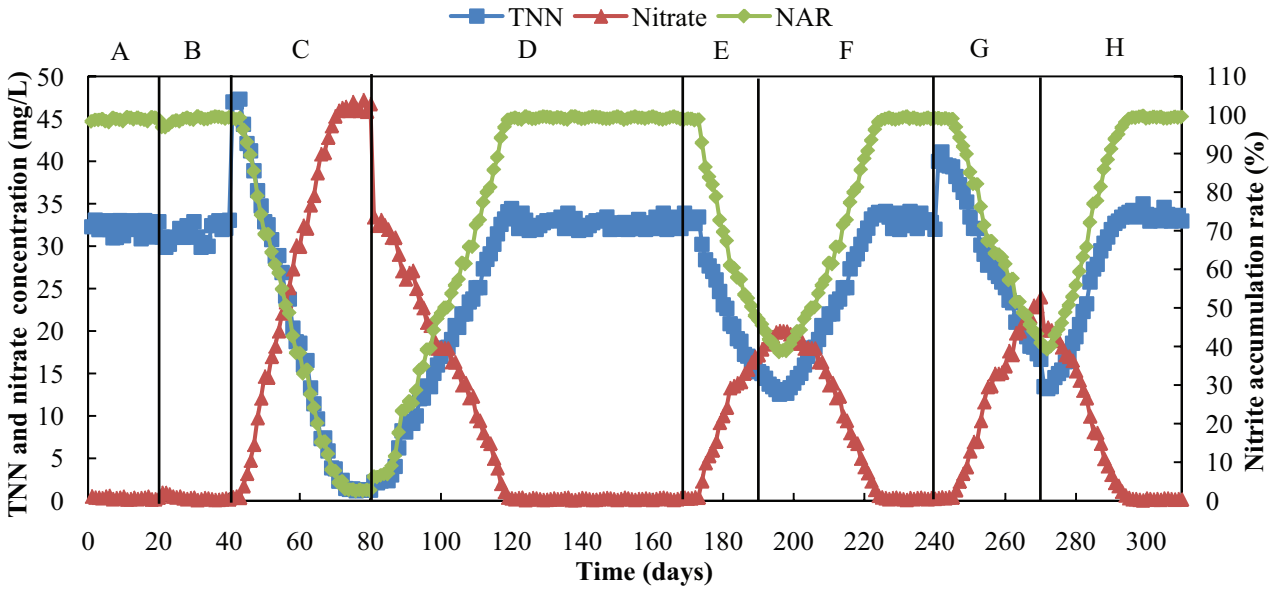

Fig. 2. Nitritation performance of MBBR from the 1st to the 310th day. Period A: 1–20; B: 21–40; C: 41–80; D: 81–170; E: 171–190; F: 191–240; G: 241–270; H: 271–310 day.

bacteria was also up to 0.18% which was about 42% of initial (day 81) share of NOB populations. Similarly, NOB activity was largely reduced during days 102–120 where NOR/NOB decreased from 19.33 to 2.50. Through following operation of 50 d, stable nitritation was always remained, until day 170 NOB populations were almost completely washed out from the reactor and NOR was almost zero. It could be concluded that during the process of achieving nitritation through ratio control, the effective repression on NOB activity was crucial and plays a leading role at the beginning. Raudkivi et al. [8] reported that the inhibition threshold for NOB is small, adaptation occurs over time. The adaptation of NOB to the environment after inhibition was not studied in this trial, the changing operating conditions provided the environment for the suppression of NOB. Afterwards, NOB populations were washed out slowly along with sustained effective repression on NOB.

These results also indicated that the action of ratio control to achieve nitritation by first limit NOB activity and then wash out NOB from the reactor, nitritation can be achieved without the need of completely washing out NOB. In addition, it could be found that the share of AOB increases together with the share of NOB decreases. The activity of AOB populations was also slightly limited when applying ratio control, showing that through ratio control both AOB and NOB are limited by oxygen and it is the lower tolerance for oxygen-limiting conditions that make NOB be washed out. Previous theoretical research involving mathematical modeling of a biofilm reactor declared that applying ratio control to obtain oxygen-limiting conditions was the innate character to achieve nitritation [15,26].

3.3. Slow wash-out process of NOB during achieving nitritation

As mentioned above, in days 81–170 nitritation was achieved through restraining and washing out NOB, nevertheless, the wash-out of NOB felled behind the achievement of nitritation. To further investigate the slow wash-out process of NOB, the spatial distributions of total bacteria, AOB and NOB were identified by FISH-CLSM techniques and the spatial distribution of live and dead bacteria were identified by FISH-CLSM techniques.

Distribution of total bacteria during whole operation was almost stable as shown in Fig. S1, while that of AOB and NOB changed significantly. The AOB populations grew slightly between days 81 and 102, after that they grew remarkably. Conversely, the NOB populations reduced slightly in days 81–102 after which that reduced dramatically and in day 170 little NOB was observed. It was remarkable that the reduction of NOB populations mainly occurred in the internal portion of the biofilm which was close to the solid phase.

Results about the live and dead bacteria in different days are described in Fig. 3. The figure reveals that the number of dead bacteria in day 120 is largely higher than that in days 81, 102 and 170. The share of dead bacteria in total bacteria (live and dead bacteria) remains nearly stable between days 81 and 102. In day 170, the number of dead bacteria decreases dramatically and the number of live bacteria increases dramatically compared with that in days 81, 102 and 120. It can be concluded that between days 102 and 170 too much bacteria was dead through ratio control. In this study, nitrification was the main process. While, as discussed above, NOB populations reduced largely from days 102 to 170. Up to here, it was convincing that NOB populations was mainly repressed not dead in days 81–102 and went dead resulting in being washed out in days 102–170.

To further investigate the wash-out route of dead NOB populations, 3D-EEM spectra of EPS samples were measured in days 81, 102, 120 and 170, respectively. Fig. S2 shows EEM fluorescence spectra of EPS in different days. All the fluorescence parameters of the spectra, including peak location, fluorescence intensity and chemical composition are summarized in Table S2.

Results showed that not only the major fluorescence substances but also the peak locations and fluorescence intensities of EPS samples have changed, and the changes indicate that EPS played an important role during the achievement of biofilm nitritation. As shown in Fig. S2, three main fluorescence peaks (peak A, B and C) were identified on day 81. Peak A was observed at Ex/Em of 275/336 nm, which was assigned to tryptophan PN-like substance belong to cell metabolites. Peak B and Peak C were identified at Ex/Em of 225/312 and 225/348 nm, respectively, which were both related to aromatic PN-like substance belong to cell component [27]. Peak A also appeared on days 102 and 120 and disappeared on day 170. Peak B even appeared on days 102, 120 and 170 while peak C disappeared on days 102 and 170 and appeared on day 120. Moreover, peak E (Ex/Em of 275/308) generated when nitritation was achieved on days 120 and 170, which was assigned to tyrosine PN-like substance belong to cell metabolites [28].

Peaks A and E assigned to tryptophan PN-like substance belong to cell metabolites, representing bacterial growth by fluorescence intensity. Meanwhile, peaks B and C were assigned to aromatic PN-like substance belong to cell component, representing bacterial decomposition by fluorescence intensity [27,28]. Fluorescence intensity of peaks A and B on day 102 was significantly lower than that on days 81, 120 and 170, I think most possibly resulted from the just repression on NOB (no obvious additional NOB death and AOB growth) through ratio control. Besides, peak D was only identified on day 102, which might have resulted from the same reason. From day 102 to 120, fluorescence intensity enhanced sharply under stable ratio control, especially for that of peaks A and B, corresponding to during which AOB populations grew and NOB populations reduced obviously. Four main fluorescence peaks (Peak A, B, C and E) were identified on day 120 all fluorescence intensity of which were enhanced, indicating

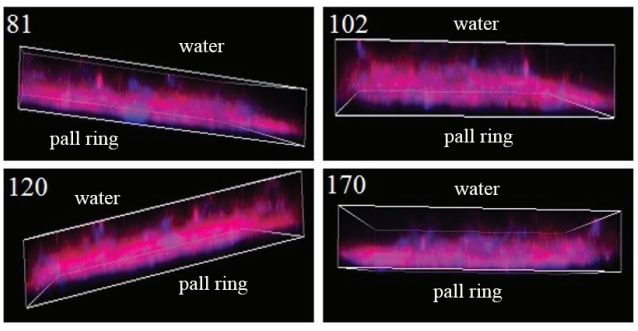

Fig. 3. Spatial distribution of live and dead bacteria in different days (blue: live bacteria; red: dead bacteria).

that the phenomenon of AOB populations growth and NOB populations decomposition was obvious.

In summary, the wash-out process of NOB from the reactor during achieving nitritation through ratio control is effective repression on NOB populations, death of NOB populations. So far, little research has focused on the wash-out process of NOB populations from the biofilm reactor, especially during achieving nitritation through ratio control.

3.4. Effect of organic carbon on nitritation performance through ratio control

Since organic carbon is the main component in domestic wastewater, it should be taken into account when treating domestic wastewater by biofilm nitritation through ratio control. As discussed in the performance of MBBR operation, an equal number of COD (C/N = 1) should be adequate to achieve stable nitritation at temperature of 8°C and more organic carbon do not make the performance of ratio control better to achieve stable nitritation.

To explain in detail, the effect of organic carbon on nitritation performance through ratio control, biofilm mass (VSS), biofilm thickness, OUR, and effluent TSS measurements was carried out in days 190 (C/N = 0), 240 (C/N = 1) and 310 (C/N = 2), respectively. Biofilm mass in days 190, 240 and 310 were 680±12, 1,990±14 and 2,010±11 mg/L, respectively. Apparently, dramatic increase of biofilm mass between days 190 and 240 must be resulted from the addition of organic carbon; however, there was no significant difference between that in days 240 and 310. Young et al. [29] has pointed out that biofilm density would fluctuate within 20% in a carbon-rich nitrifying MBBR transformed from post-carbon removal system. Hence, in this study variation of biofilm density resulted from organic carbon could be ignored compared with variation of biofilm mass (about 300%) which was same resulted from organic carbon. Thus, average biofilm thickness in days 240 and 310 could be considered as three times of that in day 190 which should be one of the main reasons for the positive effect of organic carbon on nitritation performance through ratio control. Actual obtained biofilm thickness through CLSM in days 190, 240 and 310 were 38, 84 and 88 μm, respectively. Undoubtedly, the increase of biofilm thickness must be due to the proliferation of heterotrophic bacteria which has stronger competition for oxygen compared with nitrifying bacteria [30]. Therefore, heterotrophic layer would be formed in the external of biofilm making contribution to the depletion of oxygen inside biofilm which was beneficial for repression on NOB.

Afterwards, to quantize the ability of biofilm to consume oxygen, OUR measurement was carried out in days 190, 240 and 310, respectively, which were 2.27, 6.84 and 7.05 mg/(L·min), respectively. Clearly, OUR increased dramatically when C/N improved from 0 to 1 but remained stable when C/N improved from 1 to 2. The contribution of organic carbon to achieve stable nitritation at low temperature was identical to the variation tendency of OUR in this study which should be the essential reason. Moreover, through calculation SOUR in days 190, 240 and 310 were 0.20, 0.21 and 0.21 mg/(L·VSS·min), respectively, among which there was no significant difference. It can be drawn that the increase of OUR mainly resulted from the increase of biofilm mass and not the biofilm bacterial activity.

As we all know, NOB populations would grow more easily in the case of long sludge age [15,31]. Effluent TSS values in days 190, 240 and 310 were about 13.2, 78.3 and 88.6 mg/L, respectively. Taking biofilm mass and HRT into account, calculated sludge age in days 190, 240 and 310 were about 32, 18 and 17 d, respectively. Advantageously, sludge age of biofilm mass could be shortened by nearly half which might play a positive role in repression on NOB.

3.5. Practical implications

For mainstream wastewater treatment in a continuous MBBR, PN appears to be the better choice compared with full nitritation due to the low strength of mainstream and the limit of effluent NH^{4+} concentration, especially at low temperature [20,32]. It is more applicable to achieve PN under high DO concentration compared with low DO concentration under which nitritation rate will be limited and strict control is also needed to maintain low DO concentration [10,33]. In this study, at temperature not lower than 12°C, stable PN could be achieved easily under high DO concentration (4.0 mg/L) mainly through effluent TAN concentration control. In practical operation, in order to control DO concentration not higher than 4.0 mg/L too much DO online control is not needed. Then the focus of ratio control would be the effluent TAN concentration control which is also necessary for inflow demand of subsequent anammox process.

Even at temperature of 8°C, stable PN also could be achieved easily under high DO concentration (5.0 mg/L) mainly through effluent TAN concentration control. Differently, at this temperature, an equal number of COD (C/N = 1) was needed. Normally, the C/N of actual mainstream wastewater in wastewater treatment plant (WWTP) is certainly higher than 1. Especially, at low temperature, mainstream wastewater presented in WWTP also has high organic carbon content. Even if carbon removal system is set before PN process, there will still be organic carbon presence in the inflow of PN process due to impossible 100% removal rate in carbon removal system. On the other hand, if little organic carbon was remained after pre-carbon removal system resulting in insufficient organic carbon to achieve stable PN at low temperature, operating conditions of pre-carbon removal system could be adjusted to reduce removal rate. As discussed above, it can be drawn that to achieve stable PN for actual mainstream wastewater (60 mg N/L) through ratio control, effluent TAN concentration control is the key. Strict effluent TAN concentration online control plus warning about over-DO concentration (for instance 5.0 mg/L) should be a suitable control strategy to achieve stable PN at low temperature, even at temperature of 8°C.

4. Conclusions

Stable PN for low strength wastewater (60 mg N/L) at low temperature (15°C, 12°C and 8°C) in a continuous MBBR was achieved through ratio control under high DO concentration. Slow wash-out process of NOB populations from the reactor during achieving nitritation through ratio control can be summarized as effective repression on NOB populations, death of NOB populations. Organic carbon contained in the

wastewater is largely beneficial to achievement of stable PN at low temperature (8°C) under high DO concentration. But not the more organic carbon the better nitritation performance and an equal number of COD (C/N = 1) should be adequate. In practical applications, to achieve stable PN for actual mainstream wastewater under high DO concentration at temperature not lower than 8°C, strict effluent TAN concentration online control plus warning about over-DO concentration (for instance 5.0 mg/L) should be a suitable control strategy.

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Supplementary

$$FA = \frac{17}{14} \times \frac{c(NH_4^+ - N) \times 10^{pH}}{e^{\left(\frac{6344}{273+T}\right)} + 10^{pH}} \quad (S1)$$

$$FNA = \frac{46}{14} \times \frac{c(NO_2^- - N)}{e^{\left(\frac{-2300}{273+T}\right)} + 10^{pH}} \quad (S2)$$

where T (°C) was temperature.

Table S1
Oligonucleotide probes used for FISH analysis

Probes	Target bacteria	Fluorescent dye	Colour
EUB (338 + 338II + 338III)	Total bacteria	FAM	Green
AOB_{mix} (NSO1225 + NSO190)	AOB	Cy3	Red
NOB_{mix} (NIT3 + NTSPA662)	NOB	Cy5	Violet

Table S2
Fluorescence spectra parameters and their corresponding types of chemical composition in different days

Time (day)	Peak	Ex/Em (nm)	Intensity	Chemical composition
81	A	275/336	966.3	Tryptophan PN-like
	B	225/312	1,425.11	Aromatic PN-like I
	C	225/348	1,266.04	Aromatic PN-like II
102	A	275/334	310.33	Tryptophan PN-like
	B	220/328	459.6	Aromatic PN-like I
	D	325/416	112.56	Humic acid-like
120	A	275/342	842.25	Tryptophan PN-like
	B	225/308	1,461.42	Aromatic PN-like I
	C	220/342	1,245.16	Aromatic PN-like II
	E	275/308	844.3	Tyrosine PN-like
170	B	225/310	1,353.71	Aromatic PN-like I
	E	275/306	822.9	Tyrosine PN-like

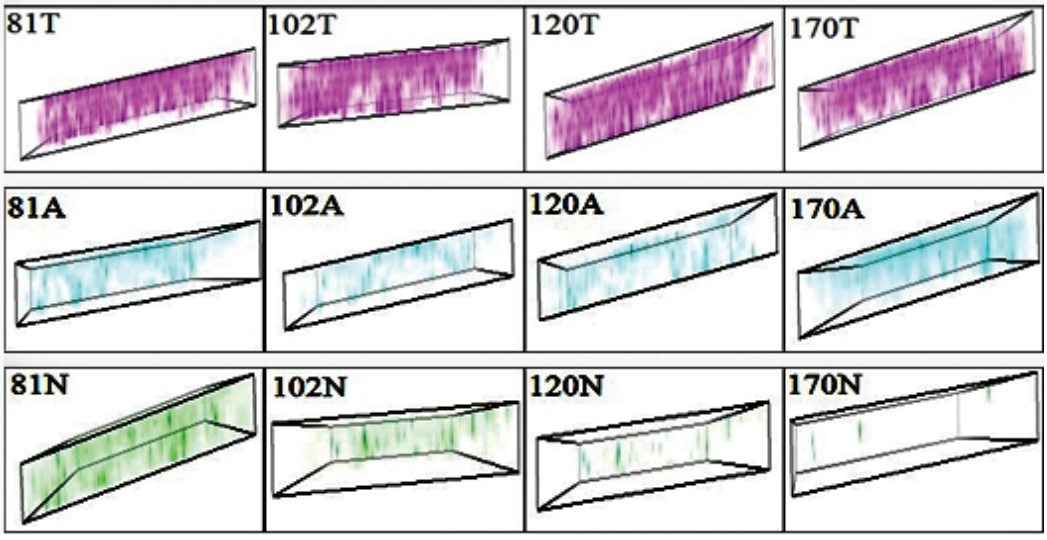

Fig. S1. Spatial distribution of total bacteria, AOB and NOB in different days (T – total bacteria; A – AOB; N – NOB; in order to see more clearly, the colour of original pictures has been adjusted).

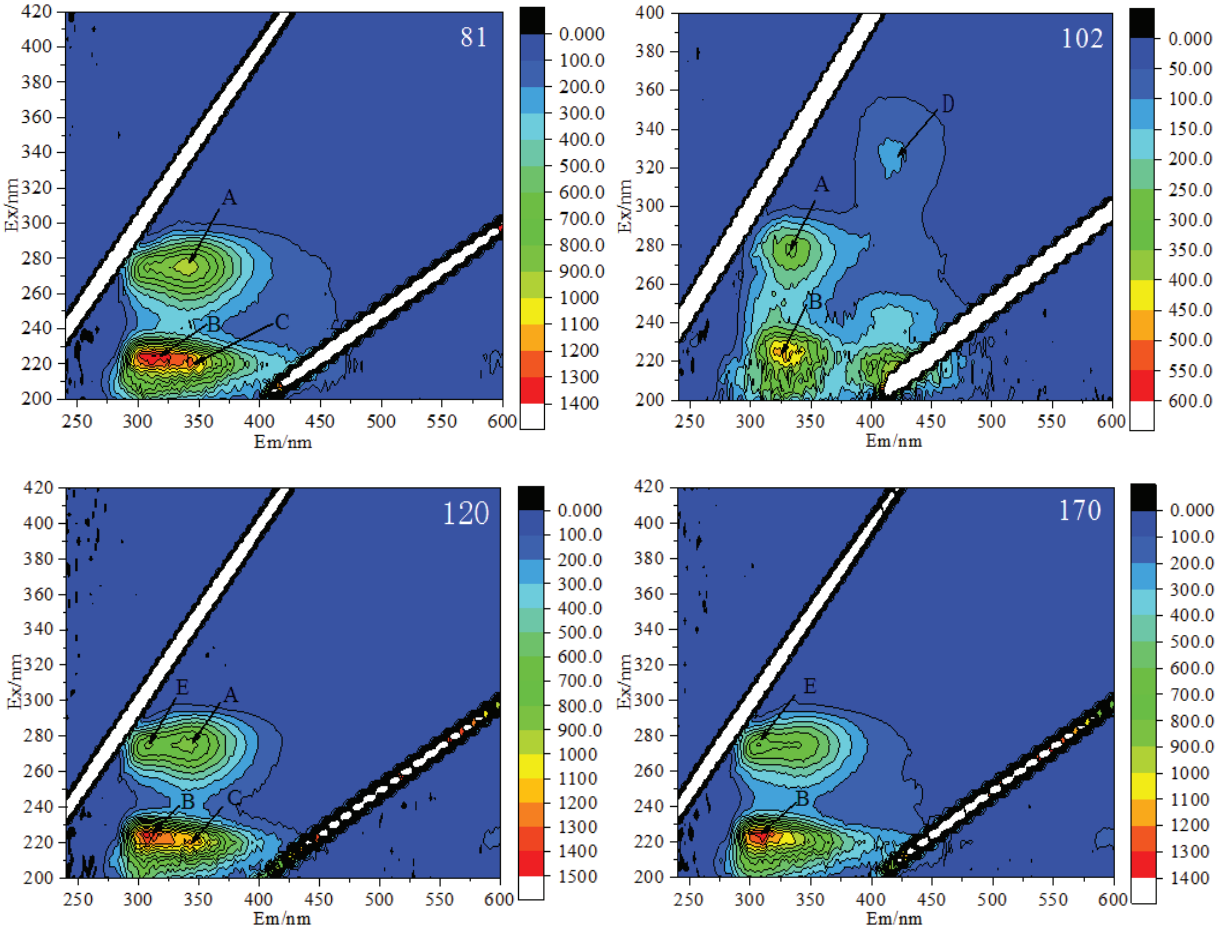

Fig. S2. 3D-EEM fluorescence spectra of EPS in different days.