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Macroscale and microscale analysis of Anammox in anaerobic rotating biological contactor

Yongtao Lv[1], Lei Wang[1,*], Xudong Wang[1], Yongzhe Yang[1], Zhiying Wang[1], Jie Li[2]

1. School of Environmental and Municipal Engineering, Xi'an University of Architecture and Technology, Xi'an 710055, China. E-mail: hybos2000@126.com
2. School of Environmental and Municipal Engineering, Lanzhou Jiaotong University, Lanzhou 730070, China

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Abstract

Inoculated with conventional anaerobic activated sludge, the Anammox process was successfully developed in an anaerobic rotating biological contactor (AnRBC) fed with a low ratio of C/N synthetic wastewater. Operated in a single point feed mode, the AnRBC removed 92.1% (n = 126) of the influent N at the highest surface load of 12 g/(m^2·day). The biomass increased by 25% and 17.1 g/(m^2·day) of maximum N removal surface load was achieved by elevating flow rate with another feed point. Fluorescence *in situ* hybridization and polymerase chain reaction analysis indicated that the Anammox genus *Candidatus Kuenenia stuttgartiensis* dominated the community. Both Anammox and denitrifying activity were detected in biofilm by the application of microelectrodes. In the outer layer of the biofilm (0–2500 μm), nitrite and ammonium consumed simultaneously in a ratio of 1.12/1, revealing the occurrence of Anammox. In the inner layer (> 2500 μm), a decrease of nitrate was caused by denitrification in the absence of nitrite and ammonium.

Key words: Anammox; AnRBC; feed mode; microelectrodes

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Introduction

The Anammox process is a biological reaction in which ammonia is oxidized to nitrogen gas using nitrite as the electron acceptor under anaerobic conditions. The stoichiometry of this reaction proposed by Strous et al. (1998) is shown in Eq. (1), in which ammonium and nitrite, in almost equimolar ratio, react to produce dinitrogen gas and a small quantity of nitrate.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \longrightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15} \quad (1)$$

The Anammox process has been put forward as a new and promising method to be combined with nitritation for nitrogen removal devoid of organic carbon. In addition, the low amount of wasted sludge would also reduce operational costs. Jetten et al. (2001) reported that the Anammox process can save up to 90% of operating cost as compared to traditional nitrogen treatment processes.

However, the low growth rate of anaerobic ammonium-oxidizing bacteria (AnAOB) (doubling time 11 days (Strous et al., 1998)) makes the start up of this process difficult. For example, the startup period of a full-scale plant built in Rotterdam was approximately two years (Van der Star et al., 2007). To start up the Anammox process, the choice of reactor type is very important. Various suspended systems, including fluidized bed reactor (van de Graaf et al., 1996), sequencing batch reactor (SBR) (Strous et al., 1998; Dapena-Mora et al., 2004; Third et al., 2005; Nutchanat and Suwanchai, 2007), and gas-lift reactor (Sliekers et al., 2003), were applied and optimized to start up the Anammox process. However, a fraction of the generated biomass was inevitably washed out with the effluent in these systems. Although membrane bioreactor (Trigo et al., 2006; Wang et al., 2009) can obtain full biomass retention, the fouling behavior of membranes would increase the operation cost significantly. An alternative for Anammox startup is anaerobic rotating biological contactor (AnRBC), a biofilm reactor with long sludge retention time, which can obtain enough biomass and operate easily. In addition, autotrophic nitrogen loss discovered in a rotating biological contactor (RBC) treating ammonium-rich leachate indicated that AnAOB was responsible for N removal (Wyffels et al., 2003), which revealed that RBC was suitable for cultivation of AnAOB.

* Corresponding author. E-mail: wl0178@126.com

Microelectrodes, i.e., needle-shaped devices with a sensitive tip, have been proven to be the most suitable and indispensable tools for micro-environmental samples measurements, especially in stratified biofilms (Satoh et al., 2005; Chae et al., 2008) and sediment mats (Jensen et al., 1993; De Beer et al., 1997a) for nitrifying and/or denitrifying activity. However, microelectrodes used to explore the nitrogen conversion of Anammox process have not been reported.

In this study, an AnRBC was performed for Anammox startup from conventional anaerobic activated sludge. The effect of feed mode on TN removal capacity was investigated. Fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR) analysis were used to analysis the existence and genus of AnAOB, and microelectrodes were employed to explore the nitrogen transformation in the biofilm.

1 Materials and methods

1.1 Experimental setup

An AnRBC with a net liquid capacity of 6.2 L was used. It has 13 polyvinyl chloride disks, with a total surface area of 0.32 m^2 and 87% submersion of the disk surface. The reactor was kept at 35 ± 5°C by a water jacket.

1.2 Origin of biomass and synthetic wastewater

The anaerobic sludge originated from a brewery wastewater treatment plant was inoculated, with mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS)/MLSS 4.2 g/L and 0.89, respectively. Synthetic wastewater was used in this study, mainly contained $NaHCO_3$, NH_4Cl, $NaNO_2$, $C_6H_{12}O_6$, and trace elements as described by van de Graaf et al. (1996).

1.3 Chemical analysis

NH_4^+-N, volatile suspended solids (VSS) and total suspended solids (TSS) were determined according to the standard methods (APHA, 1998). NO_3^--N and NO_2^--N were analyzed using a 761 basic ion chromatograph (Methohm, Switzerland).

1.4 Fluorescence *in situ* hybridization (FISH)

Biomass samples from the reactor were collected and fixed according to the procedure described by Amann (1995) with 4% paraformaldehyde solution. Hybridization was performed at 46°C for 90 min with 40% of formamide concentration. The used Amx820 probe (Schmid et al., 2001) for *in situ* hybridization was 5' labeled with the fluorochromes fluorescein isothiocyanate (FITC). An epifluorescence microscope (Nikon eclipse 90i, Japan) was used for image acquisitions.

1.5 DNA extraction and polymerase chain reaction (PCR)

Total DNA was extracted from the biofilms using Fast DNA spin kit. Primers of AMX818F/AMX1066R(5′-ATGGGCACTMRGTAGAGGGGTTT-3′, target site: 818–839, and 5′-AACGTCTCACGACACGAGCTG-3′, target site: 1047–1066) were used as described by Ikuo et al. (2007). 16S rRNA gene fragments were amplified from the extracted total DNA with a total volume of 40 μL reaction mixture containing 4 μL of buffers, 4 μL of Dntp (2.5 mmol/L), 0.8 μL of Taq DNA polymerase, 8 μL of primer, 2 μL of sample DNA and 21.2 μL of sterilized water. The PCR conditions targeted for AnAOB were as follows: 300 sec initial denaturation at 94°C, 25 cycles of 60 sec at 94°C, 60 sec at 50°C and 70 sec at 72°C. Final extension was carried out for 300 sec at 72°C.

1.6 Microelectrodes preparations and measurements

Microelectrodes for pH, NH_4^+, NO_2^- and NO_3^- with tip diameters of approximately 15 μm were manufactured in our laboratory, and were constructed, calibrated and used according to the methods of De Beer et al. (1997b).

The biofilm was placed in a flowcell reactor and incubated for two hours in synthetic medium containing (mmol/L): NH_4Cl 0.3, $NaNO_2$ 0.3, $NaNO_3$ 0.05, Na_2HPO_4 0.57, $MgCl_2$ 0.084, $FeCl_3$ 0.185, $CaCl_2$ 0.02, $MnCl_2$ 0.01, and EDTA adjusted to pH value.

2 Results and discussion

2.1 startup and operation of Anammox

To start up the Anammox process, denitrifying biofilm was firstly cultivated and thus the COD concentration was decreased for AnAOB enrichment. Once the Anammox activity appeared, the influent nitrogen concentrations were lowered to gain higher N removal efficiency. The operational strategy consisted of increasing nitrogen loading rate (NLR) by means of elevating the influent ammonium and nitrite concentrations and shortening the HRT. Parameter settings of the AnRBC operated in a single point feed mode are summarized in Table 1.

During the first 5 days, the ammonium concentration in the effluent was a bit higher than that in the influent, which was caused by some dissolved biomass (Fig. 1a). From day 27, ammonium removal increased little. From day 120, the ammonium removal efficiency increased significantly by lowering the influent concentration. On day 142, around 80% of ammonium was removed.

Compared to ammonium removal, the effluent nitrite concentration decreased at the beginning and the removal efficiency was related to the influent COD concentrations, which was contributed to denitrification (Fig. 1b). The phenomenon has been also reported in previous literatures

Table 1 Characteristics of the AnRBC operation during different periods

Time (day)	Flow rate (L/day)	Influent concentration (mg/L)		
		NH_4^+-N	NO_2^--N	COD
1–26	4.8	100–120	100–120	200
27–119		100	100	20
120–162		70	70	20
164–305	6.2	70–280	70–305	20–80

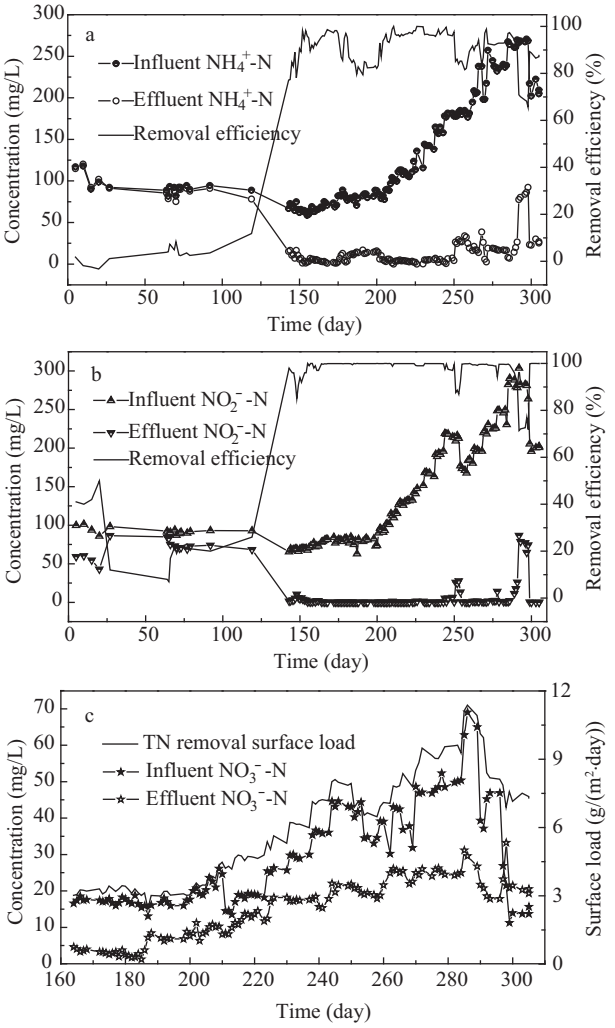

Fig. 1 Performance of the AnRBC. (a) NH_4^+-N removal; (b) NO_2^--N removal; (c) NO_3^--N removal and TN removal surface load.

(Dapena-Mora et al., 2004; Third et al., 2005; Nutchanat and Suwanchai, 2007; Wang et al., 2009). On day 142, nitrite and ammonium removed simultaneously revealed the achievement of Anammox process.

According to Anammox stoichiometry (Eq. (1)), small quantity of nitrate produced with the decrease of both ammonium and nitrite and the maximum TN removal efficiency was calculated to be 88.8%. However, in this system nitrate removal was also observed due to denitrification (Fig. 1c). From day 164 to day 290, the maximum and average TN removal efficiency reached 97.5% and 92.1%, respectively, at the highest influent surface load of 12 g/(m^2·day) demonstrating that it was more efficient for TN removal in a high N/C system than in a completely autotrophic system. At the same time, the TN removal surface load increased from 3 to 11.4 g/(m^2·day) by increasing the influent concentrations (Fig. 1c), and 0.59 kg/(m^3·day) of the maximum TN removal volume load was achieved. However, when the influent ammonium and nitrite concentrations were elevated to 270 and 290 mg/L, the removal efficiency decreased. It might be caused by nitrogen overload, and therefore, the TN removal capacity could not be improved by increasing influent concentrations at single-feed-point mode.

2.2 Effect of feed mode on Anammox process in AnRBC

When the reactor was fed in single point, the biofilm depth decreased along the flow direction and only the first three disks were filled with biofilm (Fig. 2a). From the 8th disk on, there was little biomass, which was caused by lack of substrate. To offer sufficient substrate and improve the nitrogen removal capacity, another feed point was set between the 4th and the 5th disk. Parameter settings of a two-feed-point mode are summarized in Table 2.

After 70 days of continuous operation, obvious variation of biomass was observed (Fig. 2a). The biomass increased by approximately 25% and the biofilm depth increased from the 4th to 9th disk. The TN removal surface load increased from 11 to 17 g/(m^2·day) (Fig. 2b) and 90.3% of average TN removal efficiency (n = 71), 0.71 kg/(m^3·day) of maximum TN removal volume load and 17.1 g/(m^2·day)

Table 2 Characteristics of a two-feed-point operation

Feed point 1		Feed point 2	
Flow rate (L/day)	Concentration (mg/L)	Flow rate (L/day)	Concentration (mg/L)
Q_1 = 6.2	NH_4^+-N: 220–280 NO_2^--N: 220–290 COD: 50–80	Q_2 = 3.1	NH_4^+-N: 110–150 NO_2^--N: 140–180 COD: 20–30

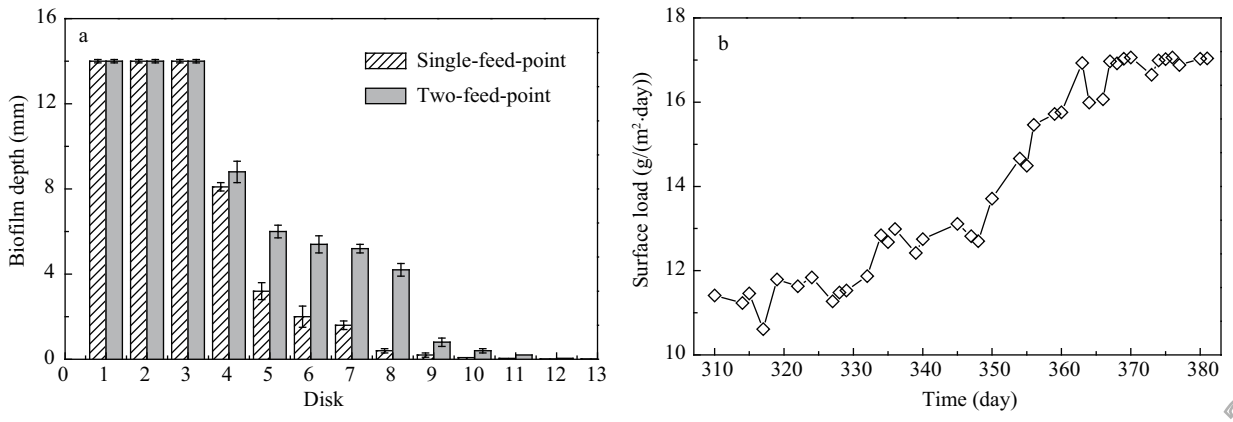

Fig. 2 Effect of feed mode on Anammox process in AnRBC system. (a) variation of biomass; (b) N removal capacity.

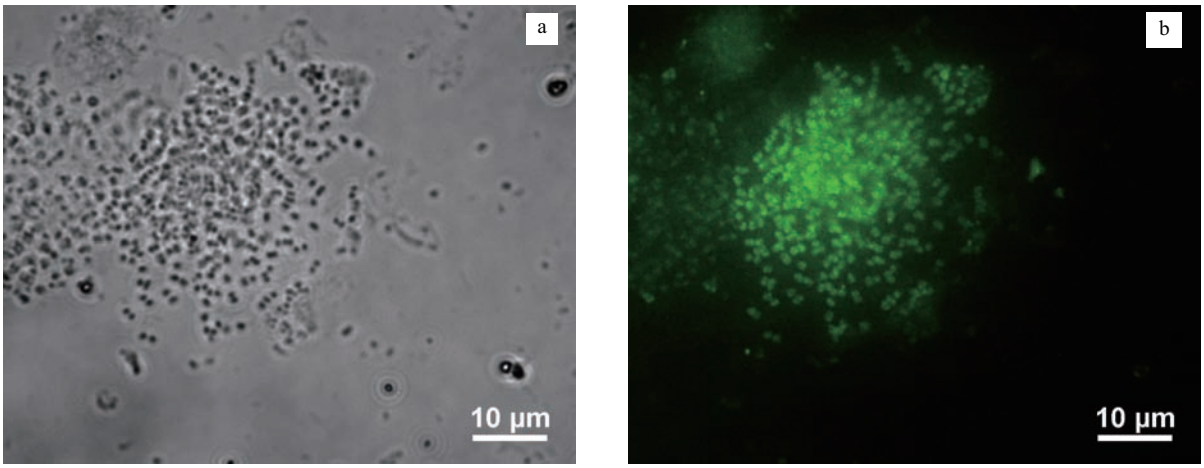

Fig. 3 FISH analysis of the biomass. (a) phase contrast-micrograph; (b) the same section of biomass showing AnAOB stained with FITC (green).

of maximum TN removal surface load were achieved. The TN removal surface load increased by 40% compared to single feed point.

2.3 FISH and PCR analysis

By the application of FISH probe, bacteria belonging to AnAOB were identified as the dominant population (Fig. 3), accounting for around 80% of the total biomass according to a rough calculation, which indicated the presence of *Candidatus Brocadia anammoxidans* and/or *Candidatus Kuenenia stuttgartiensis*.

PCR was conducted with the primers AMX818F/AMX1066R. Products of 16SrDNA with full length of 241 bp (TGCGGAGCTAaCGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAAGAATTGACGGGGGCTCGCACAAGCGGTGGAGCATGTGGCTTAATTCGATGCAACGCGAAGAACCTTACCGGGGCTTGACATGGTAGAAGTAGAATCCTGAAAGGGTGACGATCGGTATCCAGTCCGAAGCTATCACAGGTGTTGCATGGCTGTCGTCAGCTCGTGTCGtGAGACGTTAA) were obtained after purification. Contrasted with Anammox species reported, the sequence was 100% similar to *Candidatus Kuenenia stuttgartiensis* (Schmid et al., 2000), which confirmed the genus of AnAOB.

2.4 Transformation of nitrogen compounds in biofilm

The nitrogen conversion in the biofilm was measured by the application of microelectrodes (Fig. 4). In the outer layer of the biofilm (0–2500 μm), nitrite and ammonium consumed simultaneously in ratio of 1.12 and both of them depleted at about 2500 μm, while nitrate increased slightly and a peak of 60 μmol/L was observed at 2000 μm. It is in contrast to observations of nitrification or denitrification in biofilm (Satoh et al., 2005; Chae et al., 2008). The pH value increased slightly from 8.13 to 8.19. All of these revealed the occurrence of Anammox. The ratio of nitrate produced to ammonium consumed was only 0.04, stoichiometrically, denitrification occurred simultaneous in this region. However, in the inner layer of the biofilm (> 2500 μm) nitrate decreased in the absence of nitrite and ammonium, showing the activity of denitrifying bacteria.

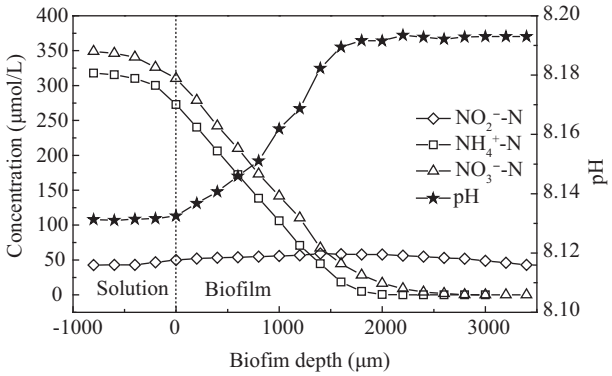

Fig. 4 Micro-profiles of nitrogen compounds inside the biofilm.

3 Conclusions

This study showed that AnRBC was a suitable reactor for AnAOB cultivation and enrichment. A low ratio of C/N system was more efficient for nitrogen removal than a completely autotrophic system, and 92.1% of an average TN removal efficiency was achieved at the highest influent surface load of 12 g/(m^2·day). The TN removal capacity could be elevated to 17.1 g/(m^2·day) by adding another feed point. The activity of both AnAOB and denitrifying bacteria were detected in biofilm using microelectrodes. In the outer layer of the biofilm, nitrite and ammonium were consumed in a ratio of 1.12/1 with slight increment of nitrate, revealing the occurrence of Anammox. In the inner layer, denitrification was responsible for the decrease of nitrate in the absence of nitrite and ammonium.

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