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Nitrogen removal performance and microbial community structure in the start-up and substrate inhibition stages of an anammox reactor

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In this study, the nitrogen removal performance and microbial community structure were investigated during the start-up, instability, and recovery stages of an anaerobic ammonium oxidation (anammox) reactor loaded with compound carriers (shale ceramsite and suspended ball carrier). The results indicated that the anammox reactor successfully started up on 116th d when the nitrogen loading rate (NLR) reached 0.72 ± 0.05 kg N m^{-3} d^{-1}. The anammox reactor ran well with free ammonia (FA) at 13.65 ± 2.69 mg/L and free nitrous acid (FNA) at 39.49 ± 10.95 μg/L, indicating that its tolerance for FA and FNA was higher than that of granular sludge anammox reactors. The anammox system was inhibited when FA and FNA reached 29.65 mg/L and 77.02 μg/L, respectively. The tolerance of anammox bacteria towards FA and FNA decreased after this inhibition. The nitrogen removal performance could be efficiently recovered by decreasing the influent substrate concentration and increasing the hydraulic retention time (HRT). *Candidatus* Brocadia and *Candidatus* Jettenia, two genus-level anammox bacteria, were detected in this reactor using a high-throughput sequencing technique. After high substrate shock, the abundance of *Candidatus* Brocadia decreased while that of *Candidatus* Jettenia increased, which might be due to the competition between *Candidatus* Jettenia and *Candidatus* Brocadia. The relationships between anammox communities and operational factors were investigated via redundancy analysis (RDA), which showed that FA was the principal factor affecting the microbial community structure during the operation stage.

[**Key words:** Anammox; Carrier; Start-up; Nitrogen removal; Inhibition and recovery; Microbial community]

The anaerobic ammonium oxidation (anammox) process can directly convert ammonium into nitrogen gas, with nitrite as the electron acceptor, under anoxic conditions, according to Eq. 1. Compared to the nitrification-denitrification process, anammox reduces exogenous electron donors and sludge production and has a lower operating costs (1). Therefore, using the anammox process to treat high-strength ammonium wastewater has become increasingly popular in the field of environmental engineering.

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

Owing to the low cellular yield and slow growth rate of anammox bacteria, longer start-up periods were considered an important challenge in engineering applications. Efforts to enrich the anammox bacteria and shorten the start-up periods have been made by selecting suitable reactor types (2,3), using sludge washout strategies (4), enhancing the activity of ammonium oxidation (5), and using different types of carriers (6,7). Among these strategies, introducing specific carriers into the reactor seems to be the best method to enrich anammox bacteria. Moreover, anammox bacteria are very sensitive to environmental factors, such as the presence of organic matter, salinity level, and high substrate concentrations. The low growth rate of anammox microorganisms in combination with the inhibition effects and operational problems complicates the start-up of the anammox process. Although the inhibition caused by high substrate concentrations is known (8–10), it is necessary to further classify the differences in substrate tolerance and restorability degree after inhibition among different reactors. High substrate concentrations could also result in the increase of free ammonia (FA) and free nitrous acid (FNA), which would further inhibit the anammox system (11), meaning high substrate concentrations could affect the anammox process directly and indirectly. Thus, there is a need to study the effects of high substrate concentrations and associated inhibitions caused by FA and FNA on the anammox process.

To date, bacterial species with anaerobic ammonium-oxidizing functions have been found in five candidate genera, that is, *Candidatus* Brocadia, *Candidatus* Kuenenia, *Candidatus* Anammoxoglobus, *Candidatus* Jettenia, and *Candidatus* Scalindua (12–14). A shift in environmental conditions alters the microbial communities and thus affects the performance of the anammox process. Therefore, researching microbial community structure characteristics is of utmost importance because it can provide avenues for understanding nitrogen removal processes occurring in the anammox system.

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In this study, an up-flow anaerobic sludge bed (UASB) loaded with simple and inexpensive compound carriers (shale ceramsite and suspended ball carrier) was used to set up an anammox reactor. The aim of this study was twofold: to investigate and evaluate the start-up and recovery performance after substrate inhibition and to analyze the changes of microbial communities in order to further clarify the biological process in the anammox reactor.

MATERIALS AND METHODS

Experimental setup The experimental work was carried out in a laboratory-scale UASB reactor (8.8 L working volume) with an internal diameter of 100 mm and a height of 900 mm (Fig. 1). The reactor was loaded with simple and inexpensive compound carriers composed of shale ceramsite and suspended ball carrier, which increased the microbial biofilm packing area and enlarged the contact area between the current and ceramsite. The loading volume of the packing accounted for approximately 70% of the effective volume. The reactor was covered with black rubber sponge insulation board to prevent light interaction and preserve heat. It was also equipped with an external water bath to maintain the temperature at 33–35°C. The influent was continuously supplied to the reactor via a peristaltic pump at varying hydraulic retention times (HRTs). A three-phase separator was installed at the top of the reactor. After the gas and effluent were separated using the three-phase separator, the gas was extracted from the gas chamber and the effluent flowed out through the water outlet.

Operating conditions The loading of the anammox system was changed by varying the substrate concentration and HRT throughout the operation process. Table 1 shows the specific operation parameters of the reactor, which was operated over a period of 187 d.

Influent and seed sludge The influent was synthetic wastewater composed of three parts: substrates, mineral medium, and trace elements. In this study, the growth substrates, ammonium nitrogen (NH_4^+-N) and nitrite nitrogen (NO_2^--N), were supplied by adding NH_4Cl and $NaNO_2$. The other components of the synthetic wastewater were $CaCl_2{\cdot}2H_2O$ (0.0056 g/L), $MgSO_4{\cdot}7H_2O$ (0.3 g/L), $KHCO_3$ (1.25 g/L), KH_2PO_4 (0.01 g/L), trace element solution I (1 mL/L), and trace element solution II (1 mL/L). Trace element solution I was composed of EDTA (15 g/L) and $FeSO_4$ (5 g/L). Trace element solution II contained EDTA (15 g/L), $ZnSO_4{\cdot}7H_2O$ (0.43 g/L), $CoCl_2{\cdot}6H_2O$ (0.24 g/L), $MnCl_2{\cdot}4H_2O$ (0.99 g/L), $CuSO_4{\cdot}5H_2O$ (0.25 g/L), $Na_2MoO_4{\cdot}2H_2O$ (0.22 g/L), and $NiCl_2{\cdot}6H_2O$ (0.19 g/L) (15). The seed sludge was a 1:1 mixture of denitrifying and digested sludge (sludge concentration of about 10 g mixed liquor suspended solids (MLSS)/L). The denitrifying sludge was harvested from the anoxic tank of the wastewater treatment plant located on the Nanhu campus of China University of Mining and Technology (Jiangsu, China), and the digested sludge was harvested from the digestive basin of a municipal solid waste incineration plant in Jiangsu. The mixed sludge was repeatedly washed with nutrient solution to remove impurities (such as slag) and residual pollutants (organic matter, NH_4^+-N, NO_2^--N, and so on) before inoculation.

TABLE 1. Operating conditions of the anammox reactor.

Period		Days	HRT (d)	Circulating water	Influent concentration	
					NH_4^+-N (mg/L)	NO_2^--N (mg/L)
Start-up stage	I	1–50	1	Yes	50.93 ± 3.72	65.87 ± 3.54
	II	51–69	1	–	50.93 ± 3.72	65.87 ± 3.54
	III	70–79	0.5	–	72.58 ± 4.10	87.31 ± 8.88
		80–86			97.57 ± 3.52	131.21 ± 3.05
		87–116			147.62 ± 6.51	188.89 ± 9.5
Operation stage	IV	117–142	0.5	–	198.82 ± 1.35	261.79 ± 1.42
		143–154			298.06 ± 4.92	390.88 ± 4.59
	V	155–158	0.5	–	385.76 ± 3.54	519.75 ± 2.05
		159–161	0.375	–	299.08 ± 1.52	379.03 ± 1.45
	VI	162–170	0.5	–	110.35 ± 14.2	132 ± 16.43
		171–175		–	223 ± 2.51	294.32 ± 0.45
	VII	176–180	0.417	–	103.63 ± 0.25	120 ± 1.36
	VIII	181–187	0.5	–	125.88 ± 0.13	170.42 ± 0.59

Analytical methods for standard wastewater quality indicators Influent and effluent samples were routinely collected and either analyzed immediately or stored in the refrigerator at 4°C until they were analyzed. NH_4^+-N, NO_2^--N, nitrate nitrogen (NO_3^--N), MLSS, and mixed liquor volatile suspended solids (MLVSS) were measured according to standard methods (16). A pH meter was used to measure the pH.

Microbial diversity analysis In this study, 16S rRNA gene high-throughput sequencing was used to investigate the diversity and structural dynamics of the microbial community in the anammox system. Biomass samples (S1, S2, S3, S4, S5, S6, S7, and S8) were extracted during different phases of the operational process (on 1th, 69th, 86th, 116th, 139th, 151th, 170th, and 183th d, respectively). Although S5 and S6 were in the same stage, the substrate concentrations on 139th and 151th d were considerably different, thus it was necessary to extract these samples (S5, S6). DNA was extracted from these samples using the FastDNA Spin Kit for Soil (MP Biomedicals, LLC, Solon, OH, USA). Amplification of the 16S rRNA sequence in the V4 region was carried out using the bacterial primer set of 515F (5′-GTGCC-AGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′). Multiplexing was possible through the addition of 12-nucleotide barcodes to the 5′ end of 907R. Amplifications were performed in a 20 μL mixture containing 4 μL of 5×FastPfu Buffer, 2 μL of dNTPs (2.5 mM), 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, and 10 ng of template DNA. Polymerase chain reaction (PCR) conditions consisted of the following steps: 3 min at 95°C; followed by 27 cycles of 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C; and ending with a final extension at 72°C for 10 min. The resulting PCR products were purified via a UNIQ-10 PCR Purification Kit (Majorbio, Shanghai, China). After purification, the 16S rRNA V4-region PCR products were quantified using a TBS-380 mini-fluorometer (Turner BioSystems, Sunnyvale, CA, USA). A DNA library was then constructed and run on an Illumina MiSeq platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Those without an exact match to the forward primer were eliminated to remove low-quality sequences and minimize the effects of random sequencing error. All the raw reads have been deposited at NCBI Sequence Read Archive (SRA) database under the accession number SRP133428.

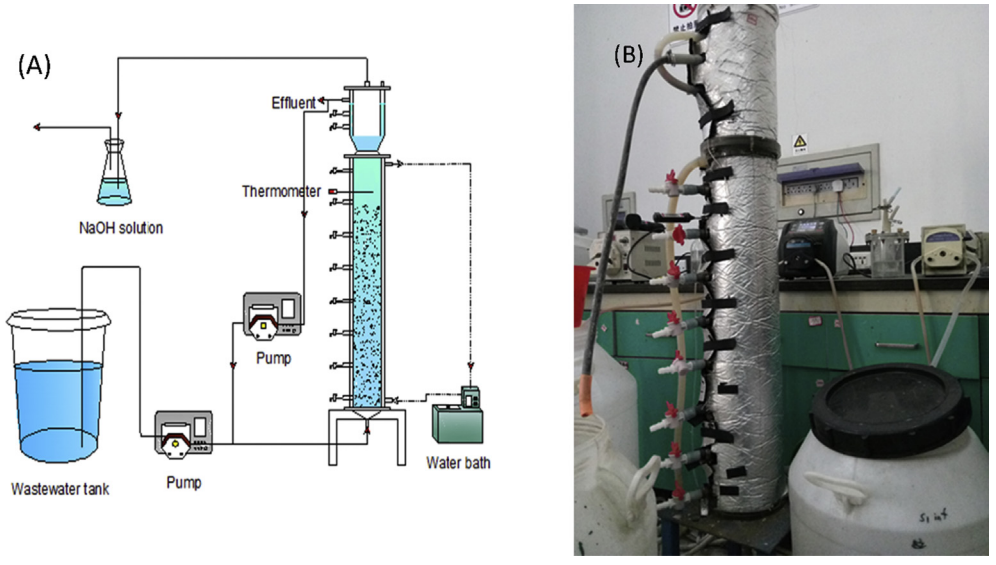

FIG. 1. Schematic diagram (A) and photograph (B) of the anammox–UASB system.

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