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Continuous nitrogen removal by a single-stage reactor packed with ring-laced string medium

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The efficiency of nitrogen removal by a partial-nitritation/anammox (PNA) reaction was investigated using a packed-bed reactor in which ring-laced strings were used as the supporting medium. A stable population of PNA microorganisms was established from typical activated sludge, after less than two months of acclimation in the packed-bed reactor, by applying a high nitrogen-loading rate (NLR: 0.53 kg/m^3/d) and short hydraulic retention time (HRT: 1.8 h). The stability of reactor performance was confirmed in industrial wastewater (IW), demonstrating a nitrogen removal efficiency (NRE) of greater than 77% during 260 days of continuous operation, between 0.19 and 0.53 kg/m^3/d of NLR. Partial nitrification was adequately controlled by low-level oxygen supply to the reactor. Pyro-tag sequencing analysis of the biofilm revealed a clear abundance of anammox bacteria in the inner part of the biofilm and ammonium-oxidizing bacteria in the outer part. In the synthetic inorganic medium (SIM), the microbial community structure did not change drastically between the early and late phases of the experiment's continuous operation, which lasted over 200 days. In IW, however, the existence ratio of anammox bacteria decreased to 4% on day 249 of continuous operation. The number of detected operational taxonomic units (OTUs) increased in the IW, implying that the community structure was widely diversified. However, anammox bacteria could propagate sufficiently to catalyze nitrogen removal under this condition because the NRE was stable at approximately 88%.

[**Keywords:** Anammox; Ammonia oxidizing bacteria; Nitritation; Deammonification; Denitrification; Packed-bed reactor]

Nitrogen removal from wastewater through anaerobic ammonium oxidation (anammox) has recently attracted much attention as a new treatment process. In this process, anammox bacteria chemoautotrophically oxidize ammonium using nitrite as an electron acceptor. Nitrite can be supplied through the oxidation of ammonium (1) and the reduction of nitrate by partial denitrification (2,3).

The anammox process offers many advantages over the conventional nitrification–denitrification process. Denitrification does not require external electron donors (e.g., methanol and acetate) as hydrogen donors, and oxygen demand is reduced due to the partial nitrification of ammonium to nitrite. Additionally, surplus sludge production is minimal because of the low cell production rate of anammox bacteria (4,5). Moreover, this process emits smaller quantities of greenhouse gasses than those emitted by conventional systems because nitrous oxide is not an intermediate in the anammox reaction (6).

Due to these advantages, by 2014, more than 100 full-scale plants had been developed and had implemented single-stage processes, which combine partial nitritation and anammox in one reactor and are also known as partial-nitritation/anammox (PNA) processes (7). In the PNA process, aerobic ammonium-oxidizing bacteria (AOB) oxidize a portion of the available ammonium to nitrite. Subsequently, anammox bacteria reduce the remaining ammonium using nitrite to produce dinitrogen gas in the absence of oxygen (8,9).

The single PNA reactor can be achieved in a functionally developed biofilm on a carrier or in granular biomass (10,11). Achieving the right balance in the activities of different microbial groups is required in biofilms, where spatially structured communities of microbes function in a complex web of symbiotic interactions. Aerobic organisms, in the outer layer of the biofilm, consume available oxygen and create anaerobic conditions in the interior of the biofilm or granule, which are suitable for anammox (12). Partial oxidation of ammonium to nitrite is also required for the successful operation of the PNA process. Simultaneously, however, nitrite has an inhibitory effect on the anammox reaction, which means that it must be controlled below threshold concentrations. In addition to the growth and maintenance of the slow-growing anammox bacteria, the activity of aerobic AOB must also be established at the right level to balance the suppression or out-selection of nitrite-oxidizing bacteria.

It has been reported that in full-scale PNA installations, the sequencing batch reactor (SBR) technology (13,14) is the most commonly applied type (more than 50% of all PNA systems), followed by granular systems (15) and moving bed biofilm reactors (MBBRs) (16). A few rotating biological contactors (17) and activated sludge systems (18) are also in operation. Only a few studies have been conducted on the continuous-flow operation of PNA processes.

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The packed-bed reactor was developed to maximize the amount of microbial biomass inside the reactor. A microbial biofilm was generated and immobilized on the surface of the installed supporting medium/carrier. In traditional activated sludge treatment, microorganisms are suspended in the liquid in a continuous stirred-tank reactor. In a packed-bed reactor, however, they are fixed on a stationary support. Various types of supporting media have been reported in wastewater treatment processes, including granulated activated carbon (19) and porous ceramic carriers based on casting sand, sawdust, and zeolite (20). Packed-bed type reactors enable a smaller reactor size with high efficiency and robustness against hydrodynamic variations and loading shocks in the inlet concentration. Authors have reported high-performance methanogenesis in the thermophilic down-flow anaerobic packed-bed reactor (TADPR) using an unwoven carbon fiber textile as the supporting medium (21).

The ring lace consists of fibrous string bundles and has mainly been used in aerobic wastewater treatment. There have been few reports, however, on PNA processes using ring-laced strings as a packed medium in the continuous-flow mode. In this study, nitrogen removal efficiency (NRE) using a single packed-bed reactor, in which ring-laced strings were installed as the supporting medium for PNA biofilm development, was examined. The reactor performance was evaluated using both synthetic inorganic medium (SIM) and industrial wastewater (IW) in continuous operation. Microbial composition in the reactor biofilm and the succession of microbial composition were also studied by pyro-tag sequencing analysis targeted on the 16S rRNA gene to evaluate NRE in this type of reactor.

MATERIALS AND METHODS

Experimental setup for anammox enrichment A synthetic inorganic medium (SIM) containing $(NH_4)_2SO_4$ and $NaNO_2$, at equal molar nitrogen concentrations, was used to evaluate the enrichment of anammox bacteria. The medium contained (per liter of demineralized water) the following: $(NH_4)_2SO_4$ 30, 165 mg/L; $NaNO_2$ 30, 195 mg/L; $KHCO_3$, 500 mg; KH_3PO_4, 27.2 mg; $MgSO_4 \cdot 7H_2O$, 300 mg; $CaCl_2 \cdot 2H_2O$, 180 mg; and 1 ml trace element solutions I and II. Trace element solution I contained (per liter of demineralized water) the following: EDTA, 5 g and $FeSO_4$, 5 g. Trace element solution II contained (per liter of demineralized water) the following: EDTA, 15 g; $ZnSO_4 \cdot 7H_2O$, 0.43 g; $CoCl_2 \cdot 6H_2O$, 0.24 g; $MnCl_2 \cdot 4H_2O$, 0.99 g; $CuSO_4 \cdot 5H_2O$, 0.25 g; $NaMoO_4 \cdot 2H_2O$, 0.22 g; $NiCl_2 \cdot 6H_2O$, 0.19 g; $NaSeO_4 \cdot 10H_2O$, 0.21 g; and H_3BO_4, 0.014 g. The nitrogen-loading rate (NLR) was adjusted by adjusting the concentrations of $(NH_4)_2SO_2$ and $NaNO_2$ in the medium.

A 140 ml of activated sludge (MLVSS: 200 mg/L) obtained from a conventional denitrification process (Toyama Green Food Recycle Center, Toyama, Japan) was filled up in a 140 ml Duran bottle, and was flushed with argon gas to remove oxygen for 5 min. The medium was continuously purged with nitrogen gas and continuously supplied into the bottle at a predetermined hydraulic retention time (HRT). The bottles were placed in an incubator at 35 °C. Mixing was not carried out through the operation.

Packed-bed single-stage PNA reactor A jar-fermentor system (Marubishi Co, Ltd., Japan) was used as the reactor. The liquid volumes in the reactor were 5.5 L in a 10 L vessel (SIM) and 1.5 L in a 3 L vessel (IW). The reactors were packed with a supporting medium of ring-laced strings made with polyvinylidene chloride (Kajima Environmental Engineering Corp., Japan). Fifteen bundles of these strings were arranged vertically with both ends spaced at equal intervals. The volume fraction of the ring-laced strings was approximately 2 vol%; the diameter of the ring lace was 25 mm (Fig. S1). The reactor was shielded with aluminum foil to block the light.

For reactor startup, SIM containing 1570 mg/L (5 mM) of $(NH_4)_2SO_4$ as the sole nitrogen source was used to enrich the PNA microorganisms. In this experimental setup, the medium was prepared without any anaerobic manipulation and was flushed with inactive gas.

Six grams of the activated sludge obtained from a conventional denitrification process were inoculated into 5.5 L of SIM. After inoculation, the reactor was mixed overnight at a moderate speed with a magnetic stirrer at 100 rpm, in order to attach microbial biomass to ring-laced media. Then the SIM was continuously supplied to the reactor via a peristaltic pump (102U peristaltic pump and 501RL pump head, Watson-Marlow, Poole, UK). The effluent was discharged through the overflow line. The influent was fed continuously. The influent-flow rate was adjusted according to the predetermined NLR. All tubing and connectors were fabricated from butyl rubber, norprene, or polyvinylchloride to limit oxygen diffusion. The contents of the reactor were mixed with a magnetic stirrer at 100 rpm. The reactors were operated at 35 °C and pH was maintained at 7.8 by automatic titration of 10% NaOH solution. The flow rate of air supply was controlled by mass flow meter in order to maintain NO_2—N concentration less than 50 mg/L throughout the experiment.

After PNA microorganisms were established in the SIM, the microorganisms were taken from the reactor and inoculated into another reactor to examine NRE from IW. The inoculation volume ranged from 0.9 g to 1.5 L of IW; therefore, the final concentration was 600 mg/L. The IW was continuously fed into the reactor at the predetermined HRT. The IW was a filtrate generated during a thermophilic methanogenic process for the manufacture of Shochu, a Japanese alcoholic beverage, which was provided by a Shochu brewery (Kirishima Shuzo Co. Ltd., Miyazaki, Japan). The average wastewater characteristics were as follows (mg/L); T-BOD, 308; T-CODcr, 739; VSS, 32; T-N, 619; NH_4—N, 538; and pH, 7.4. The IW contained less than 1 mg/L each of each NO_2—N and NO_3—N. Before the PNA reactor, the 0.5 L volume of the aeration tank, in which 7 cm of each 4 ring-laced strings were packed, was installed to capture biological oxygen demand (BOD) oxidizing microorganisms and to avoid interfusion of biomass to the subsequent PNA reactor. This process reduced T-BOD in IW to approximately 118 mg/L from 338 mg/L. The influent to the PNA reactor contained 97 mg/L of SS. Throughout the operation in IW treatment, DO concentration in the reactor was maintained at less than 0.5 mg/L by continuous aeration to control the nitritation reaction at the single-stage PNA process.

Chemical analyses To determine reactor performance, influent and effluent reactor samples were taken and their ammonium, nitrate, and nitrite content was analyzed using a liquid chromatograph (LC-8A, Shimadzu Corporation, Japan) and their dissolved organic carbon content was measured using a total organic carbon analyzer (TOC-500, Shimadzu). BOD and chemical oxygen demand (CODcr) were analyzed according to the methods of the Japanese Industrial Standard (22).

Pyro-tag sequencing analysis of biofilm samples DNA in the biofilm samples was extracted using a Soil DNA extraction kit (NucleoSpin Soil, Macherey-Nagel GmbH & Co. KG., Duren, Germany) according to the manufacturer's instructions. The extracted community DNA was then subjected to pyro-tag sequencing analysis. PCR amplification of 16S rRNA gene fragments (V4 region) was performed using the primers ad-tag-515F (5′-CGTATCGCCTCCCTCGCGCCATCAGXXXXXXXXXX GTGCCAGCMGCCGCGGTAA -3′) and ad-806R (5′-CTATGCGCCTTGCCAGCCCGCTCAG GGACTACHVGGGTWTCTAAT) (23), where the underlined sequences are adaptors for pyro-tag sequencing and XXXXXX is an arbitrary tag sequence for sample identification (24). An AmpliTaq Gold DNA Polymerase kit (Life Technologies, Carlsbad, CA, USA) was used for the PCR reactions. The PCR mixture contained 36.5 μL of sterilized water, 5 μL of PCR buffer, 5 μL of dNTP mix, 1 μL of forward primers, 1 μL of reverse primers (0.2 μM), and 1.0 μL of genomic DNA. The reactions were held at 95 °C for 10 min to denature the DNA, with amplification proceeding for 28 cycles, each consisting of 95 °C for 45 s, 55 °C for 60 s, and 72 °C for 90 s. A final extension of 10 min at 72 °C was added to ensure complete amplification. A composite sequencing sample was created by combining the equimolar ratios of amplicons from individual samples and followed by gel purification (illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare, USA). The mixed amplicons were subjected to pyro-tag sequencing using a Genome Sequencer FLX system (Macrogen, Tokyo, Japan). Phylogenetic analysis was conducted using the DDBJ 16S rRNA database (accessed March 16, 2015), the Blast program (25), and the RDP classifier ver. 2.3 (26). An operational taxonomic unit (OTU) was defined as a unique sequence, or group of sequences, with sequence homologies of over 97%.

Nucleotide sequence accession numbers The nucleotide sequences obtained in this study were deposited in the DNA Data Bank of the Japan/European Molecular Biology Laboratory/GenBank databases under accession numbers from LC191417 to LC191438.

RESULTS

Determination of enrichment conditions for anammox bacteria The well-known anammox bacterium *Candidatus Brocadia* has been detected mainly in wastewater treatment processes, where relatively high values of NLR are applied. Accordingly, the effect of NLR during the startup period of anammox enrichment was investigated. The NLR increases as the HRT decreases when a medium containing a constant nitrogen concentration is used. As shown in Fig. 1A, the high NLR operation with low HRT resulted in a fast reaction startup. At an NLR of 5 kg/m^3/day, the nitrogen removal rate (NRR) reached 1.0 kg/m^3/day within 40 days of operation (HRT of 1.8 days. At NLRs less than 1 kg/m^3/day, the reaction was confirmed at 40

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