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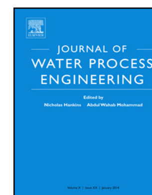

Microbial characteristics of an ANAMMOX biofilter for sewage treatment

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ABSTRACT

Anaerobic ammonium-oxidizing (anammox) process is considered to be a sustainable option for nitrogen removal. Application of this process, however, is limited by the availability of high strength nitrogen. In this study, an anaerobic ammonium oxidation (ANAMMOX) biofilter to treat sewage was deployed at ambient temperatures (18–21.5 °C), and the corresponding microbial community was investigated. The ammonia and nitrite initial loading rates were 0.154 kgN/(m^3 d) and 0.2 kgN/(m^3 d), respectively. ANAMMOX bacteria were rapidly enriched within 25 days and achieved a sustainable increase in the nitrogen removal rate (NRR) for sewage treatment. A final NRR of 0.99 kg N/(m^3 d) was seen at the 80 day point. Observations that combined fluorescence in situ hybridization (FISH) analysis with scanning electron microscopy (SEM) observations confirmed that ANAMMOX bacteria were dominant in the cultivated biofilm, accounting for 39.5 (±1.6)% of total bacteria. Denaturing gradient gel electrophoresis (DGGE) and phylogenetic analysis identified five genera of bacteria: *Pseudomonas*, *Acinetobacter*, *Nitrosomonas*, *Candidatus Brocadia fulgida* and *Ferruginibacter*. Of these, *Candidatus Brocadia fulgida* was the functional ANAMMOX bacteria in the reactor. *Candidatus Brocadia fulgida* could survive comfortably in sewage, and co-existed with *Nitrosomonas* and denitrifying bacteria.

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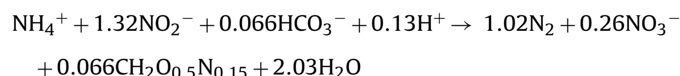
1. Introduction

Anaerobic ammonium oxidation (ANAMMOX) processes have many advantages over conventional nitrification–denitrification processes, and are recognized as highly cost-effective in removing biological nitrogen. In anoxic conditions, ANAMMOX bacteria are able to consume ammonium and nitrite under anaerobic conditions, through the following chemical reaction:



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

ANAMMOX processes can reduce aeration energy costs up to 63% compared with conventional nitrification/denitrification, and the fully autotrophic process eliminates the need for organic carbon. Furthermore, these processes significantly reduce greenhouse gas emissions, such as NO and N_2O. Moreover, the autotrophic character of the process consumes CO_2 [1], reducing sludge production. This could ultimately reduce the required footprint and size of current wastewater treatment plants (WWTPs) [2].

ANAMMOX processes have been a topic of significant research, with a focus on bacterial analysis, cultivating factors, and optimal operating conditions [3]. However, the ANAMMOX process is difficult to initiate due to ANAMMOX bacteria growth characteristics, which include a slow growth rate, low cell yield, and high sensitivity to changing environmental conditions. Because of these difficulties, the application and industrialization of ANAMMOX processes have been limited to treating a few low chemical oxygen demand (COD), ammonium-rich wastewaters at mesophilic temperatures (30–40 °C), such as landfill leachate and sludge digester liquor [4].

Recently, several ANAMMOX reactors have been built to treat wastewater with low ammonia concentrations at a lower temperature (20–25 °C)[5]. Most bioreactors have been maintained at a constant temperature in these studies, requiring additional energy consumption. However, natural sewage has a lower and more variable temperature (approximately 15–25 °C outside tropical and sub-tropical regions). Previous ANAMMOX bioreactors were frequently deployed using an Upflow Anaerobic Sludge Bed (UASB),

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Table 1
Composition of the influent wastewater for the ANAMMOX biofilter.

Temperature(°C)	pH	NH_4^+-N(mg/L)	NO_2^--N(mg/L)	NO_3^--N (mg/L)	COD_{cr}(mg/L)
21.5–18	7.5 ± 0.1	32.8 ± 2.2	42.7 ± 3.2	1.1 ± 0.3	50 ± 10

Sequencing Batch Reactor (SBR) and gaslift. Less attention has been paid to the ANAMMOX process in the biofilter. Therefore, there is great interest in developing an effective ANAMMOX process to treat low strength sewage at ambient temperatures with an anaerobic biofilter.

Previous reports characterized the microbial community of an osmotic ANAMMOXsystem. Sequencing analyses revealed that *Candidatus Brocadia* was most abundant in biofilm [6], indicating that *Ca. Brocadia* may be more tolerant of DO or elevated RSF. Dosta, J et al. investigated two-step partial nitritation/ANAMMOXprocess in granulation reactors [7]. *Planctomycetes* accounted for 7% of the global community; these are members of *Brocadia* (1.4% of the total abundance) and were the main detected anaerobic ammonium oxidizer. Wang, Yujia et al. investigated the symbiotic relationship of dominant bacteria in a lab-scale ANAMMOX UASB bioreactor [8]. Results revealed the dominance of ANAMMOX bacteria *Candidatus Kuenenia stuttgartiensis* (OTU474, 35.42%), along with heterotrophs of *Limnobacter* sp. MED105 (OTU951, 14.98%), *Anerolinea thermophila* UNI-1 (OTU465 and OTU833, 6.60 and 3.93%), *Azoarcus* sp. B72 (OTU26, 9.47%), and *Ignavibacterium* sp. JCM 16511 (OTU459, 8.33%). These results showed that microbial communities differ between different ANAMMOX processes. This may be due to the presence of heterotrophs in the autotrophic ANAMMOX system.

In this study, the role of ANAMMOX in treating sewage was investigated using a 160 L anaerobic biofilter inoculated with nitrifying sludge (added 2% v/v ANAMMOX biomass), initiated at ambient temperature (18–21.5 °C). The biofilm morphology was then assessed using SEM. The microbial compositions and community were investigated using FISH and PCR-DGGE, respectively. The goal of the research was to characterize the microbial community that became enriched in the ANAMMOX biofilter, to identify ways to enhance sewage treatment at ambient temperatures.

2. Materials and methods

2.1. Experimental setup for ANAMMOX bacteria enrichment

A 160 L anaerobic biofilter (AF) with a diameter of 50 cm and height of 120 cm was operated at ambient temperature (18–21.5 °C) in winter. It was not easy to keep temperature constant for influent wastewater, which would be an energy consumption process. Volcanic stones with particle sizes of 4–6 mm were used as carriers; these were characterized by high porosities (62.5%) and specific surface areas (11.3 m^2/kg), which facilitated bacteria attachment [9]. Sewage was pretreated using a lab-scale Anaerobic-Oxic (A/O) biological phosphorus removal (BPR) process for organic pollutants. Then, BPR effluent was fed into a lab-scale partial nitrification (PN) process, to partially oxidize ammonia to nitrite. Finally, the PN effluent was pumped to the up-flow AF reactor as the experimental substrate. Table 1 presents the influent wastewater composition; ammonia and nitrite initial loading rates were 0.154 kgN/(m^3 d) and 0.2 kgN/(m^3 d), respectively. Sodium bicarbonate was added to maintain alkalinity. Aeration-free equipment was used to maintain the anaerobic condition of the biofilter.

The seeding sludge for this study was formed by inoculating 100 L of nitrifying sludge (MLSS = 3600 mg/L) with 2 L mature ANAMMOX biomass. A peristaltic pump controlled the influent flow rate, and the hydraulic retention time (HRT) was controlled at windows between 1.3 and 5.1 h over 80 days.

2.2. Analytical methods

Duplicate samples of influent and effluent liquid were collected from the ANAMMOX reactor at the same time each day, during the 80 day test period. NH_4^+-N was analysed using the Nessler's Reagent Spectrophotometric technique; NO_2^--N was analysed using a Diazo coupled spectrophotometric technique; and NO_3^--N was measured using ultraviolet (UV) spectrophotometry methods[10]. Temperature, pH and DO were measured using a WTW pH/DO meter (WTW Multi 340i, Germany). The nitrogen removal rate (kg N m^{-3} day^{-1}) was calculated as the daily nitrogen load removed [daily flow rate × ($N_{in}-N_{out}$)] per unit volume of the reactor[11].

2.3. SEM observation

A SEM (HITACHI S-4300) was used to examine volcanic packs, inoculated sludge, and ANAMMOX biomass. Each bacteria specimen was first fixed in a 2.5% glutaraldehyde fixative solution; each was then washed three times with a phosphate buffer solution (0.1 M, pH 8.0). Each specimen was then dewatered for 15 min in serially graded ethanol at concentrations of 30%, 50%, 70%, 90% and 100%. The dewatered specimen was then replaced by isoamyl acetate, and observed with the SEM after dehydration.

2.4. Fluorescence in situ hybridization (FISH) analysis

FISH analysis was used to investigate the microbial compositions within the inoculated sludge and cultivated ANAMMOX biofilm. Samples were fixed in 4% freshly prepared paraformaldehyde solution for 2 h at 4 °C. Then, samples were rinsed twice with phosphate-buffered saline (PBS). In situ hybridizations were performed in a hybridization incubator using the procedure described by Amann et al. [12]. Table 2 lists the 16S rRNA targeted oligonucleotide probes. An AMX368 probe was used to investigate the presence of ANAMMOX bacteria [13], and EUB338, EUB338II, and EUB338III probes were used together in an equimolar mixture to detect bacteria [14,15]. Probes of NSO190 were used to detect ammonia-oxidizing β-*Proteobacteria*. NIT3 for *Nitrobacter* and Nsv443 for *Nitrospira* were used together to investigate the presence of nitrite-oxidizing bacteria (NOB) [16]. All probes were purchased from Sangon Company (China). FISH images were collected and recorded using an acquisition system (camera: DP72 and software: cellSens Dimension) coupled with an Olympus BX-51 fluorescence microscope. The average fraction was determined from 20 randomly chosen images, obtained from each of the duplicate biofilm samples, using the image analysis software cellSens Dimension.

2.5. DNA extraction and PCR-DGGE

DNA extraction and PCR-DGGE were used to investigate the microbial community of the anaerobic biofilter. Samples of 200 mg wet biomass were collected from the ANAMMOX biofilter and rinsed twice with sodium phosphate buffer (PBS, 0.1 M, pH 8.0). Total community genomic DNA was extracted using a DNA extraction liquid (100 mM Tris–HCl, 100 mM EDTA, 1.5 M NaCl, 100 mM Na_3PO_3, 1% CTAB, pH 8.0) and SDS (100 g L^{-1}) methods [17]. Bacterial universal primers GC-338F/907 R [18] directly amplified the partial 16 S rRNA gene fragments from the total DNA. The PCR products were loaded onto 8% polyacrylamide with a linear denaturing gradient ranging from 30% to 60% (100% denaturing gradient contains 7 M urea and 40% formamide). DGGE was performed using a D-code System (Bio-Rad, U.S.A), following manufacturer instructions. Electrophoresis was run at a constant voltage of 120 V for 6 h at 60 °C. Gels were subsequently silver stained, using a procedure

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