Nitrogen Removal by Granular Nitrification - Anammox in an Upflow Membrane-Aerated Biofilm Reactor

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Abstract

The nitrification-anammox process has been a promising nitrogen removal technology towards sustainable wastewater treatment, but its application in treating domestic wastewater with relatively low ammonium concentrations (mainstream) remains a great challenge. In this study, an innovative lab-scale upflow membrane-aerated biofilm reactor (UMABR) was employed to treat a synthetic wastewater containing 70 mg N L⁻¹ ammonium. With a DO level at 0.6 ± 0.1 mg O₂ L⁻¹ and HRT of 32 h, the effluent ammonium concentration was 4.8 ± 2.0 mg N L⁻¹. Increasing the nitrogen loading rate from 52.4 to 104.8 g N m⁻³ d⁻¹ with stepwise decreasing HRT from 32 to 16 h resulted in an average TN removal efficiency of 81% without nitrite accumulation. The average observed NO₃⁻-N (residue) /NH₄⁺-N (consumed) ratio of 8% was below the “theoretical ratio” of 13% and further reduction of nitrate residue needs to be addressed. Fluorescence in situ hybridization (FISH) and high-throughput sequencing analyses showed the coexistence of anammox bacteria and ammonium-oxidizing bacteria (AOB) in both biofilm and granular samples. Anammox bacteria accounted for up to 63.3% of the microbial community of the granules, with *Candidatus Jettenia* being the distinctly dominant anammox genus. In contrast, the biofilm contained abundant *Nitrosomonadaceae* (AOB, 33.1%). In addition, the brown-yellow granules exhibited a more balanced community structure with anammox bacteria and AOB accounting for 33.7% and 18.2%, respectively, which may contribute to the long-term operation of single-stage nitrification-anammox process. These results demonstrate that the nitrification-anammox UMABR could potentially be used for nitrogen removal from mainstream in some specific regions with relatively warm temperature.

42 **Keywords:** Nitritation-anammox; membrane-aerated; low nitrogen strength; biofilm; granule;
43 microbial community

1. Introduction

The increasingly stringent nitrogen discharge limit from wastewater streams with minimized energy consumption and carbon footprint has become a great challenge for water and wastewater treatment (Shi et al. 2013). Compared with the conventional biological nitrogen removal (nitrification-denitrification) process, the nitrification-anammox process is an energy-efficient and sustainable wastewater treatment technology because it significantly decreases oxygen and organic carbon consumption (Kuenen 2008, van der Star et al. 2007), resulting in cost savings in chemical additions and sludge disposal (Sliekers et al. 2002). In a nitrification-anammox system, the cooperation between ammonium-oxidizing bacteria (AOB) and anammox bacteria is essential to achieve completely autotrophic nitrogen removal under oxygen-limiting conditions in a single-stage reactor (Gong et al. 2007). Typically, AOB activities are observed mainly in the outer aerobic zone of both biofilm and aggregates, while anammox bacteria are predominant in the inner anoxic zone, thereby protecting anammox bacteria from oxygen inhibition. The nitrification-anammox process is sensitive to several operating parameters, such as dissolved oxygen (DO), nitrogen loading rate, biofilm thickness (granular size), pH, temperature, etc. (Hao and van Loosdrecht 2004). The oxygen-mass transfer efficiency and the effective biomass retention are considered as two crucial factors for successful operation of nitrification-anammox systems (Sliekers et al. 2003). Therefore, an ideal reactor configuration should facilitate oxygen transfer and have reliable biomass retention (e.g. biofilm or granular-based processes).

The membrane-aerated biofilm reactor (MABR) (Lackner et al. 2010, Li et al. 2008, Pellicer-Nacher et al. 2010), also called membrane biofilm reactor (MBfR) (Hasar et al. 2008, Hwang et al. 2010, Shin et al. 2005), is of strong interest because it provides active delivery of oxygen or

other desired gas through hydrophobic and gas-permeable membranes (Casey et al. 1999a) that also serve as biofilm support for bacterial immobilization (Ni et al. 2013). An MABR allows independent control of electron donors and acceptors due to the counter-diffusion delivery, providing a higher gas transfer efficiency and substrate utilization rate than conventional bubble diffusers (Ahmed and Semmens 1992, Casey et al. 1999a, Terada et al. 2003). Oxygen can penetrate the biofilm formed on the membrane surface, creating an oxygen concentration gradient. A higher oxygen level is in the regions close to the membrane surface while oxygen is absent in the regions near the bulk solution (Casey et al. 1999b). MABRs can favor growth of AOB and/or anammox bacteria through oxygen control strategies designed to repress NOB (nitrite oxidizing bacteria) activity, including the oxygen/nitrogen surface loading ratio (Gilmore et al. 2013, Lackner et al. 2008, Pellicer-Nacher et al. 2010), the DO concentration gradient at the membrane-biofilm interface (Downing and Nerenberg 2008), and intra-membrane air pressure (Gong et al. 2007). In recent years, MABRs were studied to achieve single-stage autotrophic nitrogen removal (Gilmore et al. 2013, Gong et al. 2007, Ni et al. 2013, Pellicer-Nacher et al. 2010). For example, a nitrogen removal rate up to $0.77 \text{ kg N m}^{-3} \text{ d}^{-1}$ was achieved in an MABR equipped with non-woven fabrics support around the gas-permeable carbon tube (Gong et al. 2007). It was also reported that the highest nitrogen removal efficiency of 72% was obtained at an oxygen to ammonium ratio of 1.96 in an intermittently-aerated MABR (Pellicer-Nacher et al. 2010).

During the past decade, the nitrification-anammox systems have been successfully implemented at a full scale for treating high nitrogen strength wastewater (Lackner et al. 2014), such as digester effluent (van der Star et al. 2007) and industrial effluent (Abma et al. 2010).

However, the application of those systems in treating municipal sewage (i.e., mainstream) with relatively low ammonium concentrations and low temperature remains a major challenge, because such conditions will lower specific activities and growth rates for both anammox bacteria and AOB (Hu et al. 2013). Typically, the total nitrogen concentrations in the municipal wastewater are ranging from 20 to 70 mg N L⁻¹ (Law et al. 2012). Very recently, it was demonstrated that a plug-flow anammox-based pilot-scale reactor of 4 m³ could treat a low nitrogen strength effluent from an aerated tank, achieving a total nitrogen removal efficiency less than 50% (Lotti et al. 2015).

In this study, a novel laboratory-scale upflow membrane-aerated biofilm reactor (UMABR) has been designed and investigated to treat low-concentration ammonia by a nitrification-anammox process. It is expected that coupling membrane aeration with upflow pattern will combine the advantages of each feature to promote oxygen mass transfer and improve biomass retention through the formation of biofilm and granules. To our knowledge, this is the first time that the nitrification-anammox process has been demonstrated to treat wastewater with low nitrogen concentrations in an MABR system with pure oxygen supply. The key objectives of this study were to: (1) evaluate the feasibility of autotrophic nitrogen removal in the UMABR system to treat synthetic wastewater with a low ammonium concentration; (2) understand the effects of ammonium loads via changing hydraulic retention time (HRT); (3) understand the effects of organic carbon addition on removal of nitrate residue; and (4) reveal microbial community structure in both biofilm and granules and identify the dominant anammox species.

2. Materials and Methods

2.1. UMABR setup and operation

The schematic of the lab-scale UMABR system is shown in Fig. 1. The reactor had a working volume of 2.5 L (height of 0.5 m, diameter of 0.08 m), and was continuously fed with a synthetic wastewater by a peristaltic pump (Cole-Parmer Instrument, Vernon Hills, IL, USA). A glass funnel was placed on top to separate gas and liquid while reducing biomass loss. The nonporous silicone membrane was installed inside the reactor, with a length of 8 m and 0.5 mm bore $\times$ 1.0 mm wall thickness (Thermo Fisher Scientific, East Grinstead, UK), resulting in a membrane area per volume of reactor (A_w/V) of $25 \text{ m}^2 \text{ m}^{-3}$. The membrane wrapped around a cylindrical stainless steel stand. The interior of the membrane was connected to a cylinder containing pure oxygen. The rate of oxygen supply was adjusted by a needle valve and a gas pressure meter based on the monitoring data of an online pH/ORP control system (Milwaukee Instruments, Rocky Mount, NC, USA) and an online DO controller/probe (Hanna Instruments Inc., Woonsocket, RI, USA). The effluent was recirculated at 100 mL min^{-1} through an overflow bottle (200 mL in liquid volume) to provide mixing, resulting in an upflow velocity of 1.2 m h^{-1} . The pH inside the reactor was maintained between 7 and 7.5 by automatically adding 0.2 M NaOH solution. To maintain a suitable temperature ($25 \pm 1 \text{ }^\circ\text{C}$), warm water from a heating circulator was recirculated into the integrated water jacket (Model 1104, VWR Scientific, West Chester, PA).

The experiment had three stages, and the operating HRT and duration for each stage with different nitrite and organic additions are shown in Table S1. In the stage I (190 days), the ammonium concentration was fixed at 70 mg N L^{-1} in the influent while gradually decreasing nitrite supply from 84 to 0 mg N L^{-1} . In the stage II (140 days), the nitrogen removal

performance under various nitrogen loading rates (or HRTs) was studied. In the stage III (40 days), the effects of organic carbon on the nitrification-anammox performance were investigated.

2.2. Synthetic medium and inoculation

The ammonium, nitrite and organic carbon in the synthetic wastewater were supplied in the forms of ammonium chloride, sodium nitrite and glucose, respectively, and their concentrations are shown in Table S1. The ammonium concentration of 70 mg N L⁻¹ in the influent was fixed to mimic the pretreated municipal nitrogenous wastewater (Hu et al. 2013). The other minerals was prepared according to a modified formula (Imajo et al. 2004): NaHCO₃ 0.42 g L⁻¹, KH₂PO₄ 0.0272 g L⁻¹, MgSO₄ 0.059 g L⁻¹, CaCl₂•2H₂O 0.18 g L⁻¹, 1 mL L⁻¹ trace elements solution I, and 1 mL L⁻¹ trace elements solution II. The trace element solution I contains (g L⁻¹): EDTA 5 and FeSO₄ 5. The trace element solution II is composed of (g L⁻¹): EDTA-2Na 15, CuSO₄•5H₂O 0.25, ZnSO₄•7H₂O 0.43, NaMoO₄•2H₂O 0.22, MnCl₂•4H₂O 0.99, NiCl₂•6H₂O 0.19, CoCl₂•6H₂O 0.24, NaSeO₄•10H₂O 0.21, and H₃BO₄ 0.014 (Imajo et al. 2004). The synthetic wastewater was deoxygenated by flushing N₂, and stored in a gas tight collapsible low density polyethylene container. The UMABR was seeded with 500 mL of inoculum that contained both anammox granules and flocs, taken from an upflow anaerobic sludge blanket (UASB) reactor operated at 30 °C that was continuously fed with 490 mg N L⁻¹ of ammonium and 588 mg N L⁻¹ of nitrite. The volatile suspended solid (VSS) at the beginning of the operation was estimated to be 8 g L⁻¹. Microbiological analysis with fluorescent *in situ* hybridization (FISH) revealed that the inoculum was dominated by anammox bacteria (~80%), and three anammox genera including *Candidatus. Brocadia*, *Ca. Jettenia* and *Ca. Kuenenia* were identified by high throughput sequencing.

2.3. Analytical methods

The concentrations of ammonium ($\text{NH}_4^+\text{-N}$), nitrite ($\text{NO}_2^-\text{-N}$), and nitrate ($\text{NO}_3^-\text{-N}$) in the effluent were regularly measured throughout the experiments to monitor the performance of the reactor. Ammonium, nitrite and nitrate concentrations were determined by a spectrophotometer (DR 890, Hach Company, Loveland, CO, USA). The concentration of chemical oxygen demand (COD) was measured using a heating block DRB200 and colorimeter DR 890 according to the manufacturer's instructions. The pH and DO were measured as previously stated.

2.4. Scanning electron microscope

Surface morphology and structural properties of the biomass samples were examined using a thermally assisted field emission (FESEM) (LEO 1550, Carl Zeiss, Oberkochen, Germany). Granule samples were fixed in 4 % paraformaldehyde and 0.1 M phosphate buffered saline (PBS) for 2 h. After washing twice with 0.1 M PBS for 10 min, the samples were dehydrated in a graded ethanol series (30%, 50%, 70%, 95%, 5 min each), and then dried with a critical point drier using liquid CO_2 (model 28000, LADD Research Industries, Williston, VT). Coatings were sputtered with a thin Au/Pd layer (~10 nm) for 1.5 min in a sputter coater (208HR, Cressington Scientific Instruments Ltd., Watford, UK) to enhance imaging. The coated samples were observed by LEO 1550 FESEM operated at an accelerating voltage of 5 kV.

2.5. Microbial community analysis

2.5.1 Fluorescence in situ hybridization

Spatial distribution and quantification of microorganisms (e.g. AOB, NOB and anammox bacteria) in the granule and biofilm were assessed using FISH. Granular or/and biofilm samples were harvested on day 190 and day 298. FISH was performed as described previously (Okabe et al. 1999). The 16S rRNA targeted-oligonucleotide probes (Sigma-Aldrich, St. Louis, MO, USA) used in this study are listed in Table S2. All probes have a synthesis scale of 0.05 μ mol, and the formamide (FA) concentration in the hybridization buffer depends on the probe types. Images of the hybridized samples were observed by confocal laser scanning microscopy (CLSM) (Zeiss LSM 880, Jena, Germany) with an Ar laser (488 nm) and two He-Ne lasers (543 nm and 633 nm).

2.5.2 DNA extraction and sequencing

Biofilm and granular biomass were harvested on day 190 and day 298 and stored immediately at -20 °C until DNA extraction. DNA was extracted using the PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA), and evaluated by spectroscopic methods (NanoDrop 2000, Thermo Fisher Scientific, Beverly, MA, USA). Bacterial 16S rRNA genes were PCR-amplified with barcoded forward primer 515F and reverse primer 806R (Caporaso et al. 2012). The thermal program was 94 °C for 5 min, and 35 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 90 s, followed by a final extension at 72 °C for 10 min. The PCR reaction mixture (25 μ L) contained 10 μ L 2.5X 5 Prime HotMaster mix (5 Prime, Gaithersburg, MD), 13 μ L PCR water (MoBio Laboratories, Carlsbad, CA, USA), 0.5 μ L of each of the primers (10 μ M), and 1 μ L of isolated DNA. All samples were amplified in triplicate on thermal cyclers (Bio-Rad, Hercules, CA, USA), pooled and visualized on agarose gels. Combined PCR products were purified using the UltraClean PCR cleanup kit (MoBio Laboratories, Carlsbad,

CA, USA) as recommended. Amplicon concentrations were quantified using Qubit 2.0 fluorometer (Invotrogen, USA).

Amplicons from all samples were pooled in equimolar ratios and bi-directionally sequenced (PE 250bp) on the Illumina MiSeq platform at Virginia Bioinformatics Institute. The forward and reverse reads were merged and filtered based on minimum length and expected errors, as specified in the USEARCH pipeline (Edgar 2010). The filtering resulted in 449990 high quality sequences from five samples of this study, averaging about 110500 sequences per sample. Sequences were then clustered to 720 bacterial operational taxonomic units (OTUs) with a 97% similarity threshold, with chimeric sequences identified and removed with UCHIME (Edgar 2013, Edgar et al. 2011). Taxonomy was assigned via the RDP classifier with the SILVA databases (Kõljalg et al. 2013, Quast et al. 2013, Wang et al. 2007).

3. Results and discussion

3.1. Development of a nitrification-anammox process

In the stage I, the ammonium concentration was fixed while the nitrite concentration was gradually decreased to zero. After inoculation, the UMABR was operated anaerobically for about one week with 70 mg N L⁻¹ ammonium and 84 mg N L⁻¹ nitrite as the substrates in the influent at the flow rate of 1.9 L d⁻¹ (HRT=32 h). Under such conditions, anammox bacteria were expected to be the major contributor for consumption of both ammonium and nitrite. The system maintained low concentrations of both ammonium (3 ± 2 mg L⁻¹) and nitrite (1.2 ± 1.0 mg L⁻¹) in its effluent, resulting in a total nitrogen removal efficiency of $94.7 \pm 1.7\%$ (Fig. 2A). Starting on day 12, the influent NO₂⁻-N/NH₄⁺-N ratio decreased from 1.2 to 1.1, and pure O₂ was delivered

through the membrane into the UMABR to stimulate AOB growth and promote the formation of nitrification-anammox coculture.

The intra-membrane pressure was adjusted to control the DO based on the influent and effluent ammonium concentrations and nitrate production. On day 28, the influent NO_2^- -N/ NH_4^+ -N ratio decreased to 0.8, leading to an increase in the effluent ammonium by 14 mg N L^{-1} (Fig. 2A). This was likely due to the low abundance of AOB and/or insufficient oxygen supply. When the pressure was gradually increased from 2.5 psi on day 30 to 4.8 psi on day 43 (Fig. 2B), the effluent ammonium decreased to 3 mg N L^{-1} (Fig. 2A). Decreasing the influent NO_2^- -N/ NH_4^+ -N ratio further caused an abrupt increase of the effluent ammonium (Fig. 2A). Accordingly, the intra-membrane pressure was increased slowly to facilitate the oxidation of ammonium to nitrite by AOB. Meanwhile, the DO concentration showed an upward trend in the first 90 days and afterwards became relatively stable within the range of $0.5 \sim 0.7 \text{ mg O}_2 \text{ L}^{-1}$ (Fig. 2B). The pressure control strategy based on the DO monitoring and nitrogen transformation was more reliable than DO measurement only (Joss et al. 2011), because DO could deliver misleading information under some situations. For example, the DO level could still fall in the target level when excessive oxygen is supplied beyond the requirement of AOB due to the consumption by NOB or other aerobic bacteria. On day 128, nitrite was no longer supplied to the reactor, and therefore the nitrite required for the anammox bacteria was provided by the AOB that was developed within the system. During this period (days 128 – 190), the DO level was maintained at $0.6 \pm 0.1 \text{ mg O}_2 \text{ L}^{-1}$ by applying the intra-membrane pressure of $6.4 \pm 0.3 \text{ psi}$ (Fig. 2B). As a result, the average ammonium

concentration in the effluent was $4.8 \pm 2.0 \text{ mg N L}^{-1}$ with an ammonium removal efficiency of $93.2 \pm 2.8\%$ (Fig. 2A).

The accumulation of nitrite or/and nitrate is an important issue during the operation of nitrification-anammox installations (Lackner et al. 2014). For example, the accumulation of nitrite and nitrate (~60% of supplied ammonium) was observed during the operation of oxygen-limited autotrophic nitrification/denitrification (OLAND) processes in a lab-scale rotating biological contactor (RBC) (De Clippeleir et al. 2013). The nitrite accumulation generally occurs for several days, and can be stimulated by a low operating temperature (e.g., $< 15 \text{ }^{\circ}\text{C}$) (Gilbert et al. 2014, Hu et al. 2013, Persson et al. 2014). The nitrate accumulation can account for up to 40% of the total nitrogen in the effluent of a moving bed biofilm reactor (MBBR) fed with a synthetic influent containing only ammonium (100 mg N L^{-1}), and decreasing DO from ~ 0.5 to $< 0.2 \text{ mg O}_2 \text{ L}^{-1}$ could significantly reduce nitrate production (Gilbert et al. 2014). These accumulations are associated with the shifts of microbial populations; especially, nitrite buildup is due to the decrease of anammox bacteria activities while nitrate is accumulated because of the excess of NOB activities. The UMABR developed in this study provides an effective solution to alleviate or eliminate this issue. In this UMABR, both nitrite and nitrate were maintained at the low concentrations of $0.1 \pm 0.1 \text{ mg N L}^{-1}$ and $2.8 \pm 1.5 \text{ mg N L}^{-1}$, respectively (Fig. 2A), which may be attributed to an appropriate AOB and anammox activities with adequate oxygen supply. The effluent nitrate during days 128 – 190 accounted for $4 \pm 2\%$ of the total nitrogen (without nitrite addition), significantly lower than those in the prior studies, which could be related to the occurrence of denitrification with the availability of organic carbon sources (e.g., cell lysis or decay).

3.2. Effects of nitrogen loading rates

In the stage II, the nitrogen loading rate was increased by decreasing HRT from 32 to 16 h. With the increased nitrogen load, a higher oxygen supply rate was required to stimulate the oxidation of ammonium. The stepwise decrease of HRT increased the effluent ammonium concentration, but the nitrite accumulation was not obvious with its effluent concentration of 0.1 – 0.2 mg N L⁻¹ (Fig. 3A). During days 190 – 220, the HRT was lowered from 32 h to 28 h while slightly increasing the intra-membrane pressure from 6.4 ± 0.3 to 6.6 ± 0.5 psi; this change did not obviously affect either the ammonium removal efficiency that was maintained above 90% (93.2 ± 2.8% at HRT 32 h and 92.9 ± 2.7% at HRT 28 h) or total nitrogen removal efficiency (from 89.0 ± 4.6% to 87.2 ± 4.2% due to slightly more nitrate production) (Fig. 3A and 3B). Further decreasing the HRT to 24, 20 and 16 h increased the remaining ammonium in the effluent (up to 18 mg N L⁻¹). For example, the effluent ammonium concentration after a HRT of 24 h was doubled compared with that of a higher HRT of 28 or 32 h, likely due to the insufficient dissolved oxygen with the intra-membrane pressure of 7.0 ± 0.1 psi. Ammonium accounted for the majority of the total nitrogen in the effluent during day 220 – 298 (Fig. 3B), and thus, nitrification was most likely the rate-limiting step (Cho et al. 2011, Gilbert et al. 2014). This is in accordance with the observation of the much higher abundance of anammox bacteria in the biomass than AOB in the FISH experiment and DNA sequencing results (as discussed below). After day 298, the enhanced ammonium removal efficiency (93.7 ± 2.4%) was achieved because a higher intra-membrane pressure of 9.4 psi was applied.

The metabolism of the nitrification-anammox process can result in nitrate production accounting for approximately 13% of total ammonium supply according to the stoichiometry of the proposed reaction equation (Third et al. 2001), and a “threshold line” indicating this 13% (Fig. 3A) can be used to evaluate whether the distribution and activity of microorganisms are appropriate and healthy in the system. For example, the observed ratio of $\text{NO}_3^- \text{-N} / \text{NH}_4^+ \text{-N}$ greater than 13% might indicate the overgrowth of NOB, which competes with AOB for oxygen and with anammox bacteria for the produced nitrite. In the present system, the observed $\text{NO}_3^- \text{-N} / \text{NH}_4^+ \text{-N}$ fell below the “threshold line” most of time, indicating successful repression of NOB activities and more nitrite conversion by anammox bacteria. The average ratio of 8% was lower than a previous studies (i.e., 11%), in which nitrification-anammox was employed for the treatment of high-strength ammonium wastewater (350 mg N L^{-1}) (Li and Sung 2015). Those results indicate that monitoring the effluent ammonium concentration and the observed $\text{NO}_3^- \text{-N}$ (residue) / $\text{NH}_4^+ \text{-N}$ (consumed) ratio could be an alternative strategy for oxygen control.

With a stepwise decrease in HRT from 32 to 16 h, the nitrogen loading rate (NLR) was increased from 52.4 to $104.8 \text{ g N m}^{-3} \text{ d}^{-1}$, resulting in the nitrogen removal rates (NRR) increasing from 46.7 ± 2.4 to $77.6 \pm 5.3 \text{ g N m}^{-3} \text{ d}^{-1}$ (Fig. 3C). Such a level of NLR was similar to some previous studies (Kuai and Verstraete 1998, Third et al. 2001) and is comparable with conventional municipal wastewater treatment systems (e.g., $65 \text{ g N m}^{-3} \text{ d}^{-1}$, Dapena-Mora et al. (2004)), depending on their influent quality, reactor configuration and operational conditions. A lower average ammonium removal rate of $40 \text{ g N m}^{-3} \text{ d}^{-1}$ ($13 - 95 \text{ g N m}^{-3} \text{ d}^{-1}$) was reported in a lab-scale MBBR with carrier material under HRT of 1 – 2 d and 20°C (Gilbert et al. 2014). In contrast, the recent pilot-scale nitrification-anammox reactor achieved average levels of 180 g N

$\text{m}^{-3} \text{d}^{-1}$ (Lotti et al. 2015). Several recently developed nitrification-anammox reactors have been operated for wastewater with low nitrogen concentration ($< 100 \text{ mg N L}^{-1}$), as shown in Table S3. The NLR applied in this study was generally lower than the previous studies but achieved a higher TN removal efficiency of $\sim 81\%$ (after day 128). The low NLR and relatively high NRR achieved in this study suggest that UMABR could be an alternative configuration for developing nitrification-anammox process to remove low concentration ammonium in the wastewater. At HRT of 16 h the biomass specific conversion rate was estimated as $50 \pm 4 \text{ mg NH}_4^+-\text{N g VSS}^{-1} \text{d}^{-1}$ (based on the dry weight of the inoculum), which is close to a previous report that achieved $50 \pm 7 \text{ mg N g VSS}^{-1} \text{d}^{-1}$ in a granular sludge fluidized bed lab-scale reactor continuously fed with nitrite addition (Lotti et al. 2014). The actual biomass-specific conversion rate in the present system should be lower than the estimated value because the amount of biomass was expected to be higher after a long-term operation (no biomass discharge except for sampling purpose).

3.3. Effects of organic carbon

The anammox bacteria cannot compete with denitrifying bacteria in the presence of a high concentration of organic carbon due to their slower growth rates (Udert et al. 2008). On day 331, a limited amount of organic carbon in form of glucose (40 mg COD L^{-1}) was supplied in the influent in the stage III. The additional COD was expected to enhance denitrification to reduce nitrate residue. The COD/N ratio of 0.57 was comparable to those of side-streams (< 0.5 , e.g., digester supernatant) treated by the full-scale nitrification – anammox process (Joss et al. 2009, Wett 2007), but lower than that of mainstream wastewater. The high COD/N ratio in mainstream wastewater would favor heterotrophic denitrification over anammox, and thus, removal of organic compounds in pretreatment could be important to mainstream anammox application. As

shown in Fig. S2, the COD removal efficiency of $59 \pm 17\%$ was achieved while the effluent nitrate remained $8.2 \pm 3.5 \text{ mg N L}^{-1}$, which was more than that observed under HRT of 16 h without COD additional ($5.0 \pm 2.3 \text{ mg N L}^{-1}$). Denitrifying bacteria are usually facultative anaerobes and prefer to utilize oxygen as an electron acceptor when it is available (Probian et al. 2003). The reduction in COD may be the result of aerobic heterotrophic bacteria that can survive under the provided conditions. In addition, biodegradable organic carbon (e.g., glucose) could stimulate the growth of heterotrophic bacteria which compete with AOB for oxygen and with anammox bacteria for nitrite (Jenni et al. 2014). The detailed role of organic carbon in the present system and inefficient reduction of nitrate with organic addition warrants further investigation.

3.4. Morphology of the granule and membrane biofilm

The UMABR was inoculated with the granules, and it was observed that a biofilm was also formed on the membrane (Fig. S1A and S1B). Both the granules and the membrane biofilm help achieve effective biomass retention for high removal efficiency and avoid the use of additional materials for bacterial immobilization (e.g. non-woven fibers (Gong et al. 2007)). After long-term operation, the DO concentrations were able to maintain at $0.8 \pm 0.2 \text{ mg O}_2 \text{ L}^{-1}$ with the applied intra-membrane pressure of $9.2 \pm 0.6 \text{ psi}$ in the stage III, indicating no significant fouling occurred despite the growing biofilm, probably due to less suspended biomass in the liquid phase of the reactor as well as no solids in the influent. Biomass samples were collected on day 298, and it can be seen that the majority of the nitrification-anammox granules have diameters between 2.0 and 5.0 mm (Fig. 4A), resulting in a high settling rate for excellent retention of the functional microorganisms in the reactor. The dense structure of the granules might indicate high contents of extracellular polymer substrates (EPS) (Ni et al. 2010) because EPS was primarily

responsible for the structural and functional integrity of the aggregates and essential to their physicochemical and biological properties (Flemming and Wingender 2001). The unique structure may also be associated with the shear forces resulting from the internal liquor recirculation. The granules observed in this study have lighter color than those in a previously developed nitrification-anammox UASB reactor (Li and Sung 2015), likely related to the lower nitrogen loading rates in the present study. The SEM micrographs show a porous structure of the granule that might be caused by N₂ release from anammox process (Fig. 4B-4D), and such a structure could benefit substrate dispersion. The smooth coccoid-shaped cells that were predominant in the granule had diameters less than 1 µm and were presumably anammox bacteria (Kuenen 2008, van Niftrik et al. 2004), which was confirmed by FISH and DNA sequencing. These bacteria formed clusters (microcolonies) with various sizes and small interspaces (Fig. 4C), which favored tight and stable preservation of the granules. Interestingly, wispy filamentous connections were observed among the bacteria, but their function remains unknown (Fig. 4D).

3.5. Analysis of microbial community

The spatial distribution of AOB and anammox bacteria in the granules was analyzed by FISH. EUB338, NSO190, and AMX368 probes were used to target all the bacteria, ammonia-oxidizing β-Proteobacteria, and all anammox bacteria, respectively. The FISH images show that AOB and anammox bacteria co-existed in the granule related to the distribution of oxygen facilitated in this UMABR, and anammox bacteria appeared to be the dominant microorganisms (Fig. 5). The specific growth zone (e.g., inner or outer layer) for either anammox bacteria or AOB was not observed; instead, both were distributed homogenously, regardless of differences in their abundance, which is likely related to the porous structure that promoted oxygen transfer

into the center of the granules. This finding agrees with a previous study in which AOB and anammox showed an overlapping spatial distribution (Li and Sung 2015), but differed from other reports in which AOB were observed only in the surface area of granules (Gong et al. 2007, Okabe et al. 2011, Winkler et al. 2012).

Biomass samples for sequencing analysis were harvested on day 190 (granules with red color) and day 298 (biofilm on the membrane, granules with brown-yellow, red, and brownish-red colors, Fig. 4A), representing the stable operating conditions during Stage I (HRT of 32 h) and Stage II (HRT of 16 h), respectively. The microbial community structures are shown in Fig. 6 and S3 (samples are marked as G-R190, Biofilm, G-BY, G-R and G-BR). As expected, the anammox bacteria were dominant in all granular samples (up to 63.3%), which agrees with the FISH observations. Three genera affiliated with anammox – *Ca. Brocadia*, *C. Jettenia* and *C. Kuenenia* - were detected, and *C. Jettenia* was the distinctly dominant anammox genus (accounting for 87.0 – 99.5% of all granules, Fig. S3A). These three genera all belong to the family *Ca. Brocadiaceae* within the phylum *Planctomycetes* (Fig. 6). G-R190, G-R and G-BR had comparable relative abundances of anammox bacteria while much many more AOB were observed in G-R and G-BR. Compared with other granular samples, G-BY showed remarkable differences in the abundances of anammox bacteria and *Nitrosomonadaceae* within *Betaproteobacteria* class (Fig. 6A and S3B), accounting for 33.7% and 18.3%, respectively. Such community composition suggests the evolution of anammox granules under long term operation with appropriate oxygen supply could be a desirable feature for the development of single-stage nitrification-anammox process (Li and Sung 2015).

In contrast to anammox being the predominant bacteria in the granules, the AOB *Nitrosomonadaceae* was the most abundant bacteria in the Biofilm sample (~ 33.1%), likely due to the readily available oxygen on the membrane surface, where meanwhile anammox bacteria accounted for only 4.8% (Fig. 6A). Additionally, *Nitrospira* was detected as high as 5.4% in the Biofilm sample (Fig. S3A), indicating the potential occurrence of nitrite oxidation associated with the elevated oxygen supply in the stage II. *Nitrospira* was not detectable in G-R190 while the other granules had a low abundance varying from 0.1 to 0.2%, which was similar to a recent report of 0.1 – 0.4% in an anammox reactor (Pereira et al. 2014). Small amounts of NOB could benefit anammox bacteria by eliminating the inhibitions of accumulated nitrite and excess DO on anammox process (Pereira et al. 2014). The community in all samples included 0.6 – 2% of genus Dok59, a member of the family *Rhodocyclaceae* in the class *Betaproteobacteria* (Fig. 6A and S3B). They are mainly rod-shaped bacteria involved capable of denitrification (Ginige et al. 2005), which can partially explain low nitrate production achieved in the UMABR (Fig. 3A).

4. Conclusions

In the present study, a nitrification-anammox UMABR was developed to treat wastewater with low ammonium concentrations for over 360 days. The nitrogen removal was evaluated under various operating conditions. The morphology and community structure of the colonizing microorganisms were also investigated. The following conclusions could be drawn from the results:

- The effluent ammonium concentration was $4.8 \pm 2.0 \text{ mg N L}^{-1}$ with a DO level at $0.6 \pm 0.1 \text{ mg O}_2 \text{ L}^{-1}$ and HRT of 32 h. Increasing the nitrogen loading rate from 52.4 to 104.8 g N m⁻³ d⁻¹ (HRT from 32 to 16 h) resulted in an average TN removal efficiency of 81%

without nitrite accumulation. The average observed $\text{NO}_3^- \text{-N} / \text{NH}_4^+ \text{-N}$ ratio of 8% was below the “theoretical ratio” of 13%.

- COD addition did not remove the nitrate residue, probably related to the activities of aerobic heterotrophic bacteria. The detailed role of organic carbon and further nitrate reduction in the present system warrants further investigation.
- The FISH images show that AOB and anammox bacteria co-existed in the granule due to the appropriate oxygen level, and anammox bacteria appeared to be the dominant microorganisms. No specific spatial distributions for growth of anammox bacteria and AOB were observed.
- Sequencing analysis confirmed the FISH observations that anammox bacteria accounted for up to 63% of the total microbial community in the granules and *Ca. Jettenia* was the dominant anammox genus. In contrast, the biofilm on the membrane surface was primarily comprised of *Nitrosomonadaceae* (33.1%).
- The proposed nitrification-anammox UMABR could potentially be used for nitrogen removal from wastewater mainstream in the regions with relatively warm temperature.
- Future studies on the quantification of N_2O emission and the actual mainstream wastewater treatment in the UMABR system are recommended.

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Figure Captions

Figure 1. The schematic of the membrane-aerated biofilm reactor (UMABR) system.

Figure 2. Reactor performance and oxygen control in the stage I (startup): (A) Profiles of NH_4^+ -N, NO_2^- -N, NO_3^- -N and TN removal efficiency, and (B) the profile of intra-membrane pressure, DO concentration and the ratios of influent NO_2^- -N/ NH_4^+ -N.

Figure 3. Reactor performance under different HRTs in the stage II: (A) the profiles of NH_4^+ -N, NO_2^- -N, NO_3^- -N and TN removal efficiency; observed NO_3^- -N/ NH_4^+ -N = (effluent [NO_3^- -N]) / (influent [NH_4^+ -N] - effluent [NH_4^+ -N]); the green dot line (threshold line) indicates the “theoretical ratio” of 13%, (B) the average concentrations of nitrogen species in the effluent with intra-membrane pressure control, and (C) the profiles of nitrogen loading rate (NLR) and nitrogen removal rate (NRR).

Figure 4. Scanning electron micrographs of a typical granule cultivated in UMABR (samples taken on day 298): (A) the fresh granules, (B) the whole granule (magnification=10 X), (C) Region I (magnification=30000 X), and (D) Region II (magnification=30000 X). Red arrows indicate different bacterial morphology.

Figure 5. FISH micrographs (samples taken on day 298): (A) EUB338 probe targeting most bacteria was labeled with FLC (green), (B) AMX368 probe targeting all anammox bacteria was labeled with Cyanine3 (red), (C) Nso190 probe targeting ammonia-oxidizing *β -Proteobacteria* was labeled with Cyanine5 (purple), and (D) Three-color merged image. A, B, C and D represent the same zone in the cross-section of a nitrification-anammox granule. Bars are 20 μm for A-D. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Figure 6. Bacterial community composition by sequencing: (A) at the family level, (B) at the phylum level. “Other” represents all classified taxa that were <1% in all samples. “G-R190”: granules with red color sampled on day 190 (at the end of stage I); “Biofilm”: biofilm on the membrane sampled on day 298; “G-BY”: granules with brown-yellow color sampled on day 298; “G-R”: granules with red color sampled on day 298; “G-BR”: granules with brownish-red color sampled on day 298.

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Inf. $\text{NH}_4^+\text{-N} = 70$
 $\text{DO} \leq 1 \text{ mg O}_2 \text{ L}^{-1}$

Microbial community structure in granules/biofilm taken on day 190 and day 298