Enhanced Nitrogen Removal by Membrane-Aerated Nitritation- Anammox in a Bioelectrochemical System

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Abstract

A bioelectrochemical system (BES) containing membrane-aerated nitrification-anammox in its cathode has been developed for enhancing nitrogen removal. Long-term performance and microbial community structure were investigated. The BES using loop operation and external voltage achieved the highest total nitrogen removal efficiency of $94.8 \pm 7.7\%$, and COD removal of $98.2 \pm 3.3\%$ at hydraulic retention time of 60 h and the lumen pressure of 10 psi. The energy consumption of the system was $0.90 \text{ kWh kg N}^{-1}$ or $0.38 \text{ kWh kg COD}^{-1}$. Sequencing analyses revealed that ammonia oxidizing bacteria (0.2–7.4%), anammox bacteria (0.4–10.3%), denitrifying bacteria (5.8–13.1%), and electrogenic bacteria (4.6–12.8%) were in abundance of the microbial community in the cathode chamber, and their distributions were affected by the aeration and physical locations. These results encourage further investigation of membrane-aerated nitrification-anammox in BES for optimization and potential applications with actual wastewater.

Keywords: Bioelectrochemical system; Nitrogen removal; Anammox; Membrane aeration; Microbial community

1. Introduction

Ammonia is the most abundant inorganic nitrogen in various wastes and wastewaters such as municipal wastewater, landfill leachate and livestock waste. Excessive discharge of ammonia into water environment will create toxic effects to aquatic organisms and cause eutrophication. Ammonia can be removed by using physical, chemical and biological methods, and biological removal is of particular interest because of its cost effectiveness. Conventional biological ammonia removal such as nitrification and denitrification has been widely applied in full scale to treat industrial and municipal wastewater (Van Hulle et al., 2010). Discovery and application of anaerobic ammonium oxidation (anammox) coupled with nitritation has provided an innovative approach for ammonia removal with significant benefits of low energy consumption and chemical input (Kuenen, 2008).

Precise control of oxygen supply is critically important to the successful operation of nitritation-anammox process, and can be accomplished using gas-permeable membrane (Gong et al., 2007) in so-called membrane-aerated biofilm reactor (MABR). In an MABR, gas-permeable membranes deliver oxygen to biological reactions and meanwhile serve as biofilm support for bacterial immobilization to favor growth of *ammonia oxidizing bacteria (AOB)* and/or anammox bacteria but repress *nitrite oxidizing bacteria (NOB)* activity (Downing & Nerenberg, 2008). MABRs have been studied for autotrophic nitrogen removal in the recent years (Gilmore et al., 2013; Gong et al., 2007; Li et al., 2016). However, the presence of organic compounds in the typical wastewater would favor the growth of heterotrophs over anammox bacteria due to very

low specific growth rate of anammox bacteria, and thus the reduction of organic compounds becomes necessary prior to the implementation of anammox-based treatment.

As an emerging treatment concept, a bioelectrochemical system (BES) such as microbial fuel cell (MFC) or microbial electrolysis cell (MEC) has been employed to achieve simultaneous organic carbon removal and energy recovery from wastewater. BES takes advantage of microbial interaction with solid electron donors/acceptors to accomplish conversion of organic compounds to electricity (Rabaey et al., 2010). As a result of electron flow, nitrogen compounds may also be removed through ammonium transport or nitrate/nitrite reduction (Kelly & He, 2014). The integration of membrane aeration with BES has been performed to improve contaminant removal and electricity generation. For example, in a membrane-aerated MFCs, more than 99% of COD could be removed and nitrogen removal efficiency was doubled to 52% compared to that of an MFC with diffused aeration (Yu et al., 2011). Membrane aeration could also significantly decrease energy consumption due to enhanced oxygen diffusion efficiency in the MFC cathode chamber. To achieve similar electricity generation, a membrane-aerated MFC required energy input of only 0.05 kWh m^{-3} , much lower than 1.76 kWh m^{-3} of a diffused aeration MFC (Yang et al., 2016).

The advantages of both membrane aeration (e.g., precise control of oxygen supply for nitrification-anammox process) and BES (organic removal and conversion to bioenergy) intrigue the exploration of membrane-aerated nitrification-anammox in BES. Such integration may create mutual benefits like low energy consumption for system operation and removal of multiple contaminants simultaneously. In this study, a BES was designed to accommodate membrane-aerated nitrification-anammox in its cathode chamber and investigated for treating high-strength

synthetic wastewater for organic and ammonia removal. The anode would function as a pretreatment step to remove a significant portion of organic compounds and generate electrons to drive ammonium ions transporting across ion exchange membrane into the cathode chamber, where nitrification-anammox would occur. An external voltage was applied to stimulate current generation and thus improve contaminant removal. For comparison, three control experiments were operated. The effects of operation mode and hydraulic retention time (HRT) were systematically investigated for current density, treatment performance, and energy production/consumption. The microbial community at different locations in the cathode was analyzed and dominant species were identified.

2. Materials and methods

2.1. BES setup

The BES consisted of two compartments, physically separated by a piece of cation exchange membrane (CEM) (Membrane International Inc., Ringwood, NJ, USA). The anodic compartment (370 mL) was placed in a tubular container, creating a cathodic compartment of 1120 mL (Fig. 1). The anode electrode was non-wet proofed carbon brush, which was pretreated by being immersed in acetone overnight and then heated at 450 °C for 0.5 h, while the cathode electrode was wet-proofed carbon cloth (13.5 cm×30 cm, Zoltek Corporation, St. Louis, MO, USA) containing 4 mg cm⁻² activated carbon powder (Thermo Fisher Scientific, Bridgewater, NJ, USA) as a catalyst for oxygen reduction reaction (ORR). The activated carbon powder was coated to the carbon cloth by using a 15% **polytetrafluoroethylene (PTFE)** solution as a binder agent and heat-treated at 370 °C for 0.5 h. A gas-permeable silicone membrane module (5 m active length, outer diameter of 3.18 mm, and inner diameter of 1.98 mm, Silastic laboratory tubing #508-009,

Dow Corning Corp., Midland, MI, USA) wrapped around the carbon cloth (Fig. 1), and was connected to a cylinder containing air (Airgas, Radnor, PA, USA). The oxygen supply rate was adjusted by a needle valve and a gas pressure meter. By adjusting the lumen pressure, the DO concentration in the cathode chamber could be controlled under an abiotic condition.

2.2. BES operation

The BES was operated in a continuous mode at room temperature ($20 \pm 1^\circ\text{C}$) with an applied voltage of 0.80 V (3644 A, Circuit Specialists, Inc., Mesa, AZ, USA) across the external resistance of 1 ohm. The anode was fed with synthetic wastewater, composed of 2532 ± 27 mg L⁻¹ sodium acetate (2000 ± 25 mg L⁻¹ COD), 500 ± 10 mg L⁻¹ NH₄⁺-N, 15 mg L⁻¹ MgSO₄, 20 mg L⁻¹ CaCl₂, 500 mg L⁻¹ NaCl, 100 mg L⁻¹ NaHCO₃, 5.35 mg L⁻¹ K₂HPO₄, 2.65 mg L⁻¹ KH₂PO₄, and trace elements (Angenent & Sung, 2001). Such a synthetic solution was intended to mimic livestock wastewater or digester effluent. The anode HRT was set at 20, 40, 60, and 90 h by varying the influent flow rate of 0.3, 0.15, 0.1, and 0.07 mL min⁻¹. The anode was inoculated with 50 mL sludge from the anaerobic digester in a local wastewater treatment plant (Pepper's Ferry Wastewater Treatment Authority, Radford, VA, USA). In the non-loop operation, the cathode was continuously fed with a mineral solution containing 420 mg L⁻¹ NaHCO₃, 27.2 mg L⁻¹ KH₂PO₄, 59 mg L⁻¹ MgSO₄, 180 mg L⁻¹ CaCl₂, 1 mL L⁻¹ trace elements solution I, and 1 mL L⁻¹ trace elements solution II (Imajo et al., 2004). The cathode was seeded with 100 mL of inoculum containing both anammox granules and flocs from an upflow anaerobic sludge blanket (UASB) anammox reactor operated at 30 °C in the lab. The pH inside the cathode was maintained between 7.0 and 7.5 by automatically adding 0.2 M HCl solution. The BES experienced four different operation modes with the same lumen pressure of 10 psi in the

cathode, including three Controls (1, 2, and 3) (Fig. 1, Fig. S1, and Table S1): Control 1 – an abiotic cathode with open circuit (one month); Control 2 – an abiotic cathode with external voltage (one month); Control 3 – loop operation of feeding the anode effluent to the cathodic compartment without an external voltage (one month); and Reactor – loop operation of feeding the anode effluent to the cathodic compartment with externally applied voltage (four months). The anode HRT was 60 h in the Control operation.

2.3. DNA extraction and sequencing

Samples were harvested 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 Ultra Clean PCR cleanup kit (MoBio Laboratories, Carlsbad, CA, USA) as recommended. Amplicon concentrations were quantified using Qubit 2.0 fluorometer (Invitrogen, USA). 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 Ultra Clean PCR cleanup kit (MoBio

Laboratories, Carlsbad, CA, USA) as recommended. Amplicon concentrations were quantified using Qubit 2.0 fluorometer (Invitrogen, USA). Amplicons from all samples were pooled in equimolar ratios and bi-directionally sequenced (PE 250bp) on the Illumina MiSeq platform at Virginia Biocomplexity Institute (Virginia Tech). The DNA sequencing data analysis are based on the description in a previous study (Li et al., 2016).

2.4. Measurement and analysis

The BES voltage was recorded by a digital multimeter (2700, Keithley Instruments, Inc., Cleveland, OH, USA) every 2 min. The concentrations of chemical oxygen demand (COD), ammonium ($\text{NH}_4^+\text{-N}$), nitrite ($\text{NO}_2^-\text{-N}$), and nitrate ($\text{NO}_3^-\text{-N}$) were measured by using a colorimeter (DR 890, Hach Company, Loveland, CO, USA). The key parameters, such as current density, Coulombic efficiency (CE), energy production, and energy consumption, were calculated according to a previous study (Yang et al., 2016). Current density (A m^{-2}) was calculated based on the liquid volume of the anode chamber. CE was defined as the total produced Coulombs over all the available Coulombs from the removed acetate:

$$CE = \frac{Q}{nFC_a} \quad (1)$$

where Q is integration of current over time, n is the number of electrons released from each molecule of acetate, F is the Faradic constant ($96,485 \text{ C mol}^{-1} \text{ e}^-$), and C_a is the mole of the removed acetate. The theoretical power requirement for the pumping system was estimated as:

$$P_{pumping} = \frac{Q\gamma E}{1000} \quad (2)$$

Where P is the pumping system power requirement (kW), Q is the flow rate ($\text{m}^3 \text{ s}^{-1}$), γ is specific weight of water (9800 N m^{-3}), and E is the hydraulic pressure head (m). In this study, the

hydraulic pressure head of 0.02 m was estimated for the recirculation pump (Kim et al., 2010).

For the membrane aeration, adiabatic compression energy was calculated following the standard method (Cote et al., 1988):

$$E_m = \frac{1}{e_b e_d e_m} \frac{RT}{(k-1)k} \left(\left(\frac{P_1}{P_2} \right)^{(k-1)k} - 1 \right) \quad (3)$$

Where R is gas law constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$), T is absolute temperature (294 K), k is ratio of specific heat of air at constant volume (1.395), P_1 is absolute pressure before compression (101325 Pa), P_2 is absolute pressure after compression (Pa), e_b is blower efficiency (0.7), e_d is drive efficiency (0.95), e_m is motor efficiency (0.92), and W_m is adiabatic compression energy ($\text{J mol}^{-1} \text{ air}$).

3. Result and discussion

3.1. Performance of the proposed system

The performance of the proposed system (“Reactor”) was described by the parameters such as current density, nitrogen removal efficiency, and COD removal efficiency, and compared with that of three control systems (Fig. 2). Among the four operation modes, the Control 2 generated the highest current density of $88.6 \pm 1.7 \text{ A m}^{-3}$, followed by the Reactor ($72.3 \pm 1.9 \text{ A m}^{-3}$) and the Control 3 ($33.9 \pm 1.5 \text{ A m}^{-3}$) ($p < 0.05$, one-tailed two-sample t-test with unequal variance at $\alpha = 0.05$ for all the following statistical tests). Current generation was not observed in the Control 1 under the open circuit condition. The difference in current generation had two implications: first, the external voltage could significantly improve current generation (“Reactor” > “Control 3”); and second, the presence of microorganisms and organic compounds in the cathode (introduced from the anode effluent) could decrease the current generation, likely due to

competition for electron acceptors (oxygen) between the cathode electrode and other electron donors (e.g., organic residues in the anolyte effluent in the Reactor) (“Reactor” < “Control 2”).

The loop operation mode clearly enhanced contaminant removal because of additional treatment in the biocathode. In general, the Reactor and the Control 3 with loop operation exhibited higher removal efficiency of both nitrogen and COD than the Control 1 and 2 with only anode treatment (Fig. 2). The Reactor achieved the highest ammonium removal efficiency of $97.6 \pm 5.4\%$, followed by $73.7 \pm 8.4\%$ of the Control 3 ($p < 0.05$). Without the cathode anammox, the ammonium removal in the Control 1 and 2 was only $2.0 \pm 1.7\%$ and $29.7 \pm 4.3\%$, respectively. The removal of total nitrogen (TN) followed the same trend as ammonium removal and the Reactor had the highest TN removal efficiency of $94.8 \pm 7.7\%$. Those results confirmed the important role of current generation in driving ammonium out of the anolyte (“Control 2” > “Control 1”, and “Reactor” > “Control 3”). In addition, the membrane-aerated nitrification-anammox in the cathode could significantly improve ammonium removal efficiency.

The COD removal efficiency was mainly affected by current generation, and the influence of loop operation mode was minor. With current generation, more than 80% COD removal was obtained in the Control 2, Control 3, and the Reactor, and the highest removal of $98.2 \pm 3.3\%$ was achieved in the Reactor. In contrast, only $25.9 \pm 2.8\%$ of soluble COD removal was observed in the Control 1. Most of organic degradation was accomplished in the anode chamber, which removed 84.5–90.6% of the COD. In addition, high Coulombic efficiency (CE) of 85.9–90.6% suggested that most of the soluble COD was converted to electricity by electrogenic

bacteria in the anode. The remaining COD in the anode effluent was further polished in the cathode by heterotrophic bacteria, such as denitrifiers.

3.2. Effect of hydraulic retention time

The above results have demonstrated that the proposed system with membrane-aerated nitrification-anammox in the cathode chamber could stimulate current generation and contaminant removal. The system performance of the Reactor was further studied by examining the effect of HRT, a key parameter that determines the loading rates of contaminants. The results showed that a higher organic loading rate with a shorter HRT led to more current generation (Fig. 3A). The COD loading rate decreased from 189.4 to 47.0 g COD m⁻³ d⁻¹ with HRT extended from 20 to 90 h, leading to a decline of the maximum current density from 77.0 ± 3.7 to 41.4 ± 2.0 A m⁻³ ($p < 0.05$). The removal efficiency of both nitrogen and COD was slightly increased with increased HRT from 20 to 40 h, but the further extension of HRT had little impact on removal efficiency (Fig. 3A, $p > 0.05$), for example, with the HRT increased from 20 to 90 h, the ammonium removal efficiency varied between 84.4 and 97.9%, the TN removal efficiency was 78.4–94.8%, and the COD removal efficiency was 95.2–99.8%. The current density and the concentrations of organic carbon and ammonium in the anode effluent under different HRTs are shown in Fig. 3B. As HRT increased, the COD concentration in the anode effluent declined from 216 ± 13 to 114 ± 8 mg L⁻¹ ($p < 0.05$), while the current density dropped rapidly due to less available organic substrates for electrogenic bacteria, resulting in a decrease in migration of ammonium across CEM to cathode (Table S2). Therefore, an slightly increase of ammonium was observed in the anode effluent with increased HRT from 20 to 90 h, but the concentration data were not significantly different ($p > 0.05$). Meanwhile, either nitrate or nitrite was detectable. For

the nitrification-anammox process to remove nitrogen in the cathode, the COD/TN ratio in the cathode influent was a key factor. The COD/TN ratio decreased from 1.1 ± 0.1 to 0.5 ± 0.1 in the anode effluent (cathode influent) with HRT increasing from 20 to 90 h (Table S3), which had the comparable performance with the full scale nitrification-anammox process with the similar COD/TN ratio (<0.5 in digester supernatant) (Joss et al., 2009).

The HRT variance also affects dissolved oxygen (DO) and then nitrogen removal performance in the cathode. DO can significantly affect the microbial activity of denitrifying bacteria, which are facultative microbes that prefer to utilize oxygen as the electron acceptor instead of nitrate and may outcompete AOB for oxygen in the cathode chamber (Probian et al., 2003). In this study, DO and inorganic nitrogen species in the cathode effluent of the Reactor are shown in Fig. 3C and Table S4. With HRT of 20 h, the TN concentration in the cathode effluent was $107.8 \text{ mg N L}^{-1}$, and the ratio of NH_4^+/TN , NO_2^-/TN , and NO_3^-/TN was 72.5%, 6.1%, and 21.4%, indicating that nitrification was likely the rate-limiting step because of insufficient oxygen supply ($0.2 \text{ mg O}_2 \text{ L}^{-1}$), leading to ammonium accumulation in the cathode. When HRT increased from 20 to 60 h, the TN concentration decreased to 25.8 mg N L^{-1} . During this stepwise increase in HRT, the nitrogen loading rate (NLR) for the system decreased from $144.9 \text{ g N m}^{-3} \text{ d}^{-1}$ to $48.3 \text{ g N m}^{-3} \text{ d}^{-1}$, and the TN removal increased from $378.9 \text{ g N m}^{-3} \text{ d}^{-1}$ to $458.3 \text{ g N m}^{-3} \text{ d}^{-1}$. Such a level of NLR was similar to a single stage nitrification–anammox SBR (e.g., $100 \text{ g N m}^{-3} \text{ d}^{-1}$), but the TN removal rate was much higher than the average level of $320 \text{ g N m}^{-3} \text{ d}^{-1}$ (Lackner & Horn, 2012). A long HRT of 90 h ($\text{DO}=1.2 \text{ O}_2 \text{ mg L}^{-1}$) resulted in an increased concentration of TN of 29.8 mg N L^{-1} , and the ratio of NH_4^+/TN , NO_2^-/TN , and NO_3^-/TN was 35.2%, 13.2%, and 51.7%, respectively, suggesting that anammox and denitrification were likely the limited processes for

nitrogen removal. Furthermore, the observed ratio of $\text{NO}_2^-/\text{NH}_4^+$ or $\text{NO}_3^-/\text{NH}_4^+$ was analyzed based on the effluent nitrite/nitrate concentration from the cathode and the ammonium concentration in the anode effluent (which was the cathode influent). A “threshold line” for the ratio of $\text{NO}_3^-/\text{NH}_4^+$ in a typical nitrification-anammox process is 13%, based on the stoichiometry of the reactions, and could be used to evaluate whether the activities of responsible organisms are balancing in the system (Third et al., 2001). The observed $\text{NO}_3^-/\text{NH}_4^+$ ratio decreased from 11.8% to 6.3% with HRT increased from 20 to 60 h, and a higher observed $\text{NO}_3^-/\text{NH}_4^+$ ratio of 7.0% was with HRT of 90 h. These results have two implications: first, all of the observed $\text{NO}_3^-/\text{NH}_4^+$ ratios fell below the “threshold line”, indicating the successful repression of NOB activities and more nitrite conversion by anammox bacteria; and second, the declined denitrification ability due to lower availability of organic compounds may contribute to a higher ratio of $\text{NO}_3^-/\text{NH}_4^+$ at HRT of 90 h. The ratio of $\text{NO}_2^-/\text{NH}_4^+$ was used to evaluate whether nitrite accumulation occurred by the abundance of AOB over anammox bacteria (Song et al., 2013). The observed $\text{NO}_2^-/\text{NH}_4^+$ ratio decreased from 3.4% to 0.6% with HRT increased from 20 to 60 h, and a higher ratio of 1.8% occurred with HRT of 90 h, suggesting that 90 h HRT was superfluous.

3.3. Energy consumption

Energy consumption of the Reactor was expressed as kilowatt hour per cubic meter of the treated wastewater (kWh m^{-3}), kilowatt hour per kilogram of the removed nitrogen (kWh kg N^{-1}), and kilowatt hour per kilogram of the removed COD (kWh kg COD^{-1}). With HRT of 60 h, the total energy consumption was 0.93 kWh m^{-3} , among which the external voltage supply had the most contribution (87.6%, 0.82 kWh m^{-3}), followed by 10.2% or 0.09 kWh m^{-3} by aeration and 2.2% or 0.02 kWh m^{-3} by the recirculation pump (Fig. 4A). Increasing the HRT from 20 to 90 h

increased the energy consumption of the Reactor (E_W) from 0.35 kWh m⁻³ to 1.06 kWh m⁻³ (Fig. 4B), because of the increased external voltage energy input. Likewise, the energy consumption for nitrogen (E_N) and COD removal (E_C) also increased from 0.39 to 1.21 kWh kg N⁻¹ and from 0.15 to 0.43 kWh kg COD⁻¹ (Fig. 4B). The specific energy consumption of the Reactor was lower than conventional biological nutrient removal (BNR) process or previous BES (Fenu et al., 2010; Li et al., 2014; Maurer et al., 2003; Zhang et al., 2014), for example, denitrification in a wastewater treatment plant could require E_N of 12.50 kWh kg N⁻¹, a membrane bioreactor (MBR) would demand E_N of 27.30 kWh kg N⁻¹ and E_C of 3.30 kWh kg COD⁻¹, and a BES could consume up to E_N of 8.58 kWh kg N⁻¹ with 0.8 V applied. Although E_N of the Reactor was comparable to 0.80–1.92 kWh kg N⁻¹ for deammonification process (DEMON) in a SBR or 1.05–4.17 kWh kg N⁻¹ for anammox process by the biofilm (Lackner et al., 2014), the present BES reactor also achieved COD removal simultaneously. Thus, the same energy was used to accomplish multiple tasks in the Reactor.

3.4. Characterization of microbial communities

To better understand the mechanism of membrane-aerated nitrification-anammox process in the cathode chamber, three amplicon libraries for sequencing analysis, namely Cathode, Biofilm, and Suspended, were obtained from carbon cloth electrode, silicone membrane surface, and cathode bulk solution at HRT of 60 h during the stable operating period (Fig. 5 and S2). At the genus level (Fig. 5A), a total of 17 sequences were detected as >1% taxa, and more than 60% of the sequences were not classified, indicating the diversity of the microbial communities in the present system. Four groups of organisms associated with nitrogen conversion were identified: AOB, NOB, anammox, and denitrifying bacteria. The anammox bacteria were dominant

microorganism in the Initial Granule sample. The abundance of AOB *Nitrosomonadaceae* was 7.4% in the Biofilm sample, while it accounted for less than 1% in both Cathode and Suspended samples (Fig. 5B). This difference was due to the readily available oxygen on the membrane surface favoring the growth of AOB. *Nitrospira* (NOB) was negligible (~0.01%) in all three samples, which was consistent with the low effluent nitrite concentration (Fig. 3C). Such abundance of NOB was much lower than the biomass enriched in the previous nitrification-anammox membrane-aerated reactor to treating synthetic wastewater with a low nitrogen strength (Li et al., 2016). All the anammox bacteria identified so far belong to the monophyletic family *Ca. Brocadiaceae* in the phylum *Planctomycetes* (Fig. 5C) (Sonthiphand et al., 2014). Anammox bacteria were dominant in the Suspended sample (10.3%) while they accounted for ~0.5% in the Cathode and Biofilm samples (Fig. 5B). Three genera affiliated with anammox – *Candidatus Brocadia* (*Ca. Brocadia*), *Ca. Kuenenia* and *Ca. Jettenia* were identified in the Suspended sample, accounting for 2.7%, 3.3% and 4.3%, respectively (Figs. 5A). In addition, *Ca. Brocadia* was only detected in the Suspended sample while *Ca. Kuenenia* was the dominant anammox genus in both Cathode and Biofilm samples (Fig. 6B).

The community structures of three samples indicated that microbial succession occurred in the different locations of the cathode chamber under long term operation (Fig. 6). In general, the abundance of denitrifying bacteria in the Cathode sample accounted for 96% of all nitrogen-converting organisms, while AOB and anammox bacteria were dominant among all nitrogen-converting organisms in the Biofilm and Suspended samples, respectively (Fig. 6A). Such structure patterns of four groups of organisms in the three samples may be associated with DO gradient, substrate gradient (e.g., COD, nitrate), etc. At least eight genus with denitrifying

capability, including *Comamonas*, *Paracoccus*, *Dechloromonas*, *Thermomonas*, *Rubrivivax*, *Simplicispira*, *Thiobacillus*, *Geobacter*, were identified and likely responsible for alleviating nitrate accumulation and meanwhile further removing COD in the cathode chamber (Throback et al., 2004). All of these denitrifying bacteria were from the phylum *Proteobacteria* (Fig. 5C), with *Comamonas* being the dominant denitrifying genus in all samples (Fig. 6C). For examples, the abundance of *Comamonas* in the Cathode sample was the highest among three samples, but the abundance of *Thermomonas* in the Suspended sample was much higher compared with the other two samples (Fig. 6C). Previous studies indicated that the *Paracoccus* genus were expressed by the *nirS* gene to achieve nitrite reduction, while the *Comamonas* were expressed by the *nosZ* gene and the *Thermomonas* were expressed by the *nar* gene to achieve nitrate reduction (Van Spanning et al., 2007). However, the distribution of these denitrifying genera in different locations and their interactions with other nitrogen-converting organisms remain unknown. There is also possibility that some denitrifying bacteria were involved in bioelectrochemical reduction of nitrate on the cathode, but this warrants further investigation. The various community structures of biomass from different sites suggest that the membrane aeration in the cathode could create a favorable condition for the development of single-stage anammox-based process.

The dominant phyla in the Cathode and Biofilm samples are *Bacteroidetes* and *Proteobacteria*, accounting for 89% in total, while a distinct difference was observed in the Suspended sample where four phyla were dominant including *Bacteroidetes* (32.80%), *Proteobacteria* (30.10%), *Planctomycetes* (12.50%), and *Chlorobi* (12.20%) (Fig. 5C). In addition, *Synergistetes*, *Firmicutes*, *Chloroflexi* and *Alphaproteobacteria* contain heterotrophic

bacteria, while *Betaproteobacteria* includes several groups of nitrifiers, denitrifiers and other N-cycle related microorganisms (Isanta et al., 2015) (Fig. S2A).

Due to the loop operation of feeding anode effluent to the cathode chamber, the electrogenic bacteria were also detected in all three sample, such as *VadinCA02*, *Arcobacter*, *Dechloromonas*, *Clostridium*, *Sphingobacterium*, *Chryseobacterium*, *Paludibacter* (Chabert et al., 2015) (Fig. 5A). They were abundant on the surface of the carbon cloth (cathode). For example, the electrogenic bacteria accounted for 12.8% of the Cathode samples, 4.6% of Biofilm sample, and 6.5% of Suspended sample. Their functions in the cathode were not clear yet, and they could be involved in catalyzing cathodic oxygen/nitrate reduction reactions or non-electrochemical reactions. In addition to electrogenic bacteria and AOB, abundance aerobic heterotrophic bacteria would also contribute to the degradation of organic compounds.

4. Conclusions

Herein, a BES with membrane-aerated nitrification-anammox was designed and studied to enhance the removal of both organic and nitrogen contaminants. Loop operation took advantages of additional treatment in the biocathode, and externally applied voltage stimulated current generation, resulting in higher nitrogen removal. The present system was potentially energy efficient and had low specific energy consumption based on either treated water or contaminant removal. AOB, anammox genus, denitrifying bacteria, and electrogenic bacteria was abundant in the cathode, and their distribution was affected by aeration and location. The results encourage further research for optimizing system design and operation with actual wastewater.

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Reference

1. Angenent, L.T., Sung, S. 2001. Development of anaerobic migrating blanket reactor (AMBR), a novel anaerobic treatment system. *Water Research*, 35(7), 1739-1747.
2. Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME Journal*, 6(8), 1621-1624.
3. Chabert, N., Ali, O.A., Achouak, W. 2015. All ecosystems potentially host electrogenic bacteria. *Bioelectrochemistry*, 106, 88-96.
4. Cote, P., Bersillon, J.-L., Huyard, A., Faup, G. 1988. Bubble-free aeration using membranes: process analysis. *Journal (Water Pollution Control Federation)*, 1986-1992.
5. Downing, L.S., Nerenberg, R. 2008. Effect of oxygen gradients on the activity and microbial community structure of a nitrifying, membrane-aerated biofilm. *Biotechnol. Bioeng.*, 101(6), 1193-1204.
6. Fenu, A., Roels, J., Wambecq, T., De Gussem, K., Thoeye, C., De Gueldre, G., Van De Steene, B. 2010. Energy audit of a full scale MBR system. *Desalination*, 262(1), 121-128.
7. Gilmore, K.R., Terada, A., Smets, B.F., Love, N.G., Garland, J.L. 2013. Autotrophic Nitrogen Removal in a Membrane-Aerated Biofilm Reactor Under Continuous Aeration: A Demonstration. *Environ. Eng. Sci.*, 30(1), 38-45.
8. Gong, Z., Yang, F., Liu, S., Bao, H., Hu, S., Furukawa, K. 2007. Feasibility of a membrane-aerated biofilm reactor to achieve single-stage autotrophic nitrogen removal based on Anammox. *Chemosphere*, 69(5), 776-784.
9. Imajo, U., Tokutomi, T., Furukawa, K. 2004. Granulation of Anammox microorganisms in up-flow reactors. *Water Science and Technology*, 49(5-6), 155-164.
10. Isanta, E., Bezerra, T., Fernandez, I., Suarez-Ojeda, M.E., Perez, J., Carrera, J. 2015. Microbial community shifts on an anammox reactor after a temperature shock using 454-pyrosequencing analysis. *Bioresour. Technol.*, 181, 207-213.
11. Joss, A., Salzgeber, D., Eugster, J., König, R., Rottermann, K., Burger, S., Fabijan, P., Leumann, S., Mohn, J., Siegrist, H. 2009. Full-scale nitrogen removal from digester liquid with partial nitrification and anammox in one SBR. *Environmental Science & Technology*, 43(14), 5301-5306.

12. Kelly, P.T., He, Z. 2014. Nutrients removal and recovery in bioelectrochemical systems: a review. *Bioresour. Technol.* , 153, 351-360.
13. Kim, J., Kim, K., Ye, H., Lee, E., Shin, C., McCarty, P.L., Bae, J. 2010. Anaerobic fluidized bed membrane bioreactor for wastewater treatment. *Environ. Sci. Technol.* , 45(2), 576-581.
14. Kuenen, J.G. 2008. Anammox bacteria: from discovery to application. *Nat. Rev. Microbiol.* , 6(4), 320-326.
15. Lackner, S., Gilbert, E.M., Vlaeminck, S.E., Joss, A., Horn, H., van Loosdrecht, M.C. 2014. Full-scale partial nitrification/anammox experiences—an application survey. *Water Res.* , 55, 292-303.
16. Lackner, S., Horn, H. 2012. Evaluating operation strategies and process stability of a single stage nitrification–anammox SBR by use of the oxidation–reduction potential (ORP). *Bioresource technology*, 107, 70-77.
17. Li, J., Ge, Z., He, Z. 2014. A fluidized bed membrane bioelectrochemical reactor for energy-efficient wastewater treatment. *Bioresour. Technol.* , 167, 310-315.
18. Li, X., Sun, S., Badgley, B.D., Sung, S., Zhang, H., He, Z. 2016. Nitrogen removal by granular nitrification–anammox in an upflow membrane-aerated biofilm reactor. *Water Res.* , 94, 23-31.
19. Maurer, M., Schwegler, P., Larsen, T. 2003. Nutrients in urine: energetic aspects of removal and recovery. *Water Science and Technology*, 48(1), 37-46.
20. Probian, C., Wülfing, A., Harder, J. 2003. Anaerobic Mineralization of Quaternary Carbon Atoms: Isolation of Denitrifying Bacteria on Pivalic Acid (2,2-Dimethylpropionic Acid). *Appl. Environ. Microbiol.*, 69(3), 1866-1870.
21. Rabaey, K., Butzer, S., Brown, S., Keller, J.r., Rozendal, R.A. 2010. High current generation coupled to caustic production using a lamellar bioelectrochemical system. *Environmental Science & Technology*, 44(11), 4315-4321.
22. Song, Y., Ishii, S., Rathnayake, L., Ito, T., Satoh, H., Okabe, S. 2013. Development and characterization of the partial nitrification aerobic granules in a sequencing batch airlift reactor. *Bioresource technology*, 139, 285-291.
23. Sonthiphand, P., Hall, M.W., Neufeld, J.D. 2014. Biogeography of anaerobic ammonia-oxidizing (anammox) bacteria. *Frontiers in microbiology*, 5, 399.

24. Third, K.A., Slikers, A.O., Kuenen, J.G., Jetten, M.S.M. 2001. The CANON System (Completely Autotrophic Nitrogen-removal Over Nitrite) under Ammonium Limitation: Interaction and Competition between Three Groups of Bacteria. *Syst. Appl. Microbiol.* , 24(4), 588-596.
25. Throbäck, I.N., Enwall, K., Jarvis, Å., Hallin, S. 2004. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol. Ecol.* , 49(3), 401-417.
26. van der Star, W.R.L., Miclea, A.I., van Dongen, U.G.J.M., Muyzer, G., Picioreanu, C., van Loosdrecht, M. 2008. The membrane bioreactor: A novel tool to grow anammox bacteria as free cells. *Biotechnol. Bioeng.* , 101(2), 286-294.
27. Van Hulle, S.W., Vandeweyer, H.J., Meesschaert, B.D., Vanrolleghem, P.A., Dejans, P., Dumoulin, A. 2010. Engineering aspects and practical application of autotrophic nitrogen removal from nitrogen rich streams. *Chem. Eng. J.* , 162(1), 1-20.
28. Van Spanning, R., Richardson, D.J., Ferguson, S.J. 2007. Introduction to the biochemistry and molecular biology of denitrification. *Biology of the Nitrogen Cycle*. 3, 20.
29. Yang, Y., Li, X., Yang, X., He, Z. 2016. Membrane aeration as an energy-efficient method for supplying oxygen to microbial fuel cells. *RSC Advances*, 6(55), 49787-49791.
30. Yu, C.-P., Liang, Z., Das, A., Hu, Z. 2011. Nitrogen removal from wastewater using membrane aerated microbial fuel cell techniques. *Water Res.* , 45(3), 1157-1164.
31. Zhang, F., Li, J., He, Z. 2014. A new method for nutrients removal and recovery from wastewater using a bioelectrochemical system. *Bioresource technology*, 166, 630-634.

Figure 1. The schematic diagram of membrane-aerated nitritation-anammox in a BES – loop operation of feeding anode effluent to cathodic chamber with an externally applied voltage. **Note:** “DC” means “direct current”.

Figure 2. The performance (current density, NH_4^+ , TN and COD removal efficiency) of control systems (Control 1, 2, and 3) and Reactor with HRT of 60 h. Note: The calculated nitrogen/COD removal in Control 1 and 2 was based on the anode, and that in Control 3 and Reactor was based on the overall system. Error bars $\pm$ SD is based on the average measured under the stable operating condition.

Figure 3. The performance of the Reactor with different HRTs: (A) the current density and contaminant removal efficiency in the system, (B) the concentration of contaminant in anode effluent, and (C) the concentration of nitrogen in cathode effluent and dissolved oxygen (DO). Error bars $\pm$ SD is based on the average measured under stable operating conditions.

Figure 4. Energy consumption by the Reactor with HRT of 60 h: (A) contribution of different energy consumers; and (B) specific energy consumption (SEC) affected by HRT. Note: E_w was expressed as kilowatt hour per cubic meter of the treated wastewater (kWh m⁻³), E_N was expressed as kilowatt hour per kilogram of the removed nitrogen (kWh kg N⁻¹), and E_C was expressed as kilowatt hour per kilogram of the removed COD (kWh kg COD⁻¹).

Figure 6. The abundance of nitrogen-converting organisms (at the genus level): (A) the relative abundance of AOB, anammox and denitrifying bacteria in all nitrogen-converting organisms, and (B) the relative abundance of each anammox genera in all anammox bacteria, and (C) the population of denitrifiers. Note: 1) All the samples were taken at the same time from Reactor with HRT of 60 h; 2) NOB was not shown in the Fig. 6A due to very low abundance ($<0.01\%$); and 3) AOB includes one genus within *Nitrosomonadaceae* family; the anammox bacteria include *Ca. Brocadia*, *Jettenia* and *Kuenenia*, and the denitrifiers include all the genera shown in Fig. 6C.