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Nitrogen removal in a single-chamber microbial fuel cell with nitrifying biofilm enriched at the air cathode

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ABSTRACT

Nitrogen removal is needed in microbial fuel cells (MFCs) for the treatment of most waste streams. Current designs couple biological denitrification with side-stream or combined nitrification sustained by upstream or direct aeration, which negates some of the energy-saving benefits of MFC technology. To achieve simultaneous nitrification and denitrification, without extra energy input for aeration, the air cathode of a single-chamber MFC was pre-enriched with a nitrifying biofilm. Diethylamine-functionalized polymer (DEA) was used as the Pt catalyst binder on the cathode to improve the differential nitrifying biofilm establishment. With pre-enriched nitrifying biofilm, MFCs with the DEA binder had an ammonia removal efficiency of up to 96.8% and a maximum power density of 900 ± 25 mW/m^2, compared to 90.7% and 945 ± 42 mW/m^2 with a Nafion binder. A control with Nafion that lacked nitrifier pre-enrichment removed less ammonia and had lower power production (54.5% initially, 750 mW/m^2). The nitrifying biofilm MFCs had lower Coulombic efficiencies (up to 27%) than the control reactor (up to 36%). The maximum total nitrogen removal efficiency reached 93.9% for MFCs with the DEA binder. The DEA binder accelerated nitrifier biofilm enrichment on the cathode, and enhanced system stability. These results demonstrated that with proper cathode pre-enrichment it is possible to simultaneously remove organics and ammonia in a single-chamber MFC without supplemental aeration.

1. Introduction

Microbial fuel cells (MFCs) use bacteria to catalyze the conversion of chemical oxygen demand (COD) in wastewater into electricity (Liu and Logan, 2004), as electrons are extracted from substrates and donated to anodes by exoelectrogens. These electrons transfer through an external circuit to the cathode electrode, where they reduce terminal electron acceptors such as oxygen (Cheng et al., 2006), ferricyanide (Pham et al., 2004; Raghavulu et al., 2009), per chlorate (Thrash et al., 2007), or nitrate (Clauwaert et al., 2007). In wastewater treatment applications, the removal of nitrogen and phosphorus, in addition to COD, are often among the treatment goals. Therefore, the design of a wastewater treatment system using MFCs should include methods for nutrient removal.

In most wastewaters, nitrogen exists in the reduced forms of ammonia (NH_3 or NH_4^+) and organic nitrogen (Rittmann and McCarty, 2001). To remove reduced nitrogen, organic nitrogen is ammonified and ammonia is first oxidized to nitrite and nitrate by the nitrification process, which requires oxygen. In

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two-chamber MFCs, oxygen was supplied either inadvertently by diffusion to the anode chamber from an aerated cathode chamber (Kim et al., 2008; Min et al., 2005; You et al., 2009) or deliberately in a side-stream aeration tank (Virdis et al., 2008). These studies reported ammonium leakage from anode to cathode chamber across the proton exchange membrane, and both approaches incurred energy costs for aeration. Electricity production associated with ammonium oxidation was suggested for a rotating-cathode MFC (He et al., 2009). Given the very low Coulombic efficiency (CE) obtained in this system (0.06%–0.34%), it is possible that the current resulted from heterotrophic exoelectrogenesis sustained by nitrifier-produced organics rather than from direct ammonium oxidation.

Following nitrification, the oxidized forms of nitrogen could be removed in MFCs by the denitrification process in conjunction with soluble electron donor oxidation or cathode oxidation in biocathodes (Clauwaert et al., 2007; Gregory et al., 2004; Virdis et al., 2008). Recent designs (Virdis et al., 2010; Xie et al., 2011) effectively incorporated both nitrification and denitrification in an aerated cathode chamber or with pre-aerated cathode influent, eliminating the ammonium leakage issue but still requiring energy for aeration.

Single-chamber air-cathode MFCs, which save aeration energy by allowing oxygen in the atmosphere to passively diffuse into the solution, were also tested for ammonia removal (Kim et al., 2008; Min et al., 2005). In tests using swine wastewater (Min et al., 2005), 83% ammonia removal with 86% soluble COD removal was reported. A slight increase in nitrate concentrations indicated occurrence of nitrification, which was sustained by oxygen diffusion through the air cathode. Subsequent testing of this design (Kim et al., 2008) demonstrated that the main mechanism for ammonia removal in the single-chamber MFCs with no buffer solution and a single PTFE layer on the cathodes was probably not due to biological processes, but rather to volatilization of ammonia at the cathode. However, in a well-buffered system, Kim et al found these physical–chemical mechanisms were not as significant. If the air cathode is coated with more PTFE diffusion layers, the ammonia volatilization might be further reduced.

Since nitrifying bacteria have considerably lower growth rates than heterotrophs, it can be difficult for them to thrive in a mixed culture system with an ample organic electron donor. Ammonia-oxidizing bacteria (AOB) were detected in the cathode biofilm of a single-chamber air-cathode MFC (Kim et al., 2008), but this system was not specifically designed or operated to promote nitrification. Two potential strategies to increase the AOB fraction in a biofilm are pre-colonization with nitrifiers (Tran et al., 2009) and material tailoring for preferential AOB enrichment. Diethylamine functionalization onto hollow-fiber membranes enhanced the adhesion of nitrifying bacteria in a continuously fed fluidized bed bioreactor, and biofilms exhibited a much higher nitrification rate per unit area than other biofilms in these studies due to the partial positive charge of the grafted membrane surface (Hibiya et al., 2000, 2003; Terada et al., 2003; Tsuneda et al., 1995). Hence, use of diethylamine-functionalized polymer as a catalyst binder as compared to a conventional Nafion-type binder might be an effective way to improve nitrifying biofilm formation on the cathode surface. In addition, altering chemical functionality of the catalyst binder, which is the outermost layer of the solution-facing side of an air cathode, does not add an additional layer to a cathode surface and thus should not increase the internal resistance.

In this study, a single-chamber MFC with a nitrifying biofilm enriched at the air cathode was investigated for simultaneous nitrification and denitrification coupled with electricity generation. Nitrifying bacteria were pre-enriched on the air cathode before exoelectrogenic bacteria were inoculated into the MFCs. To enhance the enrichment of a nitrifying biofilm, a novel catalyst binder, diethylamine-functionalized polymer (DEA), was used and compared to a regular Nafion binder system.

2. Materials and methods

2.1. Reactor setup

Single-chamber air-cathode MFC reactors consisting of an anode and cathode placed on the opposite ends of a Plexiglas cylindrical chamber 4 cm long by 3 cm in diameter (empty bed volume of 28 mL) (Liu and Logan, 2004) were used in the nitrifying biofilm enrichment stage (Fig. 1A). Anodes were made of a non-wet proof carbon cloth (type A, E-TEK), while cathodes were wet-proofed (30%) carbon cloth (type B, E-TEK) coated with a carbon base layer and four PTFE layers on the air-facing side and a 0.5 mg-Pt/cm^2 catalyst layer on the solution-facing side (Cheng et al., 2006). When enrichment reactors were changed into batch-fed MFCs, the reactor chambers and air cathodes enriched with nitrifying biofilm were preserved, while the carbon cloth anodes were replaced by brush anodes with a 2.5 cm outer diameter and a 3 cm effective length that were made of carbon fibers (PANEX33 160k, ZOLTEK) (Fig. 1B). Two different materials were used as binders for the Pt catalyst layer, either Nafion (Aldrich, 5% aliphatic alcohol solution) (reactors Nafion-1 and Nafion-2) or DEA (supplementary materials) (reactors DEA-1 and DEA-2). The DEA binder introduces partial positive charges to the cathode surface, which were hypothesized to enhance the attachment of AOB. Two abiotic controls (Nafion-control and DEA-control) were used to monitor ammonium loss through the air cathode without biological processes involved. Nafion-control and DEA-control used the same reactor design and electrodes as duplicates Nafion-1 & 2 and DEA-1 & 2, respectively. Also, the amount of binder used for a cathode, either Nafion or DEA, was the same small amount, as described in the supplementary materials. During enrichment, all the reactors were operated in open-circuit mode. Then MFCs were operated in closed circuit with an external resistance of 1000 Ω.

2.2. Inoculation

At the beginning of nitrifier enrichment, all reactors except the abiotic controls were inoculated with 2 mL of mixed liquor taken from the aerated nitrification tank of the Pennsylvania State University Wastewater Treatment Plant and 3 ml

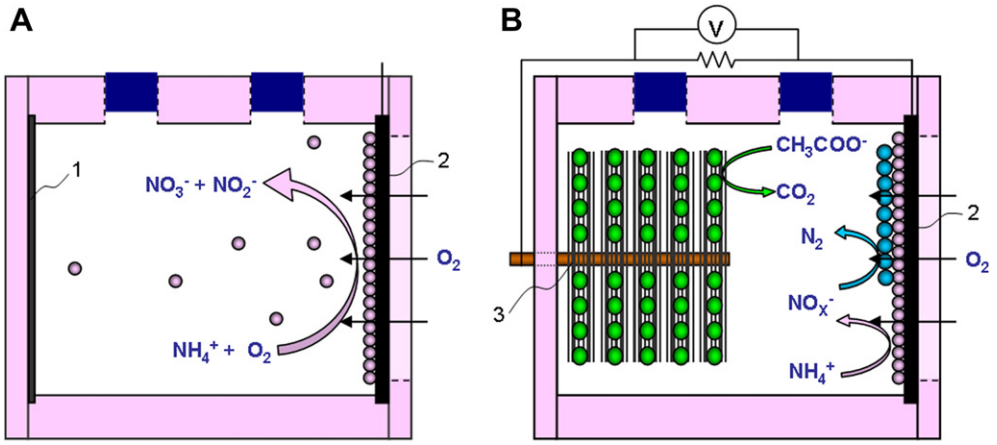

Fig. 1 – Schematics of (A) enrichment reactors and (B) single-chambered air-cathode MFCs for simultaneous nitrification and denitrification (1: carbon cloth anodes; 2: air cathodes with four PTFE layers and one 0.5 mg-Pt/cm^2 catalyst layer; 3: heat-treated brush anodes).

cultured suspension of *Nitrosomonas europaea* (ATCC 19718). Mixed liquor was included in the inoculum because we could not achieve significant cathode biofilm by enriching only the pure culture of *N. europaea*. All reactors were operated in a 30 °C thermostatic chamber in fed-batch mode, with autoclaved medium containing 0.764 g L^{-1} NH_4Cl, 1.78 mg L^{-1} Na_2CO_3, 3.12 mg L^{-1} $MgCl_2$, 0.79 mg L^{-1} $CaCl_2$, 4.26 × 10^{-2} mg L^{-1} $FeSO_4$, and 3.76 × 10^{-3} mg L^{-1} $CuSO_4$ in 50 mM phosphate buffer solution (PBS, containing 17.77 mM $NaH_2PO_4 \cdot H_2O$, 32.23 mM Na_2HPO_4, and 1.74 mM KCl, pH 7.0) (a modified recipe to the ATCC 19718 recipe). Reactors were covered to prevent the growth of photosynthetic organisms and UV inhibition of AOB. When there was above 90% ammonia removal in any reactor, all of the reactors were refilled with fresh sterile medium. The enrichment period lasted 75 days (five batches).

To move into MFC operation status, the nitrifier-enriched reactors Nafion-1&2 and DEA-1&2 as well as Nafion-control were inoculated with 14 mL of a suspended exoelectrogenic microbial consortium taken from a bottle air-cathode MFC fed with sodium acetate that had been running for more than 1 year. Operation of the DEA-control reactor was discontinued due to the detection of contamination, based on its significant nitrite/nitrate production in the last batch of the enrichment period. MFCs were operated in batch mode with medium containing 1 g L^{-1} CH_3COONa, 1.78 mg L^{-1} Na_2CO_3, 3.12 mg L^{-1} $MgCl_2$, 0.79 mg L^{-1} $CaCl_2$, 4.26 × 10^{-2} mg L^{-1} $FeSO_4$, 3.76 × 10^{-3} mg L^{-1} $CuSO_4$, and trace minerals and vitamins (Lovley and Phillips, 1988) in 50 mM PBS. The medium contained approximately 0.382 g L^{-1} NH_4Cl (100 mg NH_4^+–N L^{-1}) during the first 14 batches except for a reduced concentration of around 40 mg NH_4^+–N L^{-1} during the first two batches of the Nafion-control for testing ammonia removal by mechanisms other than the nitrification process. Therefore, the COD:N ratio of the medium for most tests was about 7.8:1, which is within the COD:N ratio range of many real wastewaters (Orhon et al., 1997). Reactors remained covered to prevent adverse effects of light. In the first batch, since the inoculum was half of the reactor volume, double-strength growth medium was used. Reactors were re-fed with complete replacement of the medium when the voltage decreased below 10 mV, giving batch durations of two to four days. Reactors did not contain any mixing device in either stage.

2.3. *Chemical analysis*

During the enrichment stage, 0.4 mL samples were taken from each reactor every two or three days using 3 mL syringes and needles (VWR International, LLC.) through the sample septum and filtered immediately through a 0.2 μm pore diameter PTFE syringe filter (VWR International, LLC.). The concentration of ammonia (which includes here both NH_4^+–N and NH_3–N) was measured using Standard Method 10031 (APHA, 1995) (HACH Company, Loveland, CO) after diluting 20 μL sample in 80 μL distilled (DI) water. Nitrite (NO_2^-–N) and nitrate (NO_3^-–N) concentrations were quantified by ion chromatography (Dionex DX-120, Dionex Co., Sunnyvale, CA) using an AS16 4-mm column with 1.0 mM NaOH eluent, following dilution of 200 μL sample in 5 mL DI water. Bulk solution pH in each MFC was tested before and after each batch using a calomel pH electrode (VWR International, LLC.) and pH meter (Fisher Scientific, AB15). In MFC mode, 1.0 ml samples were taken from reactors before and after each batch. In addition to NH_4^+–N/NH_3–N, NO_2^--N, NO_3^--N, and pH, soluble COD was also measured using Standard Method 5220 (APHA, 1995) (HACH COD system) following dilution by 4–10 times with DI water.

2.4. *Electrochemical analysis*

The voltage drop (U) across the external resistor in the circuit was measured at 10-min intervals using a multimeter (Keithley Instruments, Cleveland, OH), and current (I) was calculated using Ohm's Law. Power density (P) was obtained using $P = IU/A$, where A is the projected surface area of the cathode (7 cm^2). To obtain the maximum power densities, polarization experiments were carried out in MFC fed-batch cycles 11 and 12 using the single-cycle method (Heilmann and Logan, 2006). During these tests, the external resistance was changed from 1000 Ω to 500 Ω, 200 Ω, 150 Ω, 100 Ω, 80 Ω,

60 Ω, and 40 Ω with each resistance for 20 min and the voltage measured by a multimeter (RadioShack, Cat. No. 22-813).

2.5. Biofilm thickness analysis

Air cathodes were taken from each MFC reactor at the end of operation, and the biofilms were fixed immediately with 4% paraformaldehyde in PBS over night. Samples were then washed twice with PBS and stored in 1:1 (v/v) PBS-ethanol solution at −20 °C. Subsamples (0.5 cm × 0.7 cm) were taken from each fixed cathode using sterile scissors and dehydrated sequentially in 50, 80, and 96% (v/v) ethanol for 5 min each. The dehydrated samples were stained with 1 μg/mL DAPI reagent for 30 min and washed with deionized water twice. After air drying, the stained samples were cryo sectioned perpendicular to the cathode surface into 10 μm sections (Murga et al., 1995), which were then mounted onto a glass slide with a cover glass on top. Samples were visualized with epifluorescence (Olympus BX72, Olympus America Inc.) at a 10× magnification. Biofilm thicknesses were obtained by calculating the mean biofilm thickness of five sections from each cathode sample.

2.6. Calculations

Coulombic efficiency (CE) was calculated based on integrated current, COD removal, and reactor volume (V_r) (Logan, 2008) using $V_r = 26$ mL (the graphite fiber brush displaced approximately 2 mL water volume).

The ammonia removal efficiency ($\eta_{NH_4^+-N}$) was calculated every batch as

$$\eta_{NH_4^+-N} = \frac{[NH_4^+ - N]_0 - [NH_4^+ - N]_f}{[NH_4^+ - N]_0} \times 100\%,$$

where $[NH_4^+ - N]_0$ is the NH_4^+–N concentration in the bulk solution at the beginning and $[NH_4^+ - N]_f$ is the NH_4^+–N concentration in the bulk solution at the end of a batch.

Total nitrogen (TN) was defined as

$$TN = [NH_4^+ - N] + [NO_2^- - N] + [NO_3^- - N].$$

Since samples were all filtered before tests, insoluble organic N can be ignored. The TN removal efficiency (η_{TN}) was calculated as

$$\eta_{TN} = \frac{[TN]_0 - [TN]_f}{[TN]_0} \times 100\%.$$

To evaluate if there was an inhibition of free ammonia (FA) and free nitrous acid (FNA) on ammonia oxidation and nitrite oxidation, we also calculated FA and FNA based on pH, temperature T (K), total ammonia nitrogen (TAN, NH_3–N + NH_4^+–N), and total nitrite nitrogen (TNN, NO_2–N + HNO_2–N), which were from Park et al (Park and Bae, 2009) as

$$FA\ (mg\ NH_3 - N/L) = \frac{TAN \times 10^{pH}}{[\exp(6334/T) + 10^{pH}]}$$

$$FNA\ (mg\ HNO_2 - N/L) = \frac{TNN}{[\exp(-2300/T) \times 10^{pH}] + 1}$$

3. Results

3.1. Nitrification and total nitrogen removal

3.1.1. Enrichment stage

In the 75-day enrichment stage, the concentration of ammonia decreased by 98%–100% within a batch time of 10–20 days in DEA-1&2 reactors and 32%–93% in Nafion-1&2 reactors, compared to 0%–39% in Nafion-control and DEA-control reactors (Fig. S2). Ammonia concentration decreased in the Nafion-1&2 and DEA-1&2 reactors at an increasing rate from batch 1 to batch 3. Ammonia removal was consistently more rapid in the DEA reactors than in the Nafion reactors (Fig. S2). Nitrite and nitrate production, indicative of ammonia and nitrite oxidation, were observed in the biotic reactors, but not in the abiotic controls (Fig. S3–S4). The maximum FA and FNA that could occur in the three reactor systems were calculated to be 0.1 mg L^{-1} as NH_3–N and 9 × 10^{-6} mg L^{-1} as NO_2–N.

The duplicated Nafion-1 and Nafion-2 reactors had an increasing difference in ammonia removal during the enrichment. In the Nafion-1 reactor, the ammonia concentration decreased faster and the removal rate was close to the DEA reactors. However, ammonia removal in Nafion-2 was still slow in the last batch of enrichment, indicating incomplete formation of a nitrifying biofilm.

3.1.2. MFC operation stage

During the first 10 cycles of fed-batch MFC operation, average ammonia removal efficiencies in the DEA MFCs was 93 ± 3% and in the Nafion MFCs was 85 ± 5%, compared to 61 ± 10% in the Nafion-control MFC (Fig. 2). With an initial ammonia concentration of 104 ± 17 mg N/L in the influent medium, the ammonia concentration in the effluent of the DEA MFCs after each cycle was below 11 ± 3 mg N/L, while the Nafion-control and Nafion-1&2 MFCs had more unstable effluent ammonia

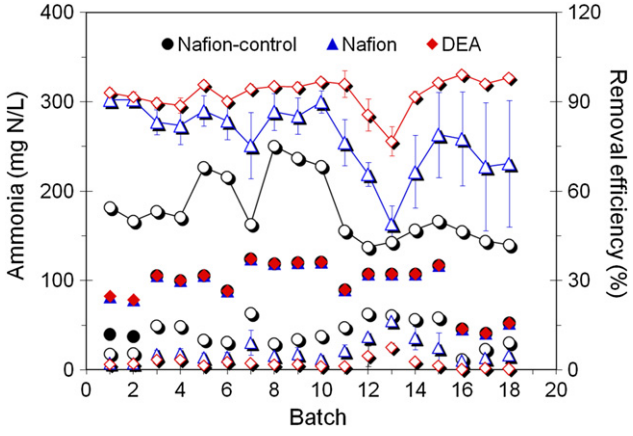

Fig. 2 – Ammonia removal efficiencies (open symbol with solid line) and ammonia concentrations in the influent (solid symbol only) and effluent (open symbol only) in MFCs with pre-enriched cathodes using different binders (Nafion, DEA) and a control lacking pre-enrichment using Nafion binder (Nafion-control).

concentrations, ranging from 18 to 63 mg N/L in Nafion-control and 7–31 mg N/L in Nafion-1&2 MFCs. The difference between Nafion-1 and Nafion-2 in ammonia removal also occurred in the MFC operation stage, with Nafion-1 having greater ammonia removal efficiency than Nafion-2.

The nitrite concentration in the Nafion-control MFC was between 6 and 11 mg N/L during cycles 3 to 6 but always below detection limit (<1.2 mg N/L) in the DEA and Nafion MFCs (Fig. 3). Nitrate concentrations of 8–30 mg N/L were found in the effluent of DEA MFCs and 14–28 mg N/L in Nafion MFCs, while in the Nafion-control MFC, net nitrate production was only detected in cycle 7 (5 mg N/L) and after cycle 10. Consistent with ammonia removal, the net production of nitrate in the duplicated Nafion MFCs also showed differences, with Nafion-1 consistently having higher nitrate concentrations than Nafion-2. The maximum TN removal efficiencies were lower in Nafion-control (75%) and Nafion MFCs (79%), compared to 94% in DEA MFCs.

The ammonia removal efficiency in each MFC dropped substantially, by 21–46% in batch 13 compared to batch 10, after polarization experiments were carried out in batches 11 and 12 (Fig. 2). After batch 13, with no additional polarization experiments, the ammonia removal abilities of both DEA reactors and Nafion-1 started to recover and finally reached their previous levels. The ammonia removal efficiency in Nafion-2 also recovered partially from batch 13; however its difference with Nafion-1 increased. In the Nafion-control MFC, ammonia removal efficiency dropped to and stayed around 40–50% after batch 11.

3.2. *pH change in enrichment and MFC stages*

During the nitrifier enrichment stage, effluent pH progressively decreased in the DEA and Nafion reactors (Fig. 4), consistent with the growth of AOB. Conversely, the Nafion- and DEA-control reactors showed a pH increase during this period. However, during the MFC status, there was generally little change in pH relative to the influent pH for all conditions, though the Nafion-control reactor showed a consistent pH drop after batch 4.

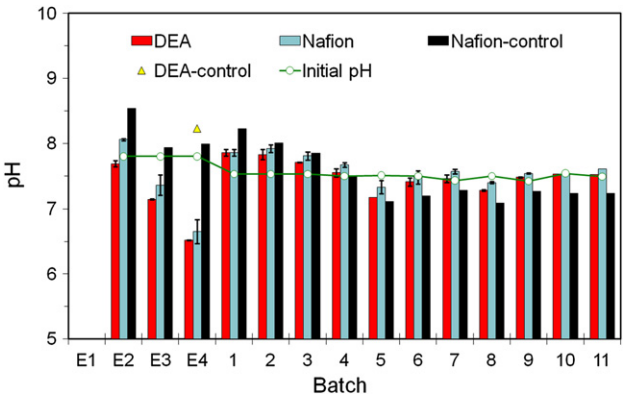

Fig. 4 – Influent and effluent pH in each batch for Nafion-control, Nafion, and DEA MFCs (E: enrichment stage).

3.3. *Electrochemical performance*

After the inoculation of exoelectrogenic bacteria and the addition of sodium acetate to the feed medium, all MFCs started to produce similar maximum cell voltages from the third batch (Fig. 5), which were 528 ± 3, 561 ± 0, and 548 ± 3 mV for DEA, Nafion, and Nafion-control MFCs, respectively. In successive batches, the batch duration (voltage > 10 mV) for the Nafion-control MFC increased from 34 to 62 h by batch 11, while in the DEA MFCs the batch duration slightly increased from 27 to 35 h. The duplicate Nafion MFCs did not show differences in batch duration in the first 11 batches.

During normal operation ($R_{external}$ = 1000 Ω), MFCs had slightly different power densities, with Nafion MFCs the highest and DEA MFCs slightly lower. Polarization tests showed that the Nafion MFCs produced the highest maximum power density (945 ± 42 mW/m^2 at $R_{external}$ = 100 Ω) (Fig. 6), with Nafion-1 higher than Nafion-2. Duplicate DEA MFCs obtained more consistent maximum power densities with each other, which was 900 ± 25 mW/m^2 at $R_{external}$ = 100 Ω. The maximum power density achieved in the Nafion-control reactor was the lowest (750 mW/m^2 at $R_{external}$ = 80 Ω).

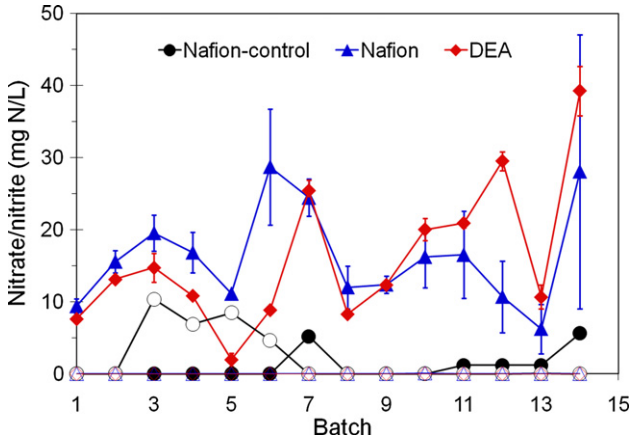

Fig. 3 – Nitrate (solid symbol) and nitrite (hollow symbol) concentrations in Nafion-control, Nafion, and DEA MFCs.

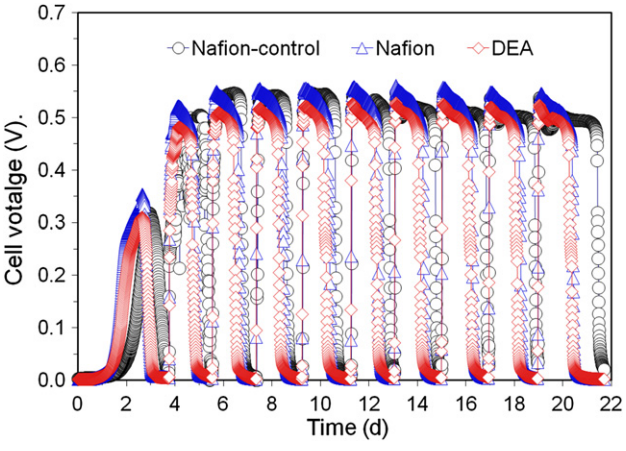

Fig. 5 – Cell voltage output in Nafion-control, Nafion, and DEA MFC.

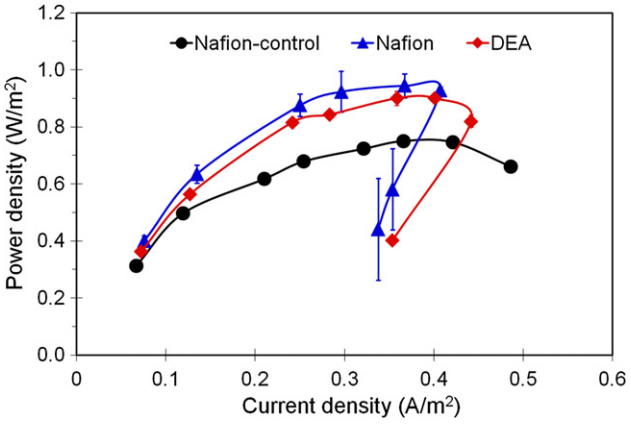

Fig. 6 – Power densities of Nafion-control, Nafion, and DEA MFCs in batch 12.

3.4. *Coulombic efficiency and COD removal*

The CEs in all the reactors started from approximately 10% in the first batch, and increased to up to 45% in Nafion-control MFCs after 10 batches. CEs in Nafion MFCs increased to 26% after 10 batches, which was similar to DEA MFCs (25%) (Fig. 7). The Nafion-control MFC consistently had a higher CE than the nitrifier-enriched MFCs, and among the nitrifier-enriched MFCs the Nafion MFCs consistently had slightly higher values than the DEA MFCs. The average COD removal efficiencies for Nafion-control, Nafion, and DEA reactors were all close to 96%, with an initial concentration of 770 mg COD/L in each batch.

3.5. *Cathode biofilm thickness*

Cathode biofilms were not visible after nitrifier enrichment, but became clearly visible during MFC fed-batch operation. At the end of the MFC stage, the mean cathode biofilm thickness ($L_{f\ mean}$) in nitrifier-enriched MFCs (254–453 μm) was significantly greater than in the Nafion-control MFC (94 μm) (Table 1) (Fig. S5). Both DEA and Nafion MFCs had different biofilm thicknesses between their duplicates.

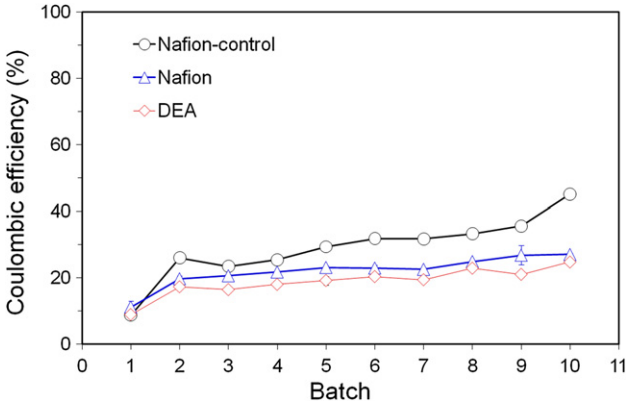

Fig. 7 – Coulombic efficiency of Nafion-control, Nafion, and DEA MFCs.

Table 1 – Cathode biofilm thickness (L_f) summary.

Sample	L_f mean (μm)	n[a]
Nafion-Control	94 ± 24	5
DEA-1	339 ± 10	5
DEA-2	453 ± 48	5
Nafion-1	359 ± 36	5
Nafion-2	254 ± 15	5

a n: The number of cryosections from each sample used for biofilm thickness calculation.

4. Discussion

4.1. *Ammonia removal*

4.1.1. *Passive diffusion of oxygen through air cathodes*

In our MFC systems, the oxygen required by the nitrification process and the cathodic oxygen reduction reaction relied on diffusion through the air cathode. Although the feeding medium might bring some dissolved oxygen to the system during the filling/drawing procedure at each cycle, the maximum amount of ammonia removal that could be supported by this external oxygen would be less than 2.3 mg NH_3–N L^{-1} (Supplementary Material). The greater ammonia removal rates in DEA and Nafion MFCs than the abiotic control indicated AOB activity in the former two systems. Furthermore, a previously reported oxygen transfer coefficient ($k = 2.3 \times 10^3$ cm s^{-1}) (Cheng et al., 2006) through the same Nafion air cathode design as we used in this study yields an oxygen diffusion rate (r_{O2}) of 2.5×10^{10} mg O_2 m^{-2} cathode h^{-1} through our air cathodes (Supplementary Material). Compared to the calculated required oxygen diffusion rate for nitrification during enrichment ($r_{O2\_enrichment} = 5.4 \times 10^3$ mg O_2 m^{-2} cathode h^{-1}) or both the oxygen reduction reaction and nitrification during MFC operation ($r_{O2\_MFC} = 3.7 \times 10^4$ mg O_2 m^{-2} cathode h^{-1}) (Supplementary Material), the oxygen diffusion rate through the Nafion air cathodes was sufficient to support these reactions. Although we do not have direct experimental data for oxygen transfer through the cathodes with DEA binder, we expect that the magnitude of the difference of oxygen diffusion rates between DEA and Nafion binders would be negligible compared to the significant difference between the rate of oxygen supply with the Nafion binder and the rate of nitrogenous and carbonaceous demand (10^7 mg O_2 m^{-2} cathode h^{-1}).

4.1.2. *Reduction of ammonia loss through air cathodes*

Ammonia removal efficiencies in both of the duplicate DEA reactors (97 ± 0%) and one of the Nafion MFCs (95%) were higher within shorter batch times than previously reported for single-chamber air-cathode MFCs at 83 ± 4% (Min et al., 2005) and 60% (Kim et al., 2008). Also, three more PTFE layers on the air-facing side of the air cathode and well-buffered systems greatly reduced ammonia loss due to volatilization at the cathode, as seen by both the early and post polarization test performance of the Nafion-control system (50–60%). Passively diffused oxygen was adequate for cathode oxidation at a current density of 76 mA/m^2 and an average ammonia oxidation rate of 18 mg N L^{-1} d^{-1}.

Ammonia oxidation was estimated by subtracting the initial ammonia removal of the Nafion-control (when there was no nitrite or nitrate detected in this system) from that of the nitrifier MFCs, assuming that the initial ammonia removal in the Nafion-control was not related with ammonia oxidation but rather only ammonia assimilation by microbes and ammonia loss through the air cathode. Since Nafion and DEA MFCs had pre-enriched nitrifying biofilm on the air cathode, there might be smaller ammonia concentrations and lower pH inside the cathode biofilm which would result in less ammonia loss. Therefore, the ammonia removal in the Nafion-control represented the maximum amount of ammonia removal due to volatilization.

4.1.3. *Nitrifier biofilm enrichment effects on nitrification, maximum power densities, and cathode biofilm thicknesses*

In single-chamber air-cathode MFCs, heterotrophic bacteria can easily form biofilms on the cathodes, where in a nitrogen removal MFC AOB and nitrite-oxidizing bacteria (NOB) are also expected to localize. The ammonia removal efficiencies in both DEA and one of the Nafion MFCs were up to 134% higher than the Nafion-control reactor, demonstrating that an enrichment of nitrifying bacteria with strictly inorganic medium before MFC status assisted AOB and NOB in the competition with heterotrophs on the cathodes. Ammonia removal in the pre-enrichment reactors during fed-batch MFC status was relatively stable over more than two months of operation (Fig. 2) except following the polarization experiment, suggesting that a well developed nitrifier biofilm would not be easily washed out or outcompeted by heterotrophs. However, the performance of Nafion-2 MFC showed that if the enrichment stage is not successful, it is difficult for a nitrifier biofilm to mature in later MFC status and recover from unexpected disturbances, like the effect of the polarization experiment on ammonia removal during batch 11 and 13. Even the Nafion-control system, which had no pre-enrichment of nitrifiers on the cathode, showed some evidence of nitrification during MFC operation through increased ammonia removal and the production of nitrite and nitrate. However, this system never established the extent of ammonia removal observed in the nitrifier-enriched systems. The inhibition constants for nitrite oxidation reported by Park et al (Park and Bae, 2009) were 46 μM for FA (0.644 mg L^{-1} as NH_3–N) and 1.7–6.8 μM for FNA (0.024–0.095 mg L^{-1} as NO_2–N), and for ammonium oxidation they were 290–1600 μM for FA (4.06–22.4 mg L^{-1} as NH_3–N) and 12 μM for FNA (0.168 mg L^{-1} as NO_2–N). The FA and FNA concentrations calculated from the initial TAN concentration and the maximum nitrite concentration in the enrichment reactors (0.1 mg L^{-1} as NH_3–N and 9×10^{-6} mg L^{-1} as NO_2–N) suggested that there should be little inhibition from FA and FNA on the ammonia oxidation and nitrite oxidation processes.

The enrichment process also seemed to improve the maximum power densities of MFCs, regardless of the catalyst binder (Fig. 6). It is presumed that proton production by AOB within the cathodic nitrifier biofilm supported proton consumption associated with oxygen reduction at the cathode, thus lowering the charge transfer resistance of the system (Xie et al., 2011; You et al., 2009).

The cathode biofilms in the DEA and Nafion MFCs were 1.7–3.8 times thicker than that in the Nafion-control MFC at the end of operation, suggesting that the enrichment of nitrifying bacteria significantly promoted total biofilm accumulation (i.e., nitrifying and heterotrophic biomass) on the cathode during the MFC fed-batch phase. However, since there was no visible biofilm detected at the end of the enrichment, the greater cathode biofilm thickness in nitrifier MFCs was not simply due to the addition of previously enriched nitrifying biofilm thickness. The nitrifier biofilm might have changed the chemical microenvironment of the cathode surface, such as creating a more favorable pH through proton production, or increased the cathode attachment surface area by its pre-attachment on the catalyst binder layer.

4.1.4. *Comparing DEA and Nafion catalyst binders*

Based on the duplicate reactor performances, the DEA binder accelerated nitrifier biofilm enrichment and enhanced the nitrification stability compared to the Nafion binder. The duplicate Nafion MFCs showed obvious differences in ammonia removal but relatively consistent electrochemical performances (i.e., voltage output and CE), suggesting that a marginally successful enrichment of nitrifier biofilm occurred in the Nafion-2 MFC. Also, although the Nafion-1 reactor eventually performed nearly as well as the DEA MFCs in ammonia removal at the end of the enrichment stage, the DEA binder system showed faster start up of AOB and NOB attachment on the cathode. This supported the hypothesis that the partial positive charge of DEA binder would enhance nitrifying biofilm enrichment on the cathode.

Based on voltage output and power generation data, the DEA binder did not improve the electrochemical performance of MFCs. On the contrary, DEA MFCs showed slightly lower maximum voltage output and power densities than Nafion MFCs. This may be associated with DEA binder having a greater hydrophobicity than Nafion, which may have hindered proton diffusion (Saito et al., 2011b). However, these differences were still within an acceptable range.

The positive charge that DEA binder provides can be adjusted by the percentage of diethylamine groups in this molecule during its preparation. Higher partial positive charge may attract more AOB onto the surface, but it is unknown whether there could be a deleterious effect that could lower the activity of attached cells (Saito et al., 2011a; Terada et al., 2006). The percentage of diethylamine groups in this research (8 wt %) was demonstrated appropriate for enhancing AOB attachment.

4.2. *Denitrification in MFC operation status*

In the presence of soluble organic electron donor during the MFC operation mode, both nitrification and denitrification likely occurred. Due to the losses of ammonia, nitrogen gas, and possibly nitric and nitrous oxides through the air cathode, it was not possible to directly calculate nitrate or nitrite reduction efficiencies and perform a nitrogen balance. However, since the enrichment of nitrifying bacteria was slow and no AOB or NOB were inoculated in the Nafion-control

MFC, we assume that the ammonia removal efficiency in the Nafion-control during the first four batches (average 52%) was not related with ammonia oxidation but rather to assimilation and volatilization losses. Therefore, by subtracting 52.3% from the ammonia removal efficiencies in the DEA and Nafion MFCs, their ammonia oxidation efficiencies can be obtained (Table 2). By comparing this estimated ammonia oxidation with the net total concentration of NO_3^- and NO_2^-, we can estimate roughly 67% of NO_3^-/NO_2^- removal in the DEA MFCs and 50% in Nafion MFCs. Here, the NO_3^- and NO_2^- removal might include denitrification, nitrate and nitrite assimilation, or other processes.

For the denitrification process in DEA and Nafion MFC systems, there are two possible pathways for denitrifiers to obtain electron donors, namely oxidizing acetate from the medium and receiving electrons from the cathode electrode. Since nitrifier biofilms were pre-enriched on the air cathodes in both DEA and Nafion MFCs, we supposed that biological denitrification with organic substrates was dominant in the early stage of MFC operation. However, during long-term operation, as we observed significant increases in cathode biofilm thicknesses, denitrifiers may have been able to penetrate the nitrifier biofilm and collocate with them on the cathode electrode surface. However, the higher oxygen concentrations adjacent to the air cathode may not be favorable for denitrification.

4.3. *pH change due to nitrification and denitrification*

Nitrification was the main process occurring in the reactors during the enrichment stage since no organic substrate was provided in the autoclaved feed medium, though reactors were initially inoculated with mixed culture. Therefore, net proton production by AOB caused a decrease of bulk solution pH. Denitrification was expected during MFC operation, hence, protons produced from nitrification might be partially offset by alkalinity production associated with nitrate and nitrite reduction, thereby tempering changes in the bulk solution pH that were observed in the enrichment stage. The oxygen reduction at the cathode also consumed protons in MFC status; however, this was accompanied by an equivalent amount of protons produced from sodium acetate oxidation at the anode, which would make the final pH in the bulk solution unchanged if the transport of protons was not obstructed by anode or cathode biofilms.

Table 2 – Comparison of ammonia oxidation with nitrate and nitrite removal.

Reaction	DEA	Nafion
NH_3/NH_4^+ oxidation efficiency[a]	41%	32%
NH_3/NH_4^+ oxidized (mg N/L)	39	31
Net NO_3^-/NO_2^- produced (mg N/L)	13	17
(NO_3^-/NO_2^- removed)/(NH_3/NH_4^+ oxidized)	67%	45%

a Estimated by subtracting assumed ammonia loss via volatilization and assimilation, derived from the early operation of the Nafion-control reactor.

4.4. *The effect of the polarization experiments on nitrification*

Polarization experiments carried out in batches 11 and 12 showed significant detrimental impacts on ammonia removal. In these tests, the larger current generated when small external resistances were connected to each MFC would promote faster oxygen consumption at the cathode and a larger pH gradient within the cathode biofilm. Under these conditions, an oxygen deficiency to AOB and NOB near the cathode might occur and inactivate nitrifiers. At the same time, the rapid elevation of pH within the biofilm would hinder nitrification. After the polarization experiments, the ammonia removal in the Nafion-control and Nafion-2 MFCs never recovered to their previous levels, which suggests that some nitrifiers on the cathode might have been killed during the polarization experiment rather than temporarily inactivated or experiencing oxygen limitation. The response of these systems in subsequent batches also supports the interpretation that ammonia removal above the initial Nafion-control performance was associated with biological processes.

4.5. *The effect of N removal processes on Coulombic efficiencies*

The CEs were typical of air-cathode systems, in which excess oxygen (calculation in 4.1.1) supports heterotrophic growth, but the difference of CEs between the Nafion-control and nitrifier-enriched MFCs might be due to the nitrogen removal processes in the latter systems. With sodium acetate (CH_3COONa) as the electron donor, the total redox reaction with cell synthesis for aerobic heterotrophs on the air cathodes is

$$0.125CH_3COO^- + 0.0875O_2 + 0.0325NH_4^+ + 0.005CO_2 = 0.0325C_5H_7O_2N + 0.0925HCO_3^- + 0.0925H_2O$$

assuming the portion of the total acetate-derived electrons used for synthesis, $f_{s,heterotrophs}$, is 0.65 (the regular range of f_s for aerobic heterotrophs is from 0.6 to 0.7 (Rittmann and McCarty, 2001)) and ammonium is their *N* source. When there is full nitrification from ammonium to nitrate and complete denitrification from nitrate to nitrogen gas in the system, the respective total redox reactions with cell synthesis are

$$0.13NH_4^+ + 0.225O_2 + 0.02CO_2 + 0.005HCO_3^- = 0.005C_5H_7O_2N + 0.25H^+ + 0.125NO_3^- + 0.12H_2O$$

and

$$0.125CH_3COO^- + 0.1277NO_3^- + 0.1277H^+ = 0.0157C_5H_7O_2N + 0.1339H_2O + 0.125HCO_3^- + 0.056N_2 + 0.0464CO_2,$$

assuming $f_{s,nitrifier} = 0.10$, $f_{s,denitrifier} = 0.44$ (Rittmann and McCarty, 2001), ammonium as the *N* source for nitrifers, and nitrate as the *N* source for denitrifiers.

Calculation according to the above equations indicated that direct oxygen reduction by aerobic heterotrophic bacteria will consume approximately 1.43 mol CH_3COO^-/mol O_2; while

full nitrification from ammonium to nitrate and complete denitrification as a competitive electron-flow pathway from nitrate to nitrogen gas, or ammonium oxidation and nitrite reduction, will consume much less COD per unit oxygen (0.54 or 0.45 mol CH_3COO^-/mol O_2, respectively). Therefore, if all systems had equivalent oxygen diffusion through the air cathode, CEs should be theoretically benefited by nitrification and denitrification processes, compared to direct oxygen reduction by aerobic heterotrophic bacteria, for a given amount of surplus oxygen diffusing through the air cathode.

However, the CEs of the DEA and Nafion MFCs were slightly lower than that of the Nafion-control. This suggested that our MFC systems had different oxygen fluxes through the different air cathodes. A possible reason for the results of CEs might be that the growth of a nitrifying biofilm on the cathode induced a higher flux of oxygen into the system, perhaps by establishing a sharper oxygen gradient at the cathode.

5. Conclusions

Single-chamber air-cathode MFCs achieved simultaneous nitrification and denitrification without aeration, with a mixed culture at the anode and a nitrifying biofilm pre-enriched at the cathode. MFCs with pre-enriched nitrifying biofilms also achieved greater maximum power densities than that of a control MFC. The greater cathode biofilm thickness in nitrifier MFCs suggested the enrichment stage aided the biofilm accumulation on the cathode during MFC fed-batch operation. Compared to Nafion, the DEA binder accelerated nitrifier biofilm enrichment and enhanced its stability. We also found a detrimental effect of polarization experiments on biological ammonia removal, and lower CEs in nitrifying biofilm MFCs than that of the control MFC.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.watres.2012.01.050.

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