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Evaluation of anammox biocathode in microbial desalination and wastewater treatment

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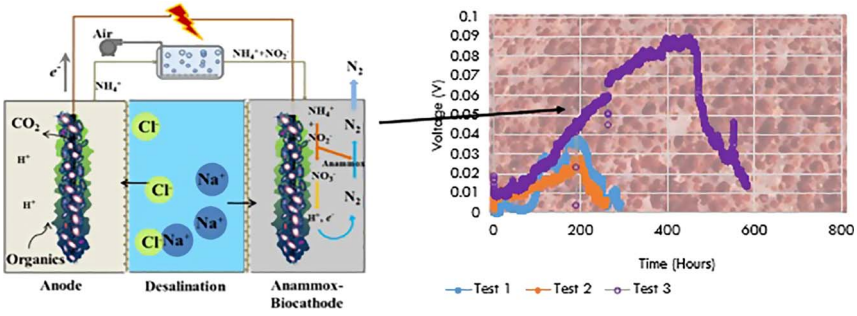
HIGHLIGHTS

- First study of anammox biocathode in microbial desalination and wastewater treatment.
- Maximum power and current densities of 0.092 W/m^3 and 0.8143 A/m^3 were obtained.
- Nitrogen removal of more than 90% was achieved in anammox biocathode compartment.

GRAPHICAL ABSTRACT



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ABSTRACT

This study presents the use of an autotrophic microorganism, Anammox bacteria, as a sustainable biocatalyst/biocathode in microbial desalination cells (MDCs) for energy-positive wastewater treatment. We report the first proof of concept study to prove that anammox mechanism can be beneficial in MDCs to provide simultaneous removal of carbon and nitrogen compounds from wastewater while producing bioelectricity. A series of experiments were conducted to enrich and evaluate the anammox mechanism and the process performance in continuous, fed-batch mode conditions. Coulombic efficiency of MDCs and nitrite and ammonium removal of wastewater increased in successive batch studies. A maximum power density of 0.092 Wm^{-3} (or a maximum current density of 0.814 A m^{-3}) with more than 90% of ammonium removal was achieved in this system. We calculated the Nernst potential for the nitrite reduction in the anammox biocathode chamber and compared with experimental values. Sequential removal of carbon and nitrogen compounds in anode and cathode chambers respectively, was also evaluated. Further, the inhibition effect of high nitrogen concentrations and the variations in microbial community profiles, especially, anammox presence was studied at different carbon and ammonia concentrations. Experimental studies and microbial community analysis are presented in detail.

1. Introduction

Ecological and environmental issues related to discharge of nitrogenous compounds in surface water bodies are manifested through excessive growth of algae and cyanobacteria resulting in low dissolved oxygen levels and massive fish kills. Current wastewater treatment processes for the removal of organic carbon using aeration technologies are energy-, chemical- and cost-intensive [1,2]. Even more burdensome are advanced treatment processes for the removal of nutrients, nitrogen and phosphorus. Nitrification followed by denitrification is the most

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common biological process applied in wastewater treatment plants. Nitrification is defined as the aerobic oxidation of ammonium to nitrite, followed by conversion of nitrite to nitrate which is typically mediated by chemo-litho-autotrophic prokaryotes such as ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA) and nitrite-oxidizing bacteria (NOB) [3]. Denitrification is the microbial reduction of nitrate to nitrogen gas performed by heterotrophic facultative anaerobic bacteria (most commonly *Alcaligenes* and *Pseudomonas* species) which require very low oxygen and organic matter for energy.

There is a growing interest to develop innovative processes to reduce the energy and chemical costs associated with the conventional nitrification-denitrification process; the anammox process is considered an ideal candidate to significantly diminish the energy and chemical inputs. Anammox bacteria are capable of achieving ANaerobic AMMonia OXidation (ANAMMOX) which results in the anaerobic conversion of ammonium to nitrogen gas with significant energy and cost savings [4]. Autotrophic bacteria create a bypass to oxidize ammonia to nitrogen gas via nitrite omitting the need for organic carbon source. Partial nitrification of ammonia to nitrite instead of nitrate enables about 40% energy savings required for aeration [5]. In addition, due to the autotrophic nature of these bacteria, their growth rate is slow and thus, results in less biosolids production. All of these benefits make anammox based nitrogen removal more cost-effective (cost reduction of up to 60%) and environment-friendly when compared with conventional nitrification-denitrification process [6]. As a result, this process has been studied intensely in recent years both at laboratory and pilot-scale levels by many researchers for wastewater treatment [7–16]. Anammox bacteria enrichment and microbial community dynamics were studied by different research groups simultaneously [8,9]. Different groups of anammox bacteria have been found in freshwater and wastewater sources. "*Candidatus* Brocadia anammoxidans" is found in freshwater, whereas bacteria found in wastewater are related to "*Candidatus* Brocadia fulgida", "*Candidatus* Kuenenia stuttgartiensis", "*Candidatus* Scalindua wagneri", and "*Candidatus* Scalindua brodae" [17].

Strous et al. [18] proposed the following stoichiometry reaction [Eq. (1)] for the anammox process.

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

According to this reaction, nitrite acts as an electron acceptor for anaerobic oxidation of ammonium. This reaction releases higher energy than aerobic ammonium oxidation. The reported energy value for the anammox process is $-358\ kJ\ mol^{-1}$ [19] and $-356\ kJ\ mol^{-1}\ NH_4^+$ [20]. This energy is substantially higher than aerobic ammonia oxidation ($\Delta G = -235\ kJ\ mol^{-1} NH_4^+$) [21].

In order to better understand the energy distribution of anammox, we need to understand the possible reactions that take place during this process. According to Van De Graaf et al. [22], the four-electron nitrite reduction forms hydroxylamine (NH_2OH) which biologically oxidizes ammonium to hydrazine (NH_2). Hydrazine is then converted to dinitrogen gas to generate the electron equivalents for the reduction of nitrite to hydroxylamine (Fig. S1) [23]. Two possible mechanisms have been proposed for reduction of nitrite and the oxidation of hydrazine, which vary depending on a single enzyme catalysis or multiple enzyme catalysis connected via electron transport chain. Either mechanism needs electrons for their first step of reduction which can be provided by cathodes in microbial desalination cells (MDCs). The electrons produced from the oxidation of wastewater at the anode will be used by a biocathode to drive nitrite/nitrate reduction. MDCs provide for simultaneous wastewater and saline water treatment facilitated by microbial biochemical reactions and ionic transport through membrane separation. This process is receiving interest in recent years due to its potential for addressing water and energy nexus issues in a single process configuration. This process can be applicable for treating high saline ground waters, wastewater treatment for water reuse and desalination in water scarce regions [24–26].

The aim of this study is to investigate the concept of using anammox bacteria as biological cathodes/catalysts in MDCs to evaluate whether it is possible to simultaneously generate electricity, desalinate salt water and remove nitrogenous compounds in the cathode chamber of a MDC process. Anammox bacteria were chosen due to their suitability/susceptibility to saline water constituents and their ability to accommodate for anaerobic ammonia oxidation process. Anammox bacteria were found to be thriving under oxygen limiting and saline water environments. For this proof of concept study, the biocathode chamber of MDC was filled with a mixed group of bacteria containing anammox bacteria and enrichment media and experiments were conducted for several batch tests to study the electricity generation potential and the biofilm formation in MDCs. The possibility of using treated wastewater from the bioanode chamber as growth medium in the biocathode chamber was also evaluated. This approach would improve the nitrogen removal process of the wastewater after its organic carbon removal in the anode chamber since wastewaters enriched in ammonium but with low organic compounds are suitable for anammox process [27,28]. Oxygen levels higher than $0.04\ mg\ L^{-1}$ and high nitrite concentrations more than $100\ mg\ L^{-1}$ have been reported to reversibly and irreversibly inhibit anammox reactions, respectively [29]. Thus, maintaining a low dissolved oxygen (DO) level is very important for anammox growth. One strategy to address this challenge is to enrich some oxygen consuming bacteria that can take up the oxygen and create the required anaerobic conditions for anammox bacteria growth. Because of these concerns, quantitative PCR (qPCR) targeting AOB, NOB and anammox bacteria was performed on the samples with increasing ammonium concentration and after using synthetic wastewater in the cathode chamber to study the microbial composition changes of this system under these conditions.

2. Materials and methods

2.1. Biomass and media preparation

Anammox biomass was provided by Hampton Roads Sanitation District wastewater treatment plant in Virginia and was divided into three 1000-mL bottles (closed to provide anaerobic conditions) in the shaker-incubator at 35 °C and 150 rpm (Fig. S2). The culture contained NH_4Cl ($382\ mg\ L^{-1}$), $NaNO_2$ ($493\ mg\ L^{-1}$), $KHCO_3$ ($200\ mg\ L^{-1}$), KH_2PO_4 ($27\ mg\ L^{-1}$), $FeSO_4{\cdot}7H_2O$ ($9.0\ mg\ L^{-1}$), EDTA ($5.0\ mg\ L^{-1}$), $MgSO_4{\cdot}7H_2O$ ($240\ mg\ L^{-1}$), $CaCl_2{\cdot}2H_2O$ ($143\ mg\ L^{-1}$) and 300 μL of trace metal solution. The trace solution contained $ZnSO_4{\cdot}7H_2O$ ($1247\ mg\ L^{-1}$), $MnSO_4{\cdot}H_2O$ ($1119\ mg\ L^{-1}$), $CuSO_4{\cdot}5H_2O$ ($44\ mg\ L^{-1}$), $Al_2(SO_4)_3{\cdot}14H_2O$ ($201.5\ mg\ L^{-1}$), $Na_2MoO_4{\cdot}2H_2O$ ($129\ mg\ L^{-1}$), $CoCl_2{\cdot}6H_2O$ ($30\ mg\ L^{-1}$), KCl ($100\ mg\ L^{-1}$), EDTA ($975\ mg\ L^{-1}$) which provided micronutrients needed for microbial growth of anammox bacteria [30]. After about two months of acclimation process, the sludge was transferred to the MDC cathode chamber (Fig. S3). The MDC anode chamber was inoculated with 30 mL of acclimatized anaerobic sludge from Starkville, Mississippi wastewater treatment plant. MDC experiments were conducted in batch mode. The working volumes of anode, desalination, and cathode chambers were 37, 28, and 37 mL, respectively. More details on MDC reactor configuration can be found in our previous publications [31–34].The synthetic wastewater composition had the following composition: Glucose ($468.7\ mg L^{-1}$), KH_2PO_4 ($4.4\ g L^{-1}$), K_2HPO_4 ($3.4\ g L^{-1}$), NH_4Cl ($1.5\ g L^{-1}$), $MgCl_2$ ($0.1\ g L^{-1}$), $CaCl_2$ ($0.1\ g L^{-1}$), KCl ($0.1\ g L^{-1}$), $MnCl_2{\cdot}4H_2O$ ($0.005\ g L^{-1}$), and $NaMoO_4{\cdot}2H_2O$ ($0.001\ g L^{-1}$) [31–34]. A salt concentration of $10\ mg\ L^{-1}$ was used in the desalination compartment for all experiments. A series of experiments were conducted to study the possibility of continuous removal of carbon and nitrogen compounds in anode and cathode chambers respectively. Further, ammonia concentrations were increased to various levels to evaluate the growth of the anammox

growth under inhibiting conditions.

2.2. Analysis and calculations

Voltage generation across a 1 KΩ external resistor was recorded every 15 min by a digital multimeter (Fluke, 287/FVF). The Current produced was calculated using Ohm's law, I = V/R. The power density was calculated (P = V/I) as per the volumes of the anode/cathode chambers. Coulombic efficiency (CE) and Coulombic recovery (CR) were calculated using the formulae as previously described [35]. The Coulombic efficiency for nitrate and nitrite reduction was calculated using Eq. (2) which is defined as the ratio of the total transferred electrons from the anode to the theoretical electrons harvested at the cathodes to remove oxidized nitrogen compounds.

$$\varepsilon_{NO_x} = 100 * \frac{\sum I(A) * t(s)}{96485\left(\frac{C}{molee^-}\right) * \Delta C_{NO_x}\left(\frac{mole}{L}\right) * n\left(\frac{molee^-}{mole}\right) * V(L)} \quad (2)$$

where "n" is the number of electrons that 1 mol of oxidized nitrogen compound presents in the cathode chamber assuming N_2 is the final product, thus it is 5 for nitrate and 3 for nitrite; ΔC_{NOx} is the difference between the initial and final concentrations of nitrite (or nitrate) and *V* is the volume of the cathode chamber (L) [36].

After observing stable voltage, polarization curves were obtained by changing the external resistance from 10 kΩ to 30 Ω (about 20 min per resistor). Collected samples were filtered through a 0.45 μm pore size cellulose acetate filter for analysis of ammonia [37], nitrite [38], NO_x (NO_2^- + NO_3^-) [39]. Chemical oxygen demand (COD) tests were carried out using standard methods. Electrical conductivity, total dissolved solids (TDS) removal, and salinity removal were recorded using a conductivity meter (Extech EC400 ExStik Waterproof Conductivity, TDS, Salinity, and Temperature Meter). The pH of the samples was measured using a pH meter (Orion 720A+ advanced ISE/pH/mV/ORP). Dissolved oxygen was measured using YSI 5100 system.

2.3. Biocathode microbial community analysis

After DNA extraction, seven different primer sets (1–7) were applied in PCR amplification for the detection of anammox, AOBs and NOBs. Table 1 lists primers used for PCR amplification in this study [27,28]. Primer sets 1 & 3 are designed to detect anammox bacteria. Primer sets 2, 4 and 5 target identification of AOB. Primers 6 and 7 were used to detect NOB. Since NOB bacteria are more phylogenetically diverse, they cannot be detected with one single primer set. Thus, two primer sets 6 and 7 were selected to detect *Nitrobacter* and *Nitrospira* respectively [40]. Polymerase chain reaction amplication was carried out as stated in each respective reference (Table 1). Otherwise, samples were amplified in a Hybaid MBS 0.2G thermal cycler with an initial DNA denaturation for 10 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and then final cycle for 10 min at 72 °C. After PCR amplification, products were loaded on 0.5TAE agarose gel, electrophoresed, and visualized on an Alpha Biotech AlphaImager. In addition to the aforementioned PCR assays, qPCR of the 16S rRNA gene was also conducted. Briefly, a 25 μL reaction mixture containing 12.5 μL ABI Syber Green Master mix, 0.5 μL of primers, and 2 μL of template DNA in conjunction with universal 16S rRNA primers as stated in [34].

Table 1
PCR primer sets used in this study.

Primer set Number	Assay	Target	References
1	hzsA526F & hzsA1829R	Anammox	[28]
2	amoA-F & amoA-2R	AOB-Ammonium-oxidizing bac	[28]
3	Pla46 & 1529	16S Anammox	[28]
4	CTO189fa_GC & CTO654r	16SrRNA-B sub AOB	[27]
5	CTO189fC_GC & CTO654r	16SrRNA-B sub AOB	[27]
6	EUB338f & NIT3	Nitrite Oxidizing bacteria (NOB)	[27]
7	EUB338f & Ntspa0685	Nitrite Oxidizing bacteria (NOB)	[27]

Table 2
Description of samples used in this study.

Sample ID	Description
A	The Initial inoculum
B	Mixed culture 10 days after (NH_4-N = 285 mg L^{-1}, NO_2-N = 63 mg L^{-1}, COD = 438.25 mg L^{-1}
C	Mixed culture 10 days after (NH_4-N = 497 mg L^{-1}, NO_2-N = 31.41 mg L^{-1}, COD = 405.42 mg L^{-1}
D	Mixed culture 10 days after (NH_4-N = 600 mg L^{-1}, NO_2-N = 0, COD = 418.08 mg L^{-1})
E	Microbial culture after being used in the cathode of MDC fed with Wastewater effluent from anode chamber

2.4. qPCR amplification

Five different samples were collected from the microbial culture at different experimental times and conditions (Table 2). These samples include sample A the original sludge we received from wastewater treatment plant as nitrogen removal culture. Sample A was used as a positive control for target genes, since each sample was based on Sample A. Sample B represents the microbial culture following addition of ammonium, collected 10 days after adding 285 mg L^{-1} ammonium to the culture. Sample C shows sample at the end of the second cycle with 500 mg L^{-1} ammonium and sample D is at the end of the third cycle after adding 600 mg L^{-1} ammonium while sample E represents the culture after using it in MDC.

3. Results and discussion

3.1. Enrichment of microbial culture

During the enrichment process, water samples were collected from the enrichment bottles and analyzed at designated intervals to evaluate the anaerobic oxidation of ammonium by bacteria. Fig. 1 shows the nitrogenous compound removal that occurred during 10 days after renewing the media described above. During the anammox reaction,

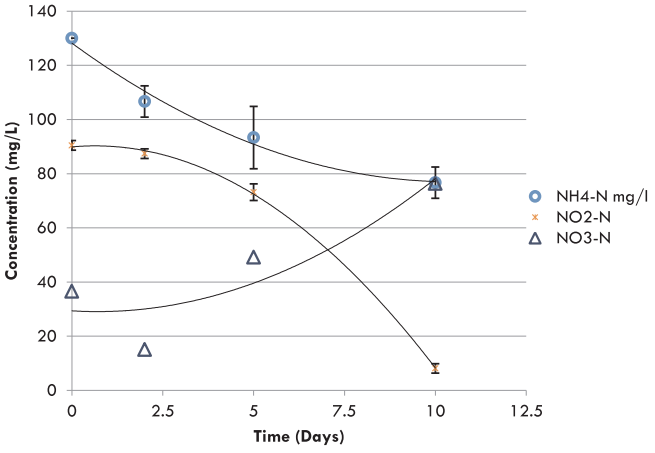

Fig. 1. Nitrite and ammonium uptake and nitrate production over time during the enrichment phase of microbial culture.

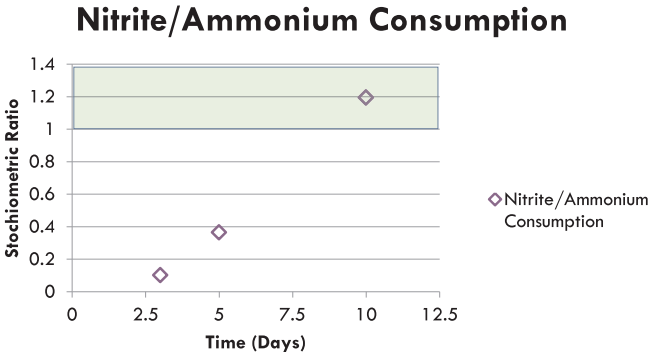

Fig. 2. Stoichiometric ratios of Nitrite to ammonium removed by reactivated anammox. The green area shows the range of the reported value from other studies so far.

ammonium and nitrite are converted to nitrogen gas, anaerobically, while nitrate is produced. A decline in ammonium and nitrite concentrations, coupled with an increase in nitrate concentrations in the samples confirmed the possibility of anammox reactions occurring in the enrichment bottles [4,41]. According to Eq. (1), the stoichiometric molar ratio of the nitrite to ammonium consumption during anammox process was approximately 1.32 [18]. However, a wide range of experimental values in various reactor types (1.28, 1.11–1.45, 1.40–1.50, and 1.00–1.18) were previously reported [42] which depends on the microbial composition and experimental conditions.

Fig. 2 shows the molar ratio of nitrite to ammonium consumption for our experimental data which indicates that anammox conditions were likely satisfied (green area on the curve) during the last days of reactivation or enrichment process. However, a higher ratio of produced nitrate to ammonium indicates that some of the nitrite was converted to nitrate through nitration by NOB. This is due to the fact that the microbial population in the cathode chamber is a mixed culture and there is a possibility that nitrifying bacteria converted nitrite to nitrate. In addition, the coexistence of nitrite consumption and nitrate generation suggests that heterotrophic denitrification did not occur significantly [17].

3.2. Anammox microbial desalination cell (AnxMDC)

Fig. 3 shows the experimental schematic while Fig. 4 shows voltage profiles generated by Anammox MDC (AnxMDC) for three batch experiments respectively. Voltage generation indicates the effective role

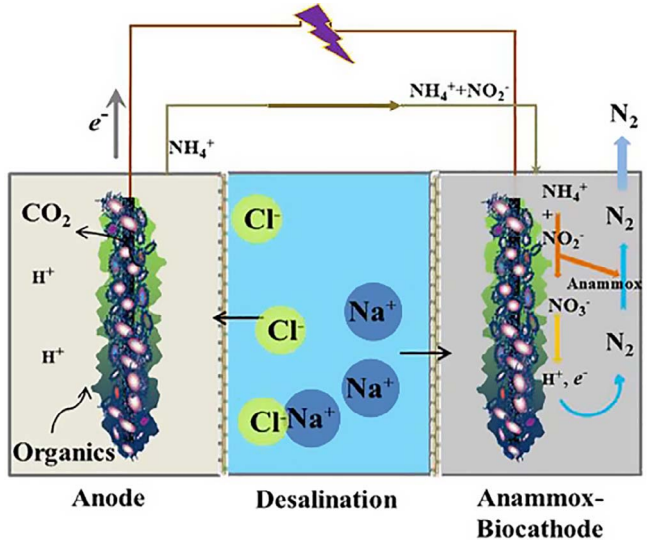

Fig. 3. Experimental scheme and working principle of the anammox microbial desalination cells.

of anammox bacteria as a biocathode and nitrite/nitrate as electron acceptors because a chemical catalyst or aeration (lack of oxygen) was not provided in the cathode chamber. Maximum power increases for the third batch of experiments, compared to the first and second batch tests, demonstrate an improvement in the biocatalytic activity of the biofilm. The maximum produced voltage was 0.0896 V which is equal to a power density of 0.114 W m^{-3} after successive batch tests. These data indicate that electricity generation can be improved with better formation of biofilms on the electrodes.

The theoretical potential of anode was calculated at the initial pH using the Nernst equation. Since just 0.0067 M of initial glucose was degraded, the expected [HCO_3^-] was determined to be 0.04 M [Eq (3)].

$$E_{\frac{Glucose}{HCO3}\_} = E_{o,Glucose} + \frac{0.05916V}{n} * \log\left(\frac{a_{ox}}{a_{red}}\right) \quad (3)$$

According to the Nernst equation the theoretical anode potential in this study would be:

$$C_6H_{12}O_6 + 12H_2O \rightarrow 6HCO_3^- + 30H^+ + 24e^-; E^O_{Glucose} = 0.104V \quad (4)$$

where E_0 is the standard potential, n is the number of moles of electrons transfered, a_{ox} and a_{red} are replaced by concentrations of oxidant and reductant. The calculated E_{anode} is −0.3919 V (pH = 6.5). In the case of cathode reactions involving nitrite and nitrate, the sequence of reactions makes it very difficult to calculate the theoretical cathodic potential for these compounds.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (5)$$

In order to simplify the calculations, the influence of intermediates was not considered in our calculation. The overall reaction of nitrate will then be [43]:

$$2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O \quad E^O_{Nitrate} = 1.246V \quad (6)$$

Since the initial concentration of NO_3^- is 0.00032 M and about 0.001 M is considered to be produced through anammox, the total concentration of nitrate which will be available for nitrate reduction would be 0.0013 M. Thus the calculated $E_{cathode}$ according to Nernst equation, in this case at pH = 7.2 is 0.706 V, which is close to the reported value of nitrate actual potentials in the bioelectrochemical systems (BESs) [43]. Therefore, the overall MDC electromotive force voltage will be:

$$E_{emf} = E_{cathode} -$$
$$E_{eanode} = 0.706 -$$
$$(-0.3919) = +1.098V \quad (7)$$

In the case of nitrite, first we need to calculate the standard potential of nitrite reduction which is calculated through the following sequencing reactions and combination of their Gibb's free energy.

$$HNO_2 + H^+ + e^- \rightarrow NO + H_2O \quad E^O_1 = 1.00V \quad (8)$$

$$NO + H^+ + e^- \rightarrow \frac{1}{2}N_2O + \frac{1}{2}H_2O \quad E^O_2 = 1.59V \quad (9)$$

$$\frac{1}{2}N_2O + H^+ + e^- \rightarrow \frac{1}{2}N_2 + \frac{1}{2}H_2O \quad E^O_3 = 1.77V \quad (10)$$

$$HNO_2 + 3H^+ + 3e^- \rightarrow \frac{1}{2}N_2 + 2H_2O \quad E^O_{1-3} = ? \quad (11)$$

$$\Delta G^O_1 = -n_1 \times FE^O_1 = -F(1.00) \quad (12)$$

$$\Delta G^O_2 = -n_2 \times FE^O_2 = -F(1.59) \quad (13)$$

$$\Delta G^O_3 = -n_3 \times FE^O_3 = -F(1.77) \quad (14)$$

$$\Delta G^O_{1-3} = -3F(E^o_{1-3}) \quad (15)$$

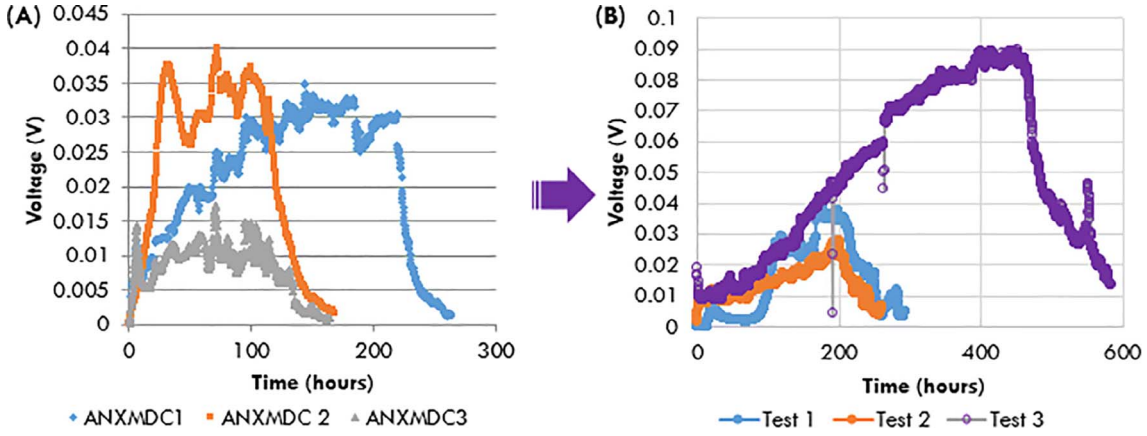

Fig. 4. ANXMDC electricity generation profiles during biofilm enrichment in fed-batch tests: A) voltage profiles of three MDCs in a single test; B) voltage profiles for a single MDC in three cycles.

$$\Delta G^{O}_{1-3} = \Delta G^{O}_{1} + \Delta G^{O}_{2} + \Delta G^{O}_{3} \rightarrow -3F(E^{o}_{1-3}) = -F(1 + 1.59 + 1.77) \quad (16)$$

$$E^{O}_{NO2} = E^{o}_{1-3} = +1.45V \quad (17)$$

Using this standard potential in the Nernst equation, the redox potential for nitrite will be E = 0.98 V which will in turn make the overall MDC electromotive force voltage to be:

$$E_{emf} = E_{cathode} - E_{eanode} = 0.980 - (-0.3919) = +1.372V \quad (18)$$

Since both E_{emf} values for nitrite and nitrate reduction were positive, their use as cathodic reactions of a microbial fuel cell (MFC) or MDC is thermodynamically favorable to release energy. The difference between this theoretical potential and the open cell potential (OCP) is energy loss. According to Cyclic Voltammetry test, the OCP for this cell is 0.286 V. Therefore, the energy losses for nitrate and nitrite as electron acceptors in our system are 0.812 and 1.086 V, respectively. These values are still high and should be reduced by improving the biofilm formation and the electron transfer mechanism from organic matter to electrode surface.

In the third batch, about 29% of the organic carbon in the anode chamber was removed to generate electrons. Table 3 shows the calculated Coulombic efficiencies and salt removals of three batch tests. The Coulombic efficiency for the third batch was much higher than the second and the first tests, which highlights the improvement of biological electrodes after several batches. Due to the higher electricity production and longer operating time, salinity removal was also higher for the third test.pH changes in the anode and cathode chambers for the three batch tests are illustrated in Fig. 5. In the cathode chamber, pH usually increases due to the hydrogen consumption in the cathode chamber associated with the nitrate reduction and due to the neutralization of acidity during anammox process [27]. Due to higher electricity production and higher efficiency of anammox process in the third test, the pH rise was even higher in the cathode chamber at the end of the experiment (Fig. 5a). pH reduction in the anode chamber, which is typical of anaerobic metabolism and release of hydrogen ions was also more significant in test 3 (Fig. 5b).

Table 3
Coulombic and Salt removal efficiencies at the end of the three batch tests.

Number of batch test	Columbic efficiencies on Glucose oxidation (%)	Salt removal %	Columbic efficiencies on nitrite/nitrate reduction (%)
Batch 1	3.4	39.4 ± 0.5	35.6
Batch 2	6.0	38.6 ± 0.2	17.5
Batch 3	52.7	53.7 ± 0.1	99.1

A comparison of nitrogen removal in the form of ammonium for three tests showed increase in removal rates (with 100% removal for test 3) during the three successive batch tests (Fig. 6a). Nitrite removal for the first, second and the third batch tests were 33.9, 52.3 and 88.7% respectively. This trend confirmed the improvement in nitrogenous compound removal process after several batches (Fig. 6b). The increased ratio of NH_4^+-N consumption to NO_2^-N consumption indicates that nitritation also occurred at the cathode where some of NH_4^+-N was converted to NO_2^-N. In the third batch, nitrogen concentration was also evident in the form of nitrate. The NO_3-N increased from 4.5 mg L^{-1} to 20.5 mg L^{-1} at the end of the experiment which happens due to the anammox reaction. These findings reaffirm that series of batch experiments can improve biofilm formation on the electrodes, which will result in better performance of the ANXMDC in terms of electricity generation and nitrogenous compounds removal. These tests were repeated several times with similar conditions and the results were reproducible, close to test 3 (Data not shown).

The Coulombic efficiency of nitrite reduction at the cathode was calculated based on the ratio of the electrons that flow across the MDC to the number of electrons that reduce all the nitrite to dinitrogen gas at the cathode. Table 3 shows that the nitrite Coulombic efficiency of test 3 was much higher than test 1 and 2. The generated coulombs transferring across the MDC for the third batch was 102.8 °C which was very close to the maximum theoretical coulombs (103.7 °C), assuming all the nitrite was reduced to nitrogen gas. Low nitrite Coulombic efficiencies for the first and second batch tests might be due to the incomplete anammox/denitrification reactions that lowers the number of electron equivalents that are used by bacteria at the electrodes [36].

A polarization curve was obtained when the cell voltage reached a steady state. Resistor was changed from low to high values and after 20 min the voltage was recorded. The results are depicted in Fig. 7. The maximum power density achieved at R = 2000 Ω, was 0.092 W/m^3. The internal resistance of the system was obtained from slope of linear polarization curve which was 3101 Ω which indicates the high internal resistance of the system and is similar to the maximum power resistance level.

3.3. Effect of ammonium and organic carbon on nitrogen removal in ANXMDC

In order to achieve sequential removal of organic carbon and nitrogen from a single wastewater stream, it is first crucial to evaluate the potential of nitrogen removal culture over high ammonium levels and organic carbon. High strength wastewaters contain high levels of ammonium (238–954.8 mg L^{-1} NH_4^+-N), low or no nitrite and some

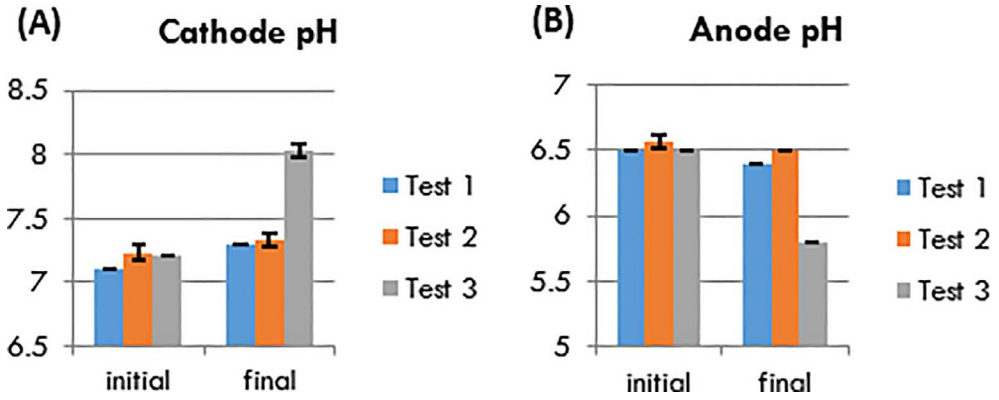

Fig. 5. (A) pH changes in the biocathode chamber and B) pH changes in the anode chamber.

levels of organic carbon. Therefore, the concentrations of ammonium were increased and nitrite levels were decreased gradually each 10 days during the current study. Because high COD inhibits anammox bacteria [42], initial COD of synthetic wastewater was kept below 450 mg L^{-1}. NH_4^+-N levels were increased up to 600 mg L^{-1} in the cathode chamber because the initial concentration of NH_4^+-N in the synthetic wastewater fed into the anode chamber was 570 mg L^{-1} and only 7% of it was removed by anode respiring bacteria indicating these bacteria are not capable of oxidizing ammonium at high concentrations.

In order to favor the growth of ammonium oxidizing bacteria (AOBs) and anammox bacteria over nitrite oxidizing bacteria, certain eco-physiological parameters such as temperature, solid retention time (SRT), free ammonia (FA) and dissolved oxygen should be regulated in the system [4]. Low DO and high temperature (30–35 °C) improve the growth of anammox bacteria [44,45]. pH and temperature were maintained at 7.7 and at 30 °C, respectively, and DO was kept around 2.5 mg L^{-1} by purging nitrogen gas into the system. Anammox bacteria grow more efficiently at pH 7.5 to 8; AOBs are not drastically affected by pH while NOB grow efficiently under pH = 7. AOB bacteria can convert part of ammonium to nitrite, which can be utilized further by anammox bacteria if the conversion of nitrite to nitrate by NOBs is inhibited [44].

$$2NH_4^+ + 1.5O_2 \rightarrow NH_4^+ + NO_2^- + H_2O + 2H^+ \quad (19)$$

Fig. 8a shows the first, second and third stages during increase of ammonium level. Due to the high concentration of ammonium, the removal efficiency is lower compared to the MDC. According to Anthonisen et al. [46], AOBs are inhibited around concentrations between 238 and 3580.56 mg L^{-1} NH_4^+-N. The increase of NO_3^- is mostly related to the anammox reaction since NOBs are more inhibited by high ammonia concentration than AOBs [46]. No nitrite was detectable in the effluent of the third cycle suggesting its consumption by anammox bacteria. This data showed that the acclimatized bacteria were able to remove high ammonium concentrations even at 600 mg L^{-1} NH_4^+-N. COD reduction in each stage indicates that some heterotrophic bacteria exist in the culture that use organic carbon as their energy source (Fig. 8b). Kartal et al. [47] showed that anammox bacteria are not strictly chemolithoautotrophic and some species can produce nitrite from nitrate in the presence of additional carbon materials. Samples were taken at the end of each stage for microbial analysis for detection of anammox, AOBs and NOBs.

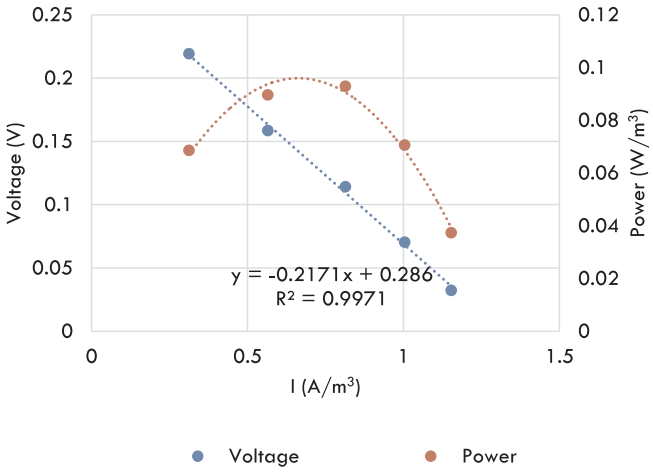

Fig. 7. Polarization curve obtained by changing external resistor of an ANXMDC.

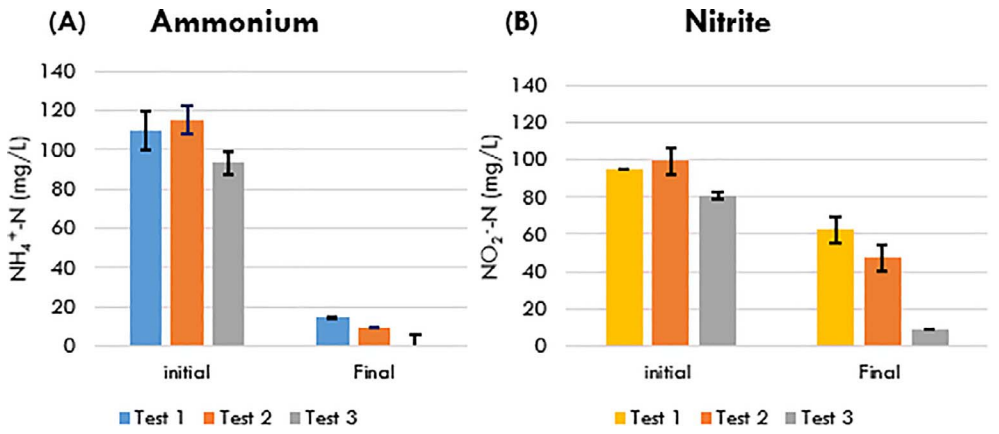

Fig. 6. (A) Ammonium removal for the three batch tests in ANXMDC; (B) Nitrite removal in ANXMDC.

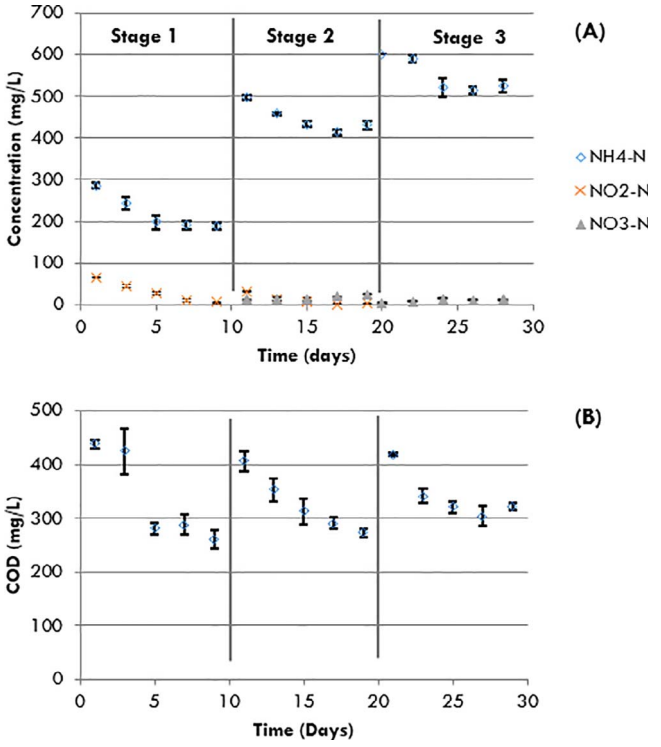

Fig. 8. A) Ammonium and B) COD concentration effects on nitrogen and COD removal of anammox microbial desalination cells.

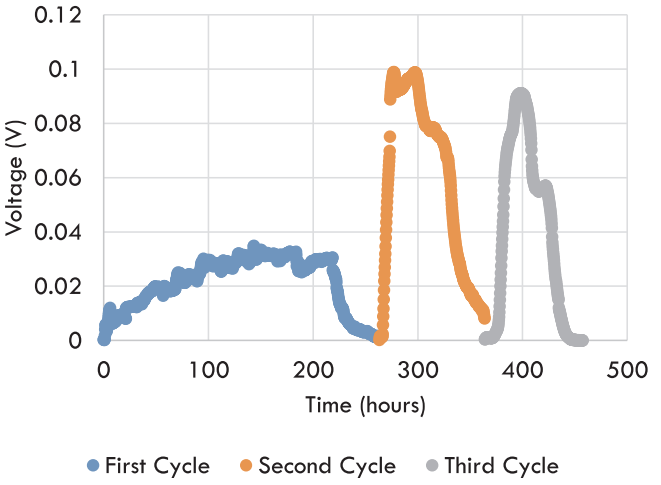

Fig. 9. Voltage generation during three batch tests in ANXMDCs with wastewater in the cathode chamber.

3.4. MDC performance with sequential carbon and nitrogen removal process

Treated wastewater from the anode chamber was transferred to the cathode chamber such that the nitrogenous compounds of wastewater stream would be removed by autotrophic bacteria. After mixing this wastewater with the new sludge, the contaminants were relatively diluted. Samples were collected to determine new initial concentrations. The anode chamber was filled with new fresh synthetic wastewater containing about 600 mg L^{-1} organic carbon. 1000 Ω resistors were applied in the electrical circuits. Fig. 9 shows electricity generation profiles for one of the MDCs during three sequential batches. In the first batch, the maximum voltage reached 0.035 V, while in the second and third batch this voltage improved to 0.0989 and 0.0911 V, respectively. Besides the improvement in voltage generation, the operating time decreased, which indicates better performance of the system.

The COD removal for the first cycle is depicted in Fig. 10a. COD measurements at the end of this cycle showed that about 30% of organic carbon was removed from the wastewater in the anode chamber while only about 10% of the organic carbon was removed from the cathode chamber. COD removal in the second and the third cycles were close to the first cycle. This indicates that the bacterial culture in the MDC cathode was not capable of removing high concentrations of organic carbon and was mostly autotrophic. On the other hand, high ammonium removal (60%) was achieved in the cathode chamber while ammonium removal in the anode chamber was not significant. Fig. 10b compares the ammonium removal between anode and cathode chambers. It was again confirmed that the microbial consortium in the cathode chamber was capable of high ammonium removal from wastewater and can be used as a secondary treatment. This removal increased by up to 77% in the second cycle. The final pH of the anolyte solution dropped as expected at the end of all three cycles. pH of the catholyte solution also dropped slightly in the cathode chamber. This drop was more significant in the second cycle (Fig. 11a). The pH drop indicates that this time nitrification was more responsible for ammonium removal than anammox reaction because pH drops significantly during nitrification reactions [48,49]. Another indication for this process is that nitrite as well as nitrate accumulated in the system at the end of the last cycle. NaCl removal for the three batch tests is shown in Fig. 11b. Although the first batch took longer (> 250 h), the final salt concentration was at similar levels in all three batches because the electricity generation was higher in the second and third tests when compared to the first test.

3.5. Microbial community variations with ammonia inhibition

Fig. 12 shows the total number of DNA copies between these five samples. As can be seen, all samples were enriched over sample E, which was taken from the MDC cathode. Samples B-D were enriched with media for growth of anammox bacteria as described by Rothrock et al. [30] with various ammonium concentrations. It appears after increasing the ammonium concentrations as high as 500 mg L^{-1} and 600 mg L^{-1} and decreasing nitrite levels to 30 and 0 mg L^{-1} (Sample C & D) the cell numbers slightly decreased suggesting inhibitory effect of high ammonium concentrations, though still above sample E. Sample E represents the MDC cathode inoculated with a small amount of the microbial culture, which was subsequently fed with treated wastewater from the anode.

Fig. 13 displays the distribution of AOBs, NOBs and Anammox bacteria. Fig. 14 shows the percentages of these bacterial groups based on the total 16S rRNA copies in each sample. It should be noted that since two primer sets were selected for detection of AOBs and anammox bacteria, the average of detected bacteria by these primers was recorded. These results showed that sample A, which represents the initial inoculum was mostly an AOB culture than anammox or NOB. After reactivation and growing this culture in a N_2-bubbled reactivation media formulated by Vanotti et al. [50] for growth of anammox bacteria which contained 100 mg L^{-1} NH_4^+-N and 100 mg L^{-1} NO_2^--N, we increased the concentration of NH_4^+-N to 285 mg L^{-1} and decreased the concentration of NO_2-N to 60 mg L^{-1}, because ammonia level in the wastewater is usually around 10–40 mg L^{-1}. According to Anthonisen et al., [46], this is equal to 238–954.81 mg L^{-1} NH_4^+-N. 10 days later (Fig. 14, Sample B) the population of AOB dropped significantly since they are usually inhibited by 238–3580.6 mg L^{-1} NH_4^+-N. The ratio of the AOBs to anammox decreased as the concentration of ammonium increased in the next samples. The number of anammox bacteria was always higher than NOBs for the samples where ammonium was increased (Samples B, C, D), indicating that high ammonium and pH were inhibitory more for NOBs than anammox. However, in our first trial anammox bacteria could not be detected in the samples from the MDC cathode where its microbial culture was fed with treated wastewater that usually is used for anaerobic and

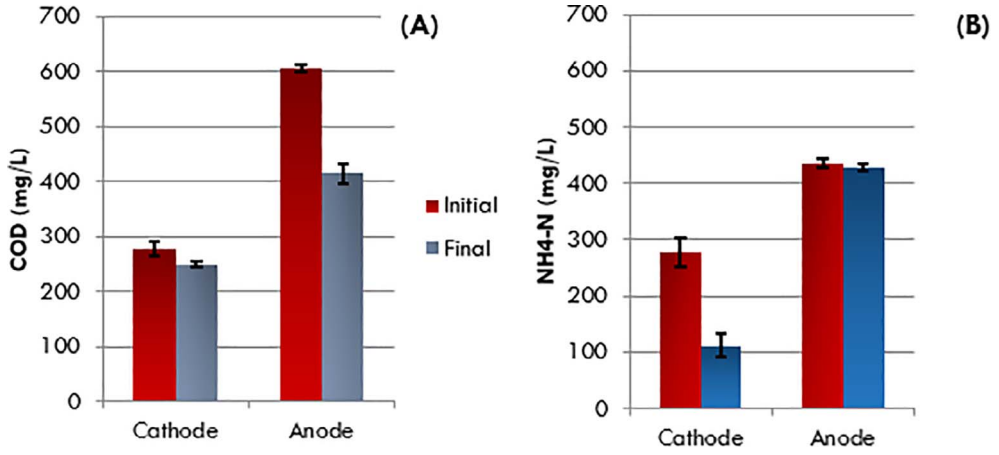

Fig. 10. A) Initial and final COD concentration in the anode and cathode chambers and B) Initial and final NH_4^+-N removal in the anode and cathode chambers.

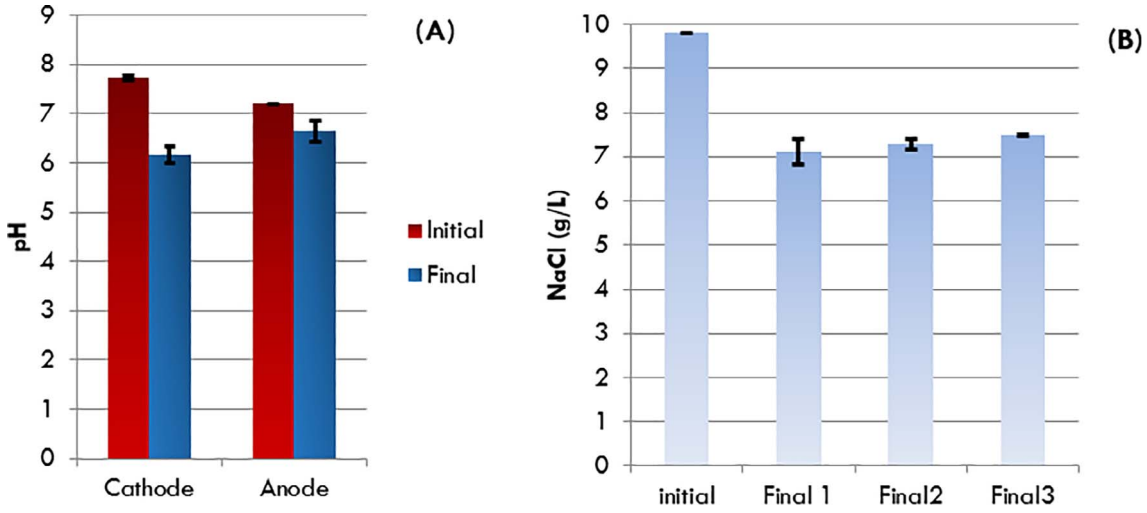

Fig. 11. pH changes in cathode and anode chamber B) Final NaCl concentration in the middle chamber for the three tests.

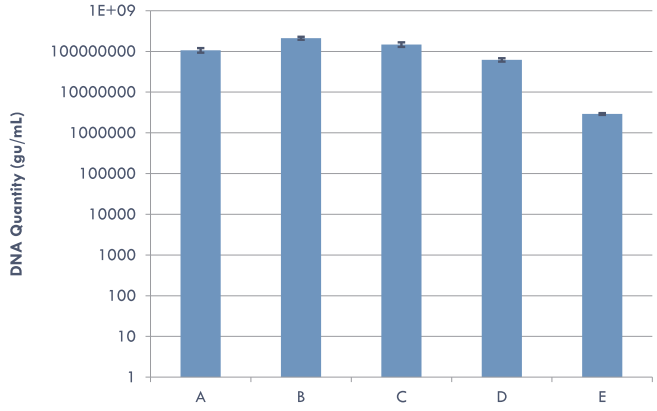

Fig. 12. Total bacterial DNA (16 SrRNA) Quantity for different samples.

methanogenic bacteria. Thus, another sample from the same culture after several days was evaluated. The anammox was detected this time, however, NOBs were still higher than anammox or AOBs. Since the number of total bacterial DNA was lower this time, probably due to the lag time period that happened between the first and second time point, we could not compare the second result dataset with the first series of data. Nevertheless, the fact that anammox was still present and that NOBs were much higher than anammox was the same for the two tests. The lower levels of bacteria in the second test, may have allowed for the preferential amplification of anammox bacteria as well. This result suggests that for complete removal of ammonium from wastewater containing organic carbon, more restricted physiological conditions should be applied so that anammox bacteria can overcome NOBs.

4. Conclusion

This study demonstrated the possibility and the proof of concept of using an autotrophic microbial culture containing anammox bacteria as the biocathode of MDC to contribute in simultaneous energy generation and wastewater treatment. Batch experiments improved the coulombic efficiency of the system as well as the nitrite and ammonium removal of the wastewater. Anammox biofilm was enriched in successive fed-batch mode experimental studies as evidenced by the voltage generation profiles in MDCs. A maximum power of 0.092 Wm^{-3} with more than 90% removal of ammonium was achieved in this system. The finding of this research showed that this system is more useful for wastewaters with low C/N ratio to suppress the possibility for the growth of heterotrophic bacteria. In this study, the activity and presence of anammox bacteria was demonstrated at high ammonium levels and relatively low oxygen concentration. Further studies should focus on transferring the treated wastewater from anode chamber to a separate container for partial nitration and possibly a new concept called reverse anammox process to provide the required nitrite followed by anammox biocathode to conduct complete anammox reaction in a continuous mode. This approach will result in optimized process performance in terms of

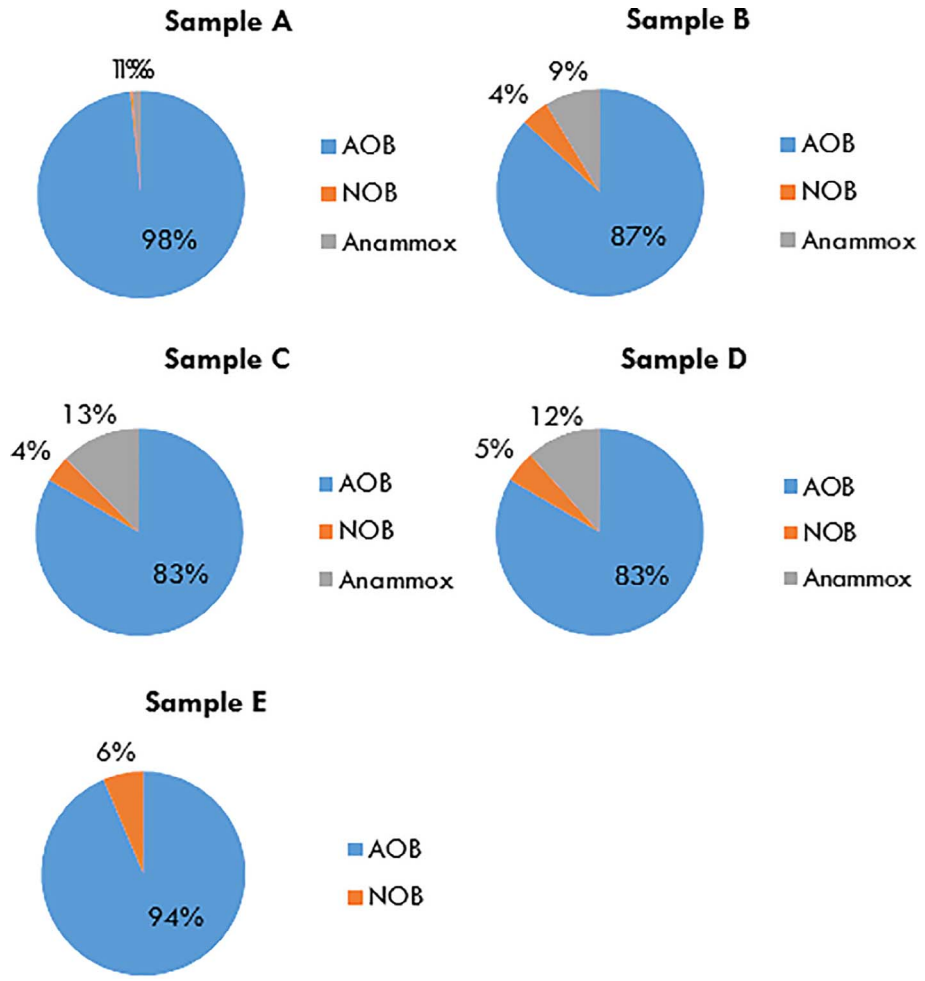

Fig. 13. Distribution of AOB, NOB and anammox bacteria in relation to each other with different ammonia concentrations.

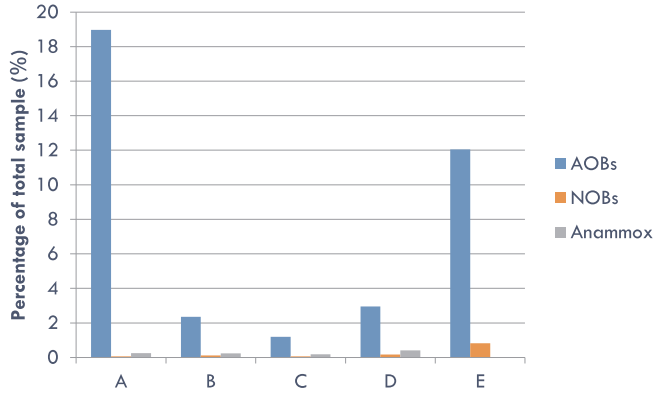

Fig. 14. Distribution of bacterial communities (AOBs, NOBs and Anammox) to total amount of bacteria at different samples.

energy recovery and water quality enhancement.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cej.2018.02.088.

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