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Nitrogen removal in anaerobic ammonium oxidation process-based bioelectrochemical system

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Nitrogen removal in anaerobic ammonium oxidation process-based bioelectrochemical system.

Nitrogen removal process was studied in a microbial electrosynthesis (BES) system at different applied voltages. Three different inoculation methods were compared and cyclic voltammograms were generated to evaluate changes on the bioelectrodes. Results from this study showed that after electrode inoculation gradually lowering the applied potential over a long period of time results in improvement of the nitrogen removal rates. Cyclic voltammetry sowed a strong correlation between the nitrogen removal efficiency of a biocathode and its specific capacitance. This study contributes to the idea that an electrical potential of -0.5 V could result in an increase of ~30% on the nitrogen removal rate of a bioelectrode using anammox process.

Keywords: wastewater treatment, nitrogen removal, bioelectrochemical systems, microbial electrosynthesis, cyclic voltammetry, bioelectrochemistry.

CERCS code: P300, P305, T490

Lämmastiku ärastus anaeroobses ammooniumi oksüdeerimise protsessipõhises bioelektrokeemilises süsteemis.

Lämmastikuärastuse protsessi uuriti mikroobse elektrosünteesi (BES) süsteemis erinevatel potentsiaalidel. Võrreldi kolme erinevat inokuleerimise meetodit ja bioelektroodide muutuste hindamiseks teostati tsüklilised voltammogrammid. Selle uuringu tulemused näitasid, et peale elektroodi inokuleerimist potensiaali järk-järguline alandamine pika aja jooksul tõhustab lämmastiku ärastamise kiirust. Tsükliline voltamperomeetria näitas tugevat seost biokatoodi lämmastiku ärastamise efektiivsuse ja katoodi mahtuvuse vahel. See uuring aitab kaasa ideele, et elektripotentsiaal -0,5 V võib tuua kaasa bioelektroodi lämmastikuärastus kiiruse suurenemise ~ 30%, kasutades anammox protsessi.

Märksõnad: reovee puhastamine, lämmastikuärastus, bioelektrokeemilised süsteemid, mikroobne elektrosüntees, tsükliline voltammeetria, bioelektrokeemia.

CERCS kood: P300, P305, T490

TABLE OF CONTENTS

ABBREVIATIONS 5

INTRODUCTION 6

1 LITERATURE REVIEW 7

1.1 Anaerobic Ammonium Oxidation (Anammox) 7

1.2 Bioelectrochemical systems (BES) 10

2 MATERIALS AND METHODS 14

2.1 MES cells 14

2.2 Inoculation 15

2.3 Preparation of synthetic media 16

2.4 Experimental setup 16

2.5 Analytical instruments 17

2.6 Analytical methods 18

2.7 Electrochemical measurements 19

2.8 Calculations 19

3 RESULTS AND DISCUSSION 20

3.1 Ammonium, Nitrite, and Nitrate removal 20

3.1.1 Negative control experiments 20

3.1.2 Set 1 22

3.1.3 Set 2 23

3.1.4 Set 3 26

3.1.5 Set 4 28

3.2 Comparison of nitrogen removal results 30

3.3 Electrochemical performance (Cyclic Voltammetry) 31

SUMMARY 36

REFERENCES 37

ANNEX 42
I. Annex 1: 16S rRNA sequencing analysis results 42
II. Annex 2: Tartu tap water composition.. 43
NON-EXCLUSIVE LICENCE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC 45

ABBREVIATIONS

Anammox-	Anaerobic Ammonium Oxidation
B. anammoxidans-	*Candidatus Brocadia anammoxidans*
BES-	Bioelectrochemical systems
CV-	Cyclic voltammetry
Cyt *c*-	Cytochrome *c*
EET-	Extracellular electron transfer
HZO-	Hydrazine-oxidizing enzyme
MEC-	Microbial electrolysis cells
MES-	Microbial electrosynthesis
PCR-	Polymerase chain reaction
TN-	Total nitrogen
WWTP-	Wastewater treatment plant

INTRODUCTION

Global population growth has increased exponentially over the last few decades causing overpopulation of our planet. This has a direct impact on the amount of wastewater that its generated which needs to be treated to minimize its effect on the environment. Nitrogen is one of the key elements which need to be removed before water can be returned to the environment with minimum impact, specifically to avoid eutrophication of water bodies.

Conventional wastewater treatment methods require high amounts of energy. Anaerobic ammonium oxidation process could help reduce the amount of energy needed in wastewater treatment since it has a lower oxygen demand than current methods. Different microbial organisms can be utilized for bioelectrosynthesis. Therefore, considerable amount of research has been made in recent years utilizing bioelectrochemical systems for different compound treatments. Exploring the combination of this systems together with the anammox process for nitrogen removal is a creative approach which can result in new alternative technologies for wastewater treatment.

The need for more resource-efficient treatment methods for wastewater is essential. Different compositions of bioelectrochemical systems have been tested, but the effect of externally applied electrical potential on the performance of the system is still unclear, which inspired this study.

The aims of this thesis:

- To determine nitrogen compounds (ammonium, nitrite, and nitrate) removal rates of nitrogen converting bacteria in a bioelectrochemical system, depending on different electrical potentials.
- To study the effect of different external electrical potentials on the anammox process:
    - Determine possible inoculation methods for anammox species in a bioelectrochemical system.
    - Define which electrical potential (-300 mV, -500 mV, -700 mV) is the best to achieve the highest nitrogen removal rate.
- To explore a bioelectrochemical alternative or supplementary method for nitrogen removal in the wastewater treatment process.

1 LITERATURE REVIEW

Wastewater treatment consumes a high amount of energy to treat organic matter. Many wastewater treatment plants (WWTP) are focused only on organic matter removal and overlook nitrogen species like ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), and phosphorus species removal, which must be also treated in order to prevent eutrophication of water bodies (Kartal et al. 2010).

Nowadays, biological nitrogen removal technologies utilize a great amount of energy to generate an aerobic environment for bacterial nitrification (Kartal et al. 2010). Around 40% of the electrical energy used by a WWTP is used only for the aeration processes (Gude 2015). In terms of total consumed power it translates to one third of the treatment plant's total costs (Drewnowski et al. 2019).

Existing wastewater treatment methods are autotrophic nitrification and heterotrophic denitrification processes to address the nitrogen removal, together with enhanced phosphorus removal process this augments the aeration energy requirements and introduces the need for organic carbon (Ghimire and Gude 2019).

It is important to develop a resource-efficient process that utilizes as low energy as possible or at least one that reduces the consumption of energy of standard treatment methods, and it is assumed that nitritation-anammox (anaerobic ammonium oxidation) process is one of the best substitutes for traditional biological nitrogen removal (Vlaeminck et al. 2012).

1.1 Anaerobic Ammonium Oxidation (Anammox)

Anaerobic ammonium oxidation (anammox) process was first described in the early 1990s. It was discovered in a denitrifying reactor where the NH_4^+ and NO_2^- consumption rates were increasing and it was determined that in anammox process NH_4^+ is oxidized under anoxic conditions, where NO_2^- acts as an electron acceptor and nitrogen gas (N_2) is produced (Equation 1) (Mulder et al. 1995).

Bacteria have been recognized as important contributors in the nitrogen cycle (Figure 1). In marine environments, at least 20% (and up to 50%) of the nitrogen turnover corresponds to the anammox process (Francis et al.2007).

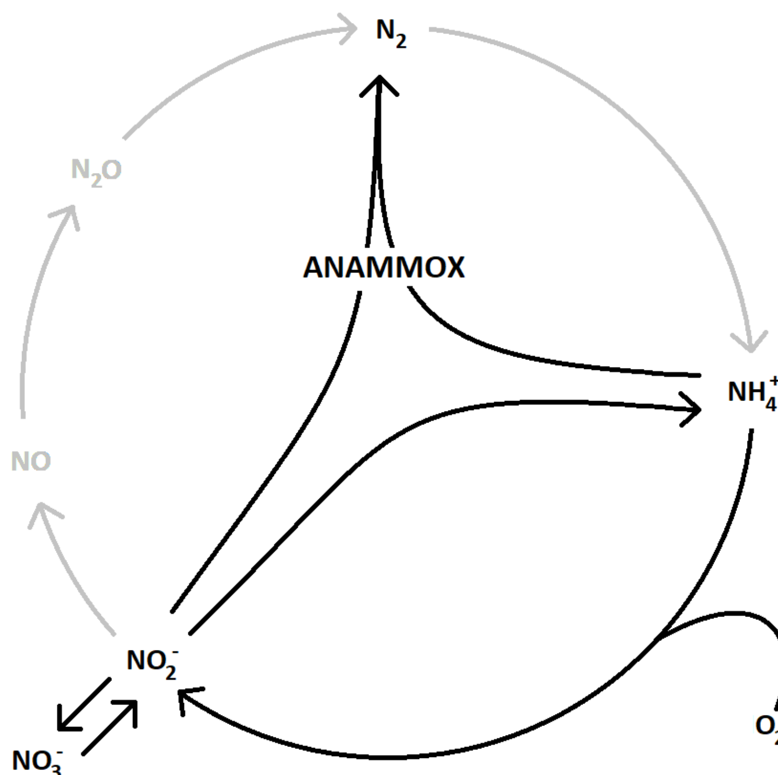


Figure 1 NH_4^+ is released from organic compounds and oxidized by aerobic nitrifying bacteria to NO_2^- and further to NO_3^-. If anaerobic conditions are present, NO_3^- and NO_2^- can be reduced to NH_4^+ or to N_2 via dissimilatory NO_3^- reduction to NH_4^+ and denitrification processes, respectively. Through anammox reaction, NO_2^- and NH_4^+ are utilized to produce N_2 (B. Kartal, Kuenen, and Van Loosdrecht 2010).

The anammox process is the sequence of processes with NH_4^+ and NO_2^- conversion into N_2 using NO and hydrazine as intermediate compounds. It corresponds to the process of forming hydroxylamine (NH_2OH) by reducing NO_2^- which anammox bacteria carry out at the cytoplasm (Strous et al. 2006). Ammonium oxidation takes place in a cytoplasmic organelle of anammox bacteria called the *anammoxosome*, where NH_4^+ with hydroxylamine (NH_2OH) produces hydrazine (N_2H_4) (Equation 2), which is an energy rich intermediate compound that bacteria use as an energy source (Kuenen 2008).

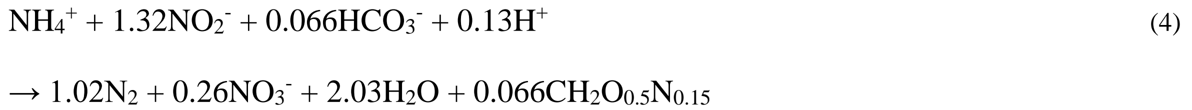
Hydrazine goes through oxidation producing N_2 and four electrons (Equation 3).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (1)$$

$$NH_2OH + NH_4^+ \rightarrow N_2H_4 + H_2O + H^+ \quad (2)$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \quad (3)$$

Through an analysis of mass balances, it was discovered that anammox organisms produce biomass ($CH_2O_{0.5}N_{0.15}$) using carbon dioxide as a carbon source. Also, NO_2^- not only works as an electron acceptor for the oxidation of NH_4^+, but it also serves as an electron donor in the reduction of carbon dioxide represented in the equilibrium balance of HCO_3^- /CO_2 (Equation 4) (Kuenen 2008).



$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \quad (4)$$

$$\rightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15}$$

A hydrazine-oxidizing protein purified from anammox bacteria biomass and was named hydrazine-oxidizing enzyme (HZO) since, the enzyme has oxidizing activity towards hydrazine utilizing cytochrome *c* (Cyt *c*) as an electron acceptor, making the HZO play an important part in the anammox process (Shimamura et al. 2007). This enzyme seems to be directly associated with the catalysis of four-electron oxidation of hydrazine (N_2H_4) to N_2, using Cyt *c* as an intermediate electron acceptor (Kartal et al. 2011).

However, anammox bacteria are not the only microorganisms that utilize nitrogen compounds. On the aerobic-anaerobic level, in a biofilm for example, interactions between aerobic ammonium and nitrite oxidizing bacteria and anoxic anammox bacteria may occur, where the anammox compete with the ammonium oxidizers for NH_4^+ and with the nitrite oxidizers for NO_2^- (Hao et al. 2002).

Anammox bacteria growth rate is slow compared with other nitrogen cycle bacteria. Anammox cultures have been reported to have a doubling time of up to 30 days (Van De Graaf et al. 1996), with the lowest doubling time reported to be 11 days (Strous et al. 1998). More recent studies recognize that the main difficulty in working with these bacteria is their extremely low growth rates, with a doubling time of roughly 2 weeks (Kuenen 2008).

The organism *Candidatus Brocadia anammoxidans* (*B. anammoxidans*) has been identified as one of the most important bacterium responsible for the anammox reaction (Strous et al. 1999).

The start-up period for a full-scale anammox reactor in Rotterdam lasted 2 years due to the slow growth of the anammox bacteria. The inoculation of the reactor was done with nitrifying sludge taken from the same treatment plant and it was monitored throughout the start-up period via real-time polymerase chain reaction (PCR). The up-flow anaerobic sludge

blanket reactor finally achieved a nitrogen conversion rate of 8-10 kg of nitrogen per m^3 per day (van der Star et al. 2007).

There are other factors that can delay or make research with anammox bacteria time consuming besides the slow growth rate. The cultivation of anammox bacteria requires significant experience and if large amounts of biomass are required the cultivation equipment becomes a critical factor (Kuenen 2008). The fact that anammox bacteria are anaerobic autotrophic organisms makes them difficult to enrich, which also limits its applications because sufficient amount of biomass required for the process might be difficult to achieve (Ni and Zhang 2013).

It has been proven that anammox bacteria in WWTP plays an important role for the treatment of wastewater that is vastly contaminated with nitrogen compounds but with a low organic content. This application is very relevant, and it has proven successful in full scale treatment plants, such as in the case of the treatment plant built in Rotterdam in the Netherlands (Kuenen 2008).

Two main methods exist when it comes to set up an anammox reactor: to start a reactor from scratch or to do an inoculation to an already running reactor with enriched anammox sludge (Ni and Zhang 2013).

1.2 Bioelectrochemical systems (BES)

Bioelectrosynthesis can be described as a process where a combination of biologically catalyzed reactions with electrochemical reactions are executed intentionally to transform a substance into a wanted product (Harnish and Holtmann 2019). Therefore systems that are capable of converting electrical energy into chemical energy, or vice versa, utilizing microorganisms as catalysts are called bioelectrochemical systems (BES) (Bajracharya et al. 2016). In recent years BES have got a lot of attention due to their application as a sustainable way to produce electricity while treating wastewater simultaneously (Patil et al. 2015). In a typical BES, the oxidation reactions at the anode and the reduction reactions at the cathode generate a potential difference that enables the electrons to flow from a low potential region to a high potential one (Venkata Mohan et al. 2010).

Various setups of BES are available depending on the purpose or objective of a research (Figure 2).

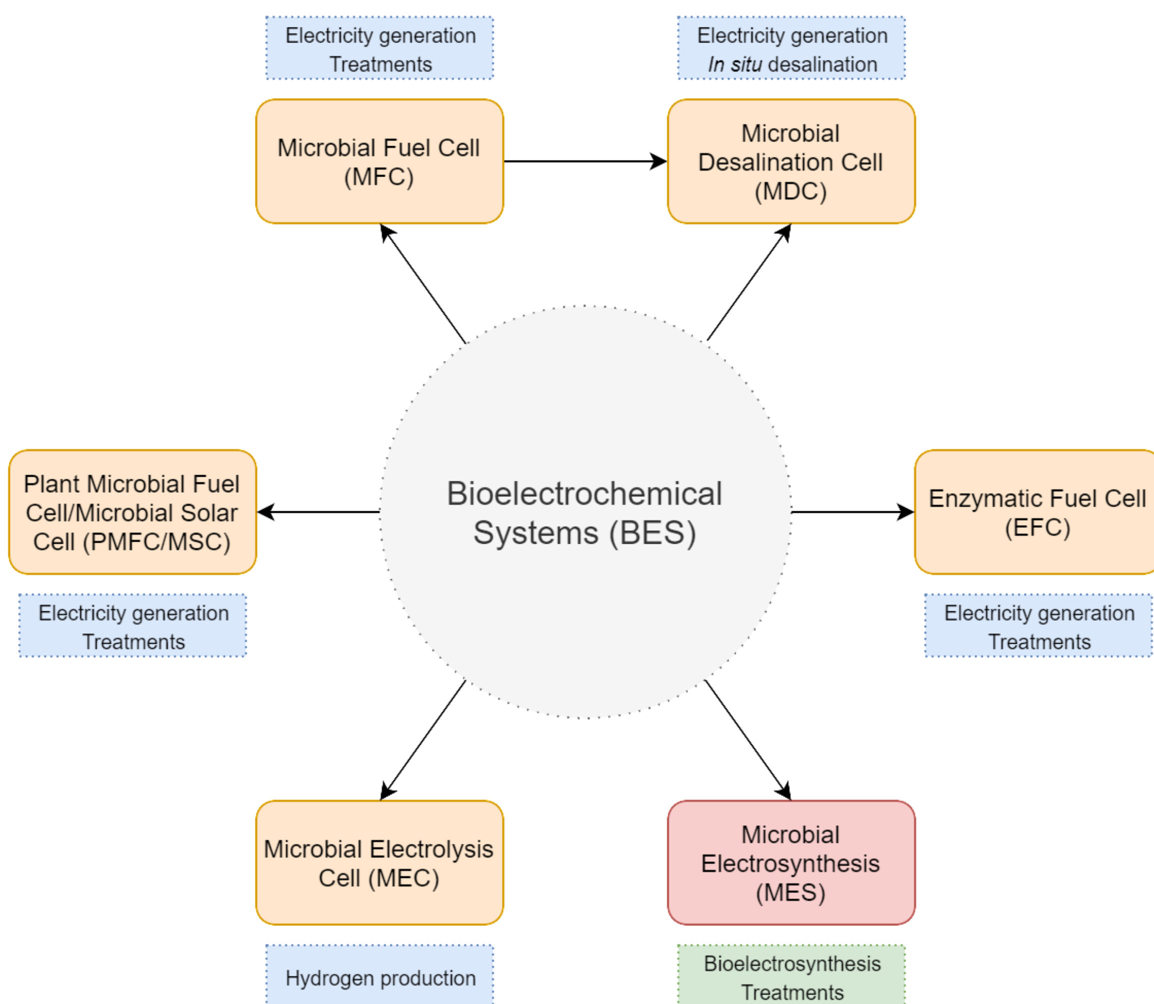


Figure 2 Overview of the different types of BES (modified from (Bajracharya et al. 2016)).

Anammox bacteria have been studied with microbial electrolysis cells (MEC) to understand if they are capable of extracellular electron transfer (EET). A recent study demonstrated that if NO_2^- is absent, and considering that cytochromes are involved in EET, oxidation of NH_4^+ can be linked with transfer of electrons to carbon-based extracellular electron acceptors like electrodes with specific potential in MEC (Shaw et al. 2019). This is strong evidence that anammox bacteria have EET capabilities. Additionally, it has been proven that there is a direct electron transfer between Cyt *c* and aqueous organic electrolyte solutions to either reduce or oxidize the Cyt *c* (Gamero-Quijano et al. 2019). Also, an autotrophic microbial culture with anammox bacteria as the biocathode in a BES has been used to provide both energy and wastewater treatment requirements such as NO_2^- and NH_4^+ removal from the wastewater (Kokabian et al. 2018). Same MEC and anammox study

reported high nitrogen removal rates suggesting that anammox bacteria were responsible for NH_4^+ removal in the MEC, linking consumed NO_2^- to consumed NH_4^+ ratios of 1.0 – 1.3 and produced NO_3^- to consumed NH_4^+ ratios of 0.12 – 0.18, which represent the theoretical ratios of anammox reaction (Shaw et al. 2019). It is worth noting that anammox bacteria were found to be the most abundant within the biofilm community according to the aforementioned study.

The latest discovery of microbial electrosynthesis (MES), also called bioelectrosynthesis, has opened new possibilities for BES. MES for example utilize the reducing power generated by the oxidation in the anode to produce desired products on the cathode (Bajracharya et al. 2016).

Different studies have emphasized different qualities of MES in terms of microbiology (Ding et al. 2018), technology and involved metabolic routes (Rabaey et al. 2011), as well as electron transfer mechanisms (Desloover et al. 2012; Kumar et al. 2017). Others have incorporated MEC and other types of BES to create a self-sustained bioelectrochemical anammox system that uses the electrons generated at the anode to improve the nitrogen removal without the need for external energy input (Li et al. 2016).

Since MES is an interdisciplinary topic, it requires knowledge about electrochemistry, microbiology, and material sciences. The performance of a biological system depends on many biological and chemical factors, electrochemical processes and the difficulties in the sense that many other factors (conductivity of electrolytes, anode and cathode potentials and conductivities, voltage losses, etc.) are involved, therefore MES might lead to complicated problems and limitations that might be difficult to detect (Bajracharya et al. 2016).

Given the previously described issues and complications surrounding anammox bacteria and BES, the use of MES with anammox bacteria is still relatively unexplored, the effect of proper electrical potential on anammox performance needs to be studied.

A moving bed biofilm reactor (MBBR) in the Institute of Chemistry in the University of Tartu has been monitored and its biofilm has been studied in MES cells showing preliminary results of 50% higher nitrogen removal efficiency to be attained at a potential of -700 mV, achieving a high specific nitrogen removal rate of 30 g of N/m^2/day (unpublished results). A 16S rRNA sequencing analysis revealed among the other bacteria the presence of denitrifying Pseudomonas and anammox bacteria *B. anammoxidans* (Annex 1). The ratio of

B. anammoxidans was found to be 5 times higher in the bioelectrodes of the MES cells than in the MBBR. Considering this initial data, the focus of this study will be to analyze the influence of an external electrical potential on the nitrogen removal rates of biocathodes while also evaluating different duration inoculation methods.

2 MATERIALS AND METHODS

2.1 MES cells

Three identical MES cells were tested parallelly throughout the experiments, which consisted of a double chambered (anode and cathode) bio-electrochemical cell with a single chamber volume of 25 mL. Chambers were separated with a proton exchange membrane (Nafion 117) (Figure 3). Polycarbonate was used as the main construction material of the MES cells. Electrodes were composed with graphite felt (ht. 12 mm, ⌀ 22.5 mm, V 4.7^{-6} m^3) coupled with titanium connection wire. Nitrile rubber "O" rings for the seals of the chambers and SYLGARD® polydimethylsiloxane (PDMS) seals were used to connect both chambers to the proton exchange membrane. Cathode chambers were connected to 500 mL bottles and circulated with peristaltic pumps.

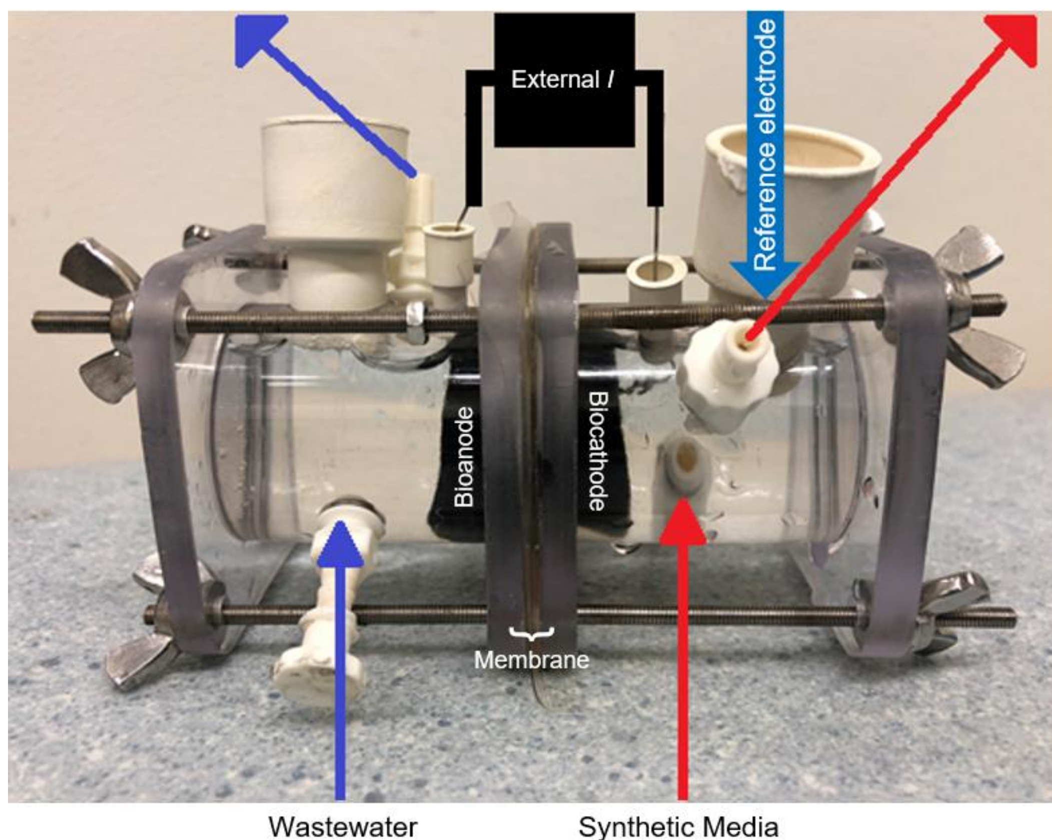


Figure 3 Schematic of experimental configuration: MES cell contained a bioelectrode with anammox bacteria in the cathode and was driven under close to anaerobic conditions.

The pretreatment of the Nafion membranes was done by boiling the 4 cm × 4 cm Nafion squares for 1 hour in 3% H_2O_2 and rinsed with deionized (DI) water and then boiled for 2 hours in DI water. After that the membranes were boiled for 1 hour in 0.5 M H_2SO_4 and finally rinsed and washed 3 times in boiling DI water. Between pretreatment and use they were stored in DI water.

Electrodes were cut with the same hole puncher and selected by mass (for set 3 and 4 masses were R1 = 0.9274 g, R2 = 0.9227 g, and R3 = 0.8761 g) the pretreatment of the electrodes was done by submerging the graphite felts in concentrated nitric acid (HNO_3) for 48 h at room temperature (24±1 °C). After that they were washed with Milli-Q water until neutral pH was achieved. Finally, they were dried in a vacuum oven (VO200, Memmert) at 1 mbar at 40 °C.

2.2 Inoculation

Electrodes were inoculated by submerging them into anammox moving bed biofilm reactors (MBBRs) (Zekker et al. 2015) for a maximum of 8 weeks. MBBRs consisted of an aeriated tank with mechanical agitation and was filled 50% with polyethylene plastic carriers that served as a surface for the biofilm to grow on. Two reactors were used for MES cell inoculation. The first one for biocathode enrichment, which held an inlet for synthetic NO_2^- influent containing solution and another one for bioanode enrichment which contained wastewater with a high NH_4^+ content (NH_4^+ = 1000-1300 mg N L^{-1}) and a moderate organic carbon level (COD = 400-700 mg L^{-1}). Nitrogen removal capabilities were tested on the MBBRs beforehand and bacteria composition analyzed (Annex 1) to ensure inoculation was viable. Three different inoculation methods were tested:

- Electrode submersion in MBBR for 2 weeks.
- Electrode submersion in MBBR for 8 weeks.
- Electrode placement into MES cathode chamber with MBBR solution circulating for 5 weeks (with and without applied electrical potential).

2.3 Preparation of synthetic media

Throughout all experiments anammox specific synthetic media solution composition was used simulating wastewater nitrogen contents. Stock solution was prepared by dissolving 1.35 g of potassium nitrate (KNO_3), 2 g of sodium bicarbonate ($NaHCO_3$), 8.5 mL of ammonium chloride (NH_4Cl), 8.24 mL of sodium nitrite ($NaNO_2$), 1 mL of phosphate buffer solution (8.5 g of KH_2PO_4, 21.75 g of K_2HPO_4, 33.4 g of $Na_2HPO_4 \cdot 7H_2O$, and 1.7 g of NH_4Cl per liter of distilled water), 1 mL of $MgSO_4$ solution (22.5 g of $MgSO_4 \cdot 7H_2O$ per liter of distilled water), 1 mL of $CaCl_2$ solution (27.5 g of $CaCl_2$ per liter of distilled water), and 1 mL of $FeCl_3$ solution (0.25 g of $FeCl_3 \cdot 6H_2O$ per liter of distilled water) (reference on composition of tap water (Annex 2)) in 5 L of either Milli-Q or tap water as well as different micro and macro elements solution according to Zhang et al. (2009). Stock solution was stored at 4 °C to avoid decomposition and pH of the synthetic wastewater was adjusted to ~7.5 at the start of each experiment.

2.4 Experimental setup

To assess the performance of anammox bacteria in the cathode chamber, all 3 MES cells (Table 1) were treated and prepared in the same way at the start of each experiment regardless of its configuration.

Table 1: MES cells configuration throughout experimental period

	Set 1			Set 2			Set 3			Set 4		
Inoculation	Old electrodes			2 weeks in MBBR			8 weeks in MBBR			5 weeks in cathode chamber		
Synthetic media	With tap water			With Milli-Q water			With tap water			With tap water (without NO_3^-)		
MES cell	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Biomass	✓	✓	✗	✓	✓	✗	✓	✓	✓	✓	✓	✓
Electrical Potential (mV)	0	0 -700	0	0 -300	0	0 -700	0 -500 -700	0 -500	0	0 -500 -700	0	0 -300 -500 -700

The following measures were taken into account to ensure same experimental conditions were achieved across the whole batch tests period:

- Bottles and magnetic stirrer were washed with demineralized water to remove any biofilm or organic growth.
- At first set, recirculation tubes were cleaned when biofilm growth was observed, later, tubes were cleaned with 70% ethanol systematically every 2 weeks to ensure no biofilm growth occurred on the inner walls of the tubes.
- Synthetic media stock solution was diluted (2-fold) by filling the bottles to 250 mL with the concentrated stock solution and adding 250 mL of tap water (only on the second set was tested preparing and diluting the stock solution with Milli-Q water).
- Bottles were purged for 20 minutes with argon gas to ensure an anerobic conditions.
- Pumps recirculation flow rate was set at approximately 16 mL/minute.
- Compensating balloons filled with argon gas to ensure anaerobic conditions were used through each experiment.
- 4 samples were taken between the 0 and 21 hours during each experiment (most of the time at the 0, 15, 17, and 19-hour samples).

All MES cells cathode chambers were fed with diluted synthetic media with a concentration in the range of 18-22 mg/L of ammonium-nitrogen (NH_4^+-N), 20-28 mg/L of nitrite-nitrogen (NO_2^--N), and 16-20 mg/L nitrate-nitrogen (NO_3^--N) (except on the last set where no NO_3^--N was added). Electrical potentials or 0 mV, -300 mV, -500 mV and -700 mV were tested. Control experiments were conducted with same concentrations on electrodes without biomass to validate the removal rates of the experiments where reactors contained only bare carbon felt electrodes. Two different controls were tested, one with no electrical potential and one with a potential of -700 mV.

2.5 Analytical instruments

The following analytical instruments were used:

- Hach Lange DR 2800 spectrophotometer (Country) for NH_4^+-N measurements
- Metrohm 930 Compact IC Flex chromatograph with a Metrohm 919 IC Autosampler plus for NO_2^--N and NO_3^--N measurements

- Eppendorf Minispin microcentrifuge for sample preparation
- IVIUM Compactstat.e potentiostat
- Jenway 3520 bench pH meter
- Radwag WPS 360/C/2 precision balance.

2.6 Analytical methods

1) NH_4^+-N determination was measured by spectrophotometry on a Hach Lange DR 2800 spectrophotometer via the Nessler method:
    i. The sample was centrifuged at 13.4 rounds per minute for 2 minutes.
    ii. 1 mL of centrifuged sample was transferred into a 25 mL volumetric flask.
    iii. The volumetric flask was filled to about one half with Milli-Q water.
    iv. 3 drops of Mineral stabilizer were added to the volumetric flask.
    v. 2 drops of Polyvinyl alcohol dispersing agent were added to the volumetric flask.
    vi. The volumetric flask was filled up to the mark with Milli-Q water, closed with a cap and shaken well to homogenize the sample.
    vii. 1 mL of Nessler reagent (0.09 mol/L solution of potassium tetraiodomercurate(II) ($K_2[HgI_4]$) in 2.5 mol/L potassium hydroxide (KOH)) was added.
    viii. The sample was left for 2 minutes during which yellow color should appear.
    ix. The sample should be measured immediately after the 2 minutes have elapsed.
    x. Spectrophotometer reading was multiplied by the dilution factor (25-fold) to obtain the NH_4^+-N concentration.

2) NO_2^--N and NO_3^--N were measured via ion chromatography on a Metrohm 930 Compact IC Flex chromatograph with a 919 IC Autosampler plus after:
    i. The sample was centrifuged at 13.4 rpm for 2 minutes.
    ii. 0.4 mL of centrifuged sample was transferred into a 25 mL volumetric flask.
    iii. The volumetric flask was filled up to the mark with Milli-Q water, closed with a cap and shaken to homogenize the sample.
    iv. The sample was transferred to a 10 mL sample tube and placed in the IC autosampler.

v. IC software considered the dilution factor (62.5-fold) to obtain the NO_2^--N and NO_3^--N concentrations.

2.7 Electrochemical measurements

IVIUM Compactstat.e potentiostat was used to maintain proper potential and to perform cyclic voltammetry (CV) measurements. Each electrode was measured at the start-up, between cycles, and at the end of the experiments. The voltage range was 0 to -500 mV vs Ag/AgCl (3M NaCl 0.209 vs SHE) with a scan rate of 1 mV s^{-1}. CV for a control electrode (abiotic) was also evaluated. Acquired CV data was used to calculate each biocathode specific capacitance.

2.8 Calculations

NH_4^+-N, NO_2^--N, NO_3^--N, and total nitrogen (TN) removal rates were calculated in terms of mg N/m^2/day (Equation 5).

$$Removal\ Rate\ (RR) = \frac{C_i - C_f}{\frac{A_{electrode}}{t}} \tag{5}$$

Where C_i and C_f are the initial and final concentrations (mg N/L), $A_{\text{electrode}}$ is the area of the electrode in the cathode chamber (m^2), and t is elapsed time of the experiment.

Specific capacitance (C_p) was calculated in terms of F/g (Equation 6).

$$Specific\ Capacitance\ (C_p) = \frac{A}{2mk(V2 - V1)} \tag{6}$$

Where A is the area inside the CV curve (AV), m is the mass of the carbon felt (g), k is the scan rate of CV (V/s), and $(V2 - V1)$ is the potential window (total voltage range) of CV (V). The areas were calculated from CV data through the Origin 2020 software from OriginLab® using the polygon area calculation tool.

3 RESULTS AND DISCUSSION

3.1 Ammonium, Nitrite, and Nitrate removal

A total of 4 sets of experiments were performed with electric potential being applied throughout the experiments at 0 mV, -300 mV, -500 mV, and -700 mV. The 1st set of experiments served as a reference point since the experiments were done already with viable bioelectrodes. These biocathodes were the same ones used to obtain the unpublished preliminary results, although it is worth mentioning that they were unattended for ~1 month before this study.

The best results for TN without electrical potential were achieved on the 1st set (25.2 mg N/m^2/day), in the case of -500 mV best TN removal rate was attained on the 4th set (6.5 mg N/m^2/day), and for -700 mV best TN removal rate results were observed on the 1st set (15.4 mg N/m^2/day). Electric potential of -300 mV was mostly used for training of the bioelectrodes and did not achieve any notable removal rates.

Replicate experiments in general showed slight variation, which standard deviation was taken from 2-3 parallel experiments. However, to better comprehend the effects of applied electrical potentials, some experiments were analyzed individually and thus no error bars could be assigned.

3.1.1 Negative control experiments

Negative control experiments with bare graphite felts were conducted in between experiments through sets 1 and 2. These controls confirmed that there was almost no nitrogen removal on abiotic electrodes (Figure 4 & 5), showing small TN removal rates (average of 1.5 mg N/m^2/day for control with no applied electrical potential and -1.3 mg N/m^2/day for -700 mV).

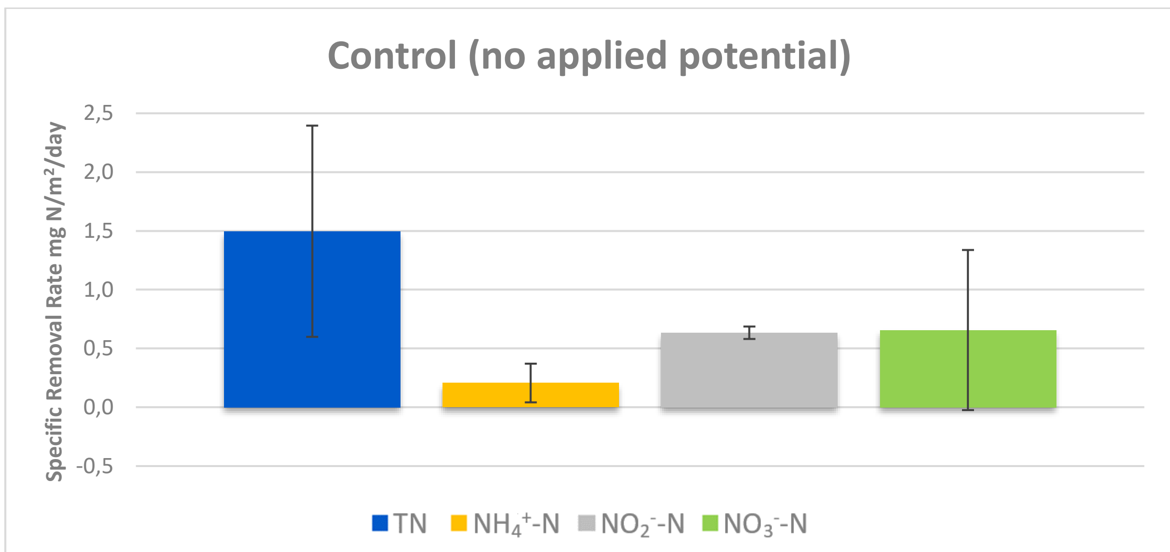


Figure 4. Control TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs without electrical potential.

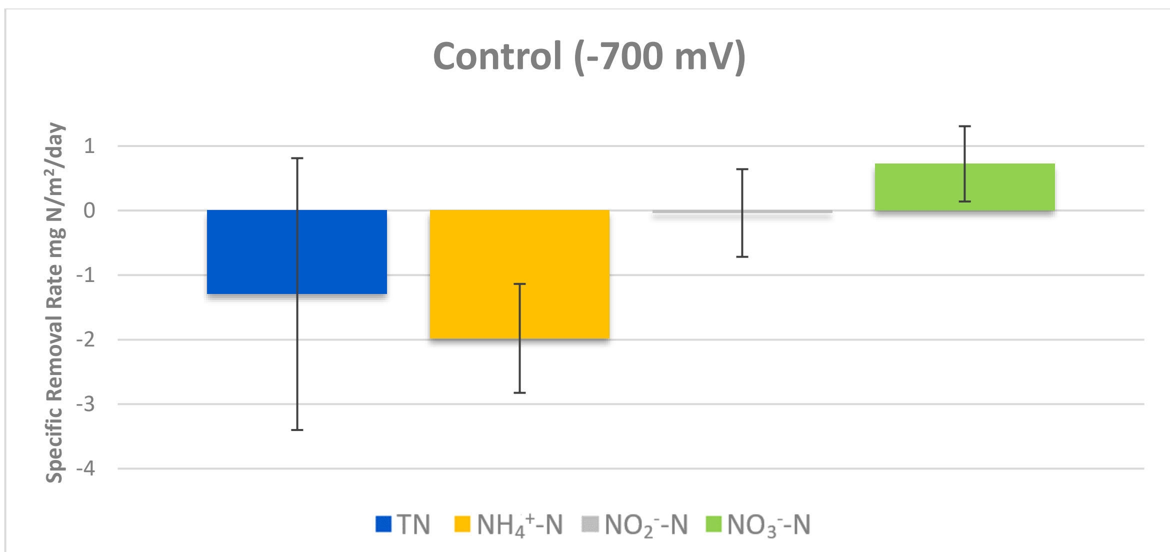


Figure 5. Control TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs with electrical potential of -700 mV.

It is important to note that these small removal rates for control without applied potential could have been due to slight bacterial contamination in the system. The recirculation tubes as well as the reactors' walls provided enough surface area where bacterial growth could occur.

No significant nitrogen removal took place for control tests with no applied electrical potential. For -700 mV nitrogen removal did not occur, nitrogen compounds rather increased with time. This proves that biocathodes activity was measured in the following results, considering the removal rates not occurring electrochemically on abiotic control cathodes.

3.1.2 Set 1

During the 1st set two reactors were analyzed: Reactor 1 (R1) without any electric potential and Reactor 2 (R2) with a potential of -700 mV. In this case, R1's average removal rate outperformed R2's (11.4 mg N/m^2/day to 5.4 mg N/m^2/day respectively) (Figure 6 & 7). R1 also removed almost double the amount of nitrogen than R2 without applying potential (1.5 mmol N/L and 0.8 mmol N/L) indicating no positive effect with this electrical potential. It was also noticed that on R2 the NO_2^--N seemed to be converted into NO_3^--N, effect that was not observed in R1 and in the negative control experiment.

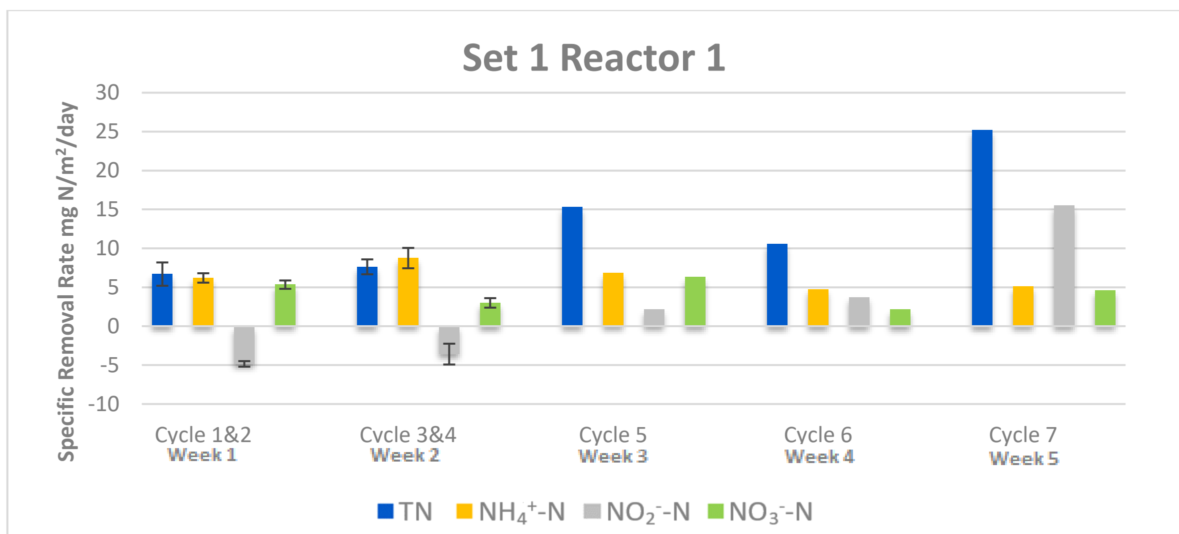


Figure 6. Set 1 Reactor 1 TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs.

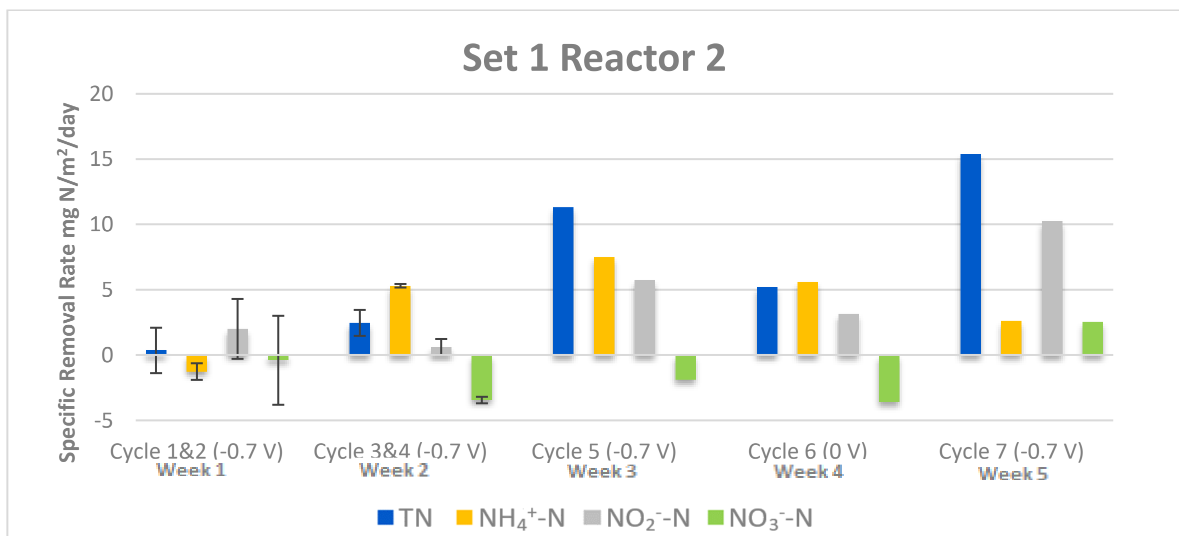


Figure 7. Set 1 Reactor 2 TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs.

Experiments showed improvement of TN removal rate for R2 from cycle 1 to cycle 7 from practically zero to 15.4 mg N/m^2/day. Also, cycle 5 and cycle 7 in R2 had significantly higher TN removal rates than in cycle 6 without applied voltage (0 mV). This could have been due to longer time needed to adapt again to a relatively high potential because reactors were unattended for ~1 month before starting the measurements of this study. Further data analysis showed that although an electrical potential of -700 mV seemed to have a negative effect on the nitrogen removal rate at earlier stages, both reactors followed a similar trend where nitrogen removal kept improving, possibly because bacterial metabolism was adapting to the applied electrical potential.

No clear advantage was observed with applied electrical potential. It is possible that a potential of -700 mV could have gone below the level of operation of Cyt *c*, which has been reported to be most active around -500 mV for denitrifiers (Gregoire et al. 2014). Because of this and considering that this set of experiments was performed with old bioelectrodes, the next sets of experiments needed to be monitored from the beginning and starting from smaller electrical potentials. Also, to have a more stable and defined synthetic media, tap water was changed to Milli-Q water in the next set.

3.1.3 Set 2

The attempt of recreating a fully synthetic environment on the 2nd set of experiments resulted in a couple of interesting repercussions. On this set the removal rates for all measured compounds were the highest achieved although with no significant difference between reactors, where TN removal rate peaked at 34.4 mg N/m^2/day for R1 without electrical potential and at 34.9 mg N/m^2/day with an electrical potential of -300 mV. Notably, it was observed that some biological growth occurred overnight in the synthetic media turning the solution cloudy (Figure 8), probably due to the fact that the synthetic media was prepared with Milli-Q water, which has lower concentration of ions of dissolved salts compared to tap water (Annex 2). Therefore, these results could not be linked strictly to nitrogen converting bacteria activity growing on the cathode and were not considered for comparisons. Taking this into account, there was a very slight increase on NH_4^+-N removal rate when -300 mV electrical potential was applied (Figure 9 & 10).

R1 results showed a relatively high nitrogen removal rate, with an average of at 29.3 mg N/m^2/day, where NH_4^+-N removal peaked at 4 mg N/m^2/day, NO_2^--N removal at 17 mg

N/m^2/day, and NO_3^--N removal at 14 mg N/m^2/day, being the highest recorded removal rates for NO_2^--N and NO_3^--N in case of low applied potential of -300 mV. In comparison, R2 removal rates were slightly lower without applying any potential at an average of 22.9 mg N/m^2/day on R2, where NH_4^+-N removal peaked at 3.5 mg N/m^2/day, NO_2^--N removal at 17 mg N/m^2/day, and NO_3^--N removal at 13.4 mg N/m^2/day.

Figure 8. Appearance of synthetic media on set 2 at start of experiment (left) and cloudiness after 15h of experiment (right).

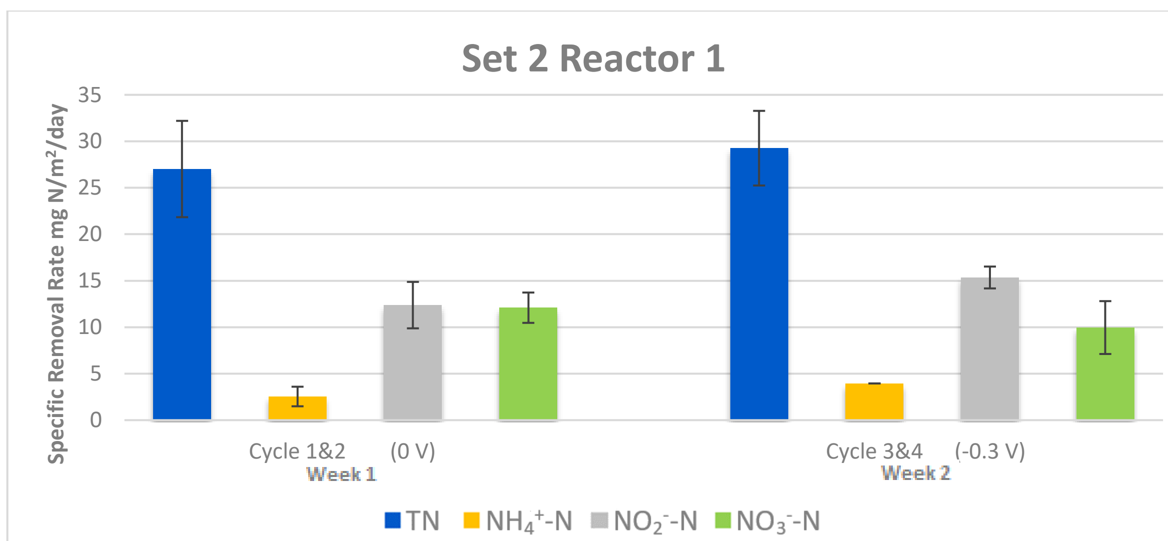


Figure 9. Set 2 Reactor 1 TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs.

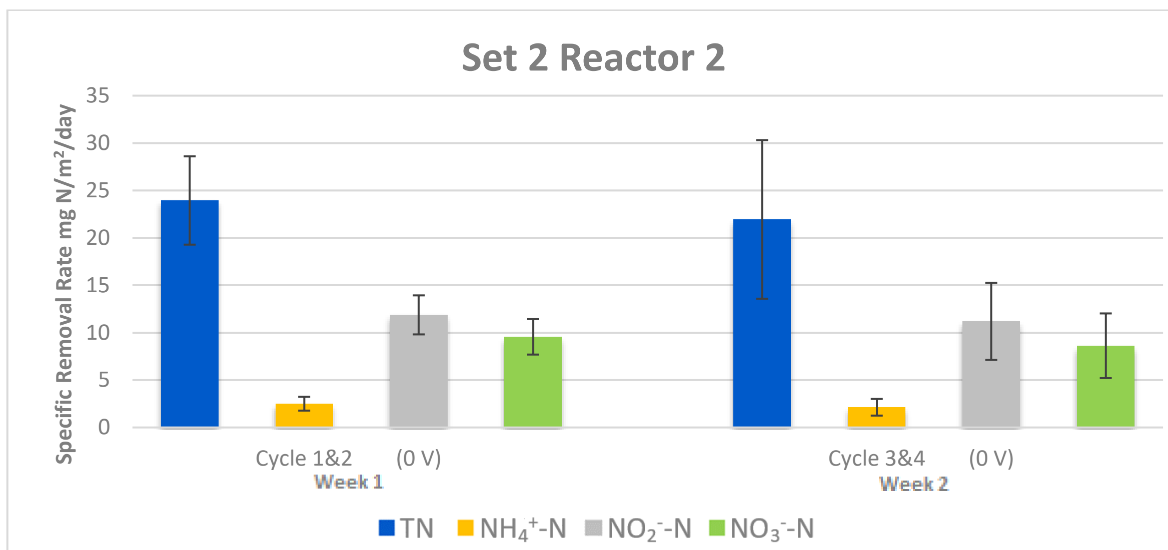


Figure 10. Set 2 Reactor 2 TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs.

However, by analyzing the removal rates of all three compounds parallelly it was observed that some of the removal rate of NH_4^+-N could be caused by anaerobic oxidation to N_2. In a similar way NO_3^--N and NO_2^--N were consumed.

To evaluate this further, the removal rates were calculated in mmol/L to analyze how much nitrogen compounds could have been in fact transformed into N_2 and it was found that in some cases in R1 up to 3.3 mmol N/L were consumed in case of applying -300 mV in R1 and in R2 without applying potential 3.1 mmol N/L were consumed.

Preparing the synthetic media with Milli-Q water resulted in more complications with contamination of the systems and interferences with the nitrogen removal rates. Because of this, and since the change on the appearance of the synthetic media was not noticed on the 1st set, tap water was used again for the preparation of the synthetic media for all further experiments. At the end of the set extra biofilm growth was noticed even on the electrodes, therefore, to avoid any possible interfering effect that the undesired biomass growth could have caused to the bioelectrodes or the measurements, new electrodes were inoculated.

3.1.4 Set 3

After 8 weeks of inoculation within three available reactors, 3 biocathodes could be tested parallelly, where one would stay without applied potential. This set-up granted a better comparison to analyze the effect of the electrical potential on the removal rates.

With the intention of increasing the nitrogen removal rate of R1, electrical potential was applied to this reactor since the first couple of cycles showed that R1 was performing the worst in comparison with R2 and R3 in terms of nitrogen removal. These results showed no positive effect at -500 mV nor -700 mV supporting the indications of the first two sets that the electrical potential was inhibiting the nitrogen removal rather than increasing it.

During the 3rd set different potentials (-500 mV and -700 mV) were tested on R1, which averaged a removal rate very close to zero (Figure 11). R2 showed an average removal rate of 3.5 mg N/m^2/day with no electrical potential except on the last experiment of the set where -500 mV were applied, and removal rate dropped indicating again no positive effect on this electrical potential (Figure 12). Reactor 3 (R3) showed the highest removal rate at an average of 7.4 mg N/m^2/day with no electrical potential applied (Figure 13).

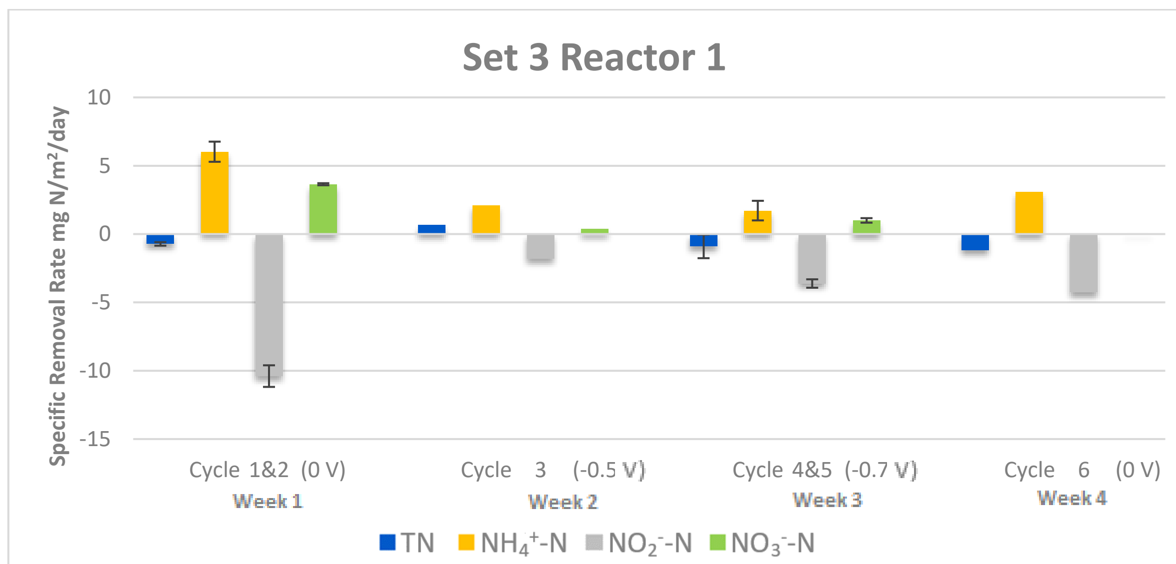


Figure 11. Set 3 Reactor 1 TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs.

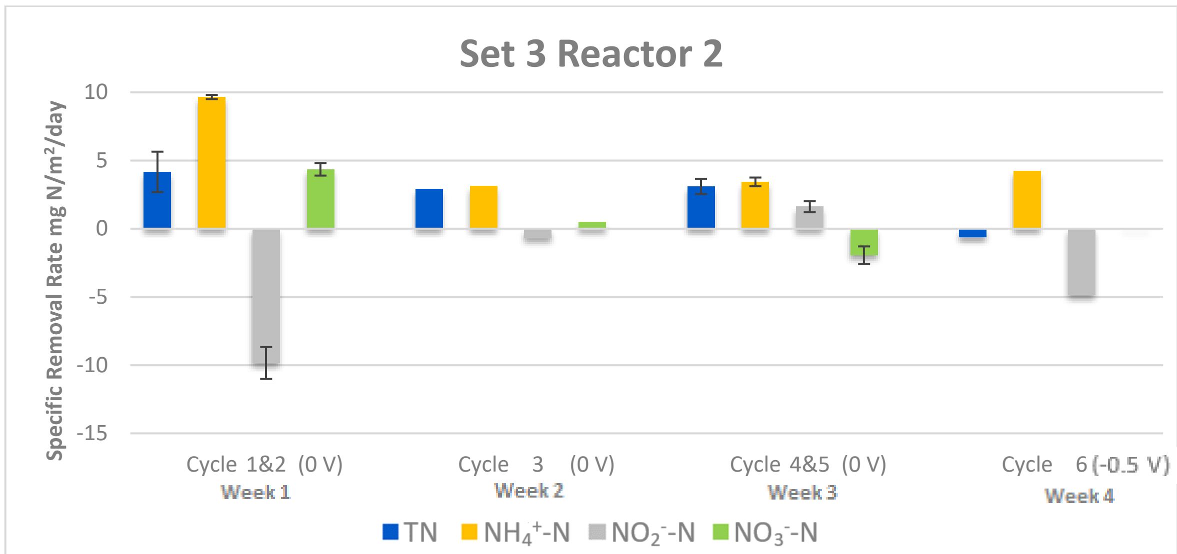


Figure 12. Set 3 Reactor 2 TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs.

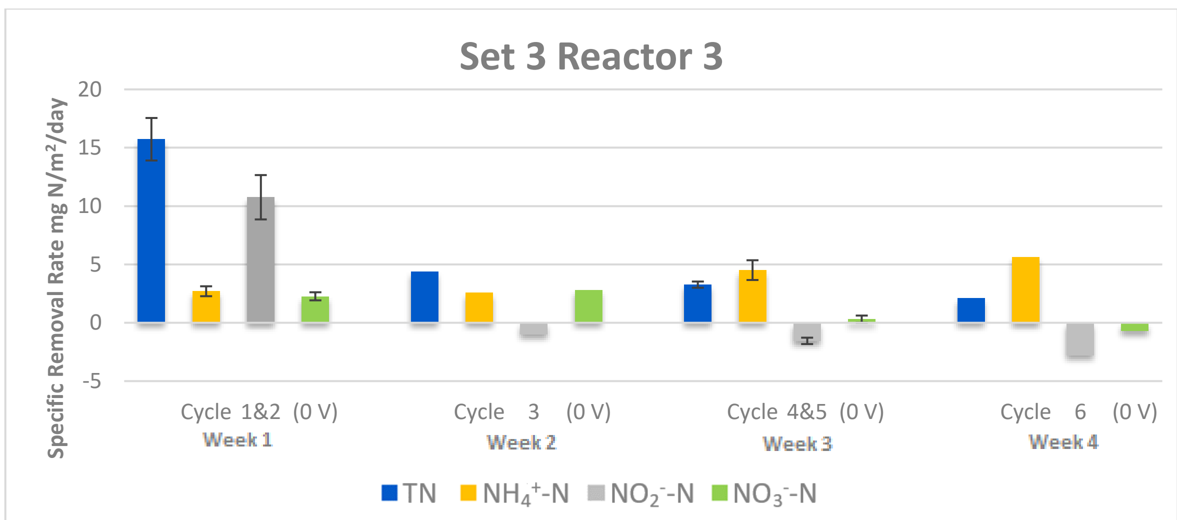


Figure 13. Set 3 Reactor 3 TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs.

At this point there were enough cases that showed no positive effect on the nitrogen removal rates by just applying an electrical potential, but there was some evidence that it could help in certain conditions. Experiments with gradual increase on the electrical potential distributed over a relatively long period of time needed to be performed. As anammox bacteria are relatively slow growers, it is possible that applying voltage just for 24 hours will result in a negative effect because the organisms do not have enough time to adapt to the new condition.

To evaluate in more detail the effect of gradually increasing electrical potential and longer adaptation period, bioelectrodes were reinoculated. This would provide comparable information not only on the nitrogen removal rates, but also for the suitable inoculation method.

3.1.5 Set 4

The same biocathodes used as in set 3 were reinoculated by circulating the MBBR biomass through the cathode chamber. R1 was applied an electrical potential of -500 mV during reinoculation.

To prove the negative influence of the electrical potential on the biocathodes, in the 4th set electrical potential was never reapplied after inoculation for R2, instead, different potentials (-500 mV and -700 mV) were applied to R1 which averaged once more almost no nitrogen removal (Figure 14). R2 showed an average removal rate of 1.1 mg N/m^2/day with no applied electrical potential (Figure 15). Similarly, R3 was performing close to those values, so different potentials were applied gradually (-300 mV, -500 mV and -700 mV) to evaluate parallelly their effect on nitrogen removal rates (Figure 16). Interestingly, same initial drop on the removal rates at all the applied potentials was noticed, although the best removal rate for R3 and for the set (6.5 mg N/m^2/day) was achieved after the reactor was exposed to -500 mV for one week.

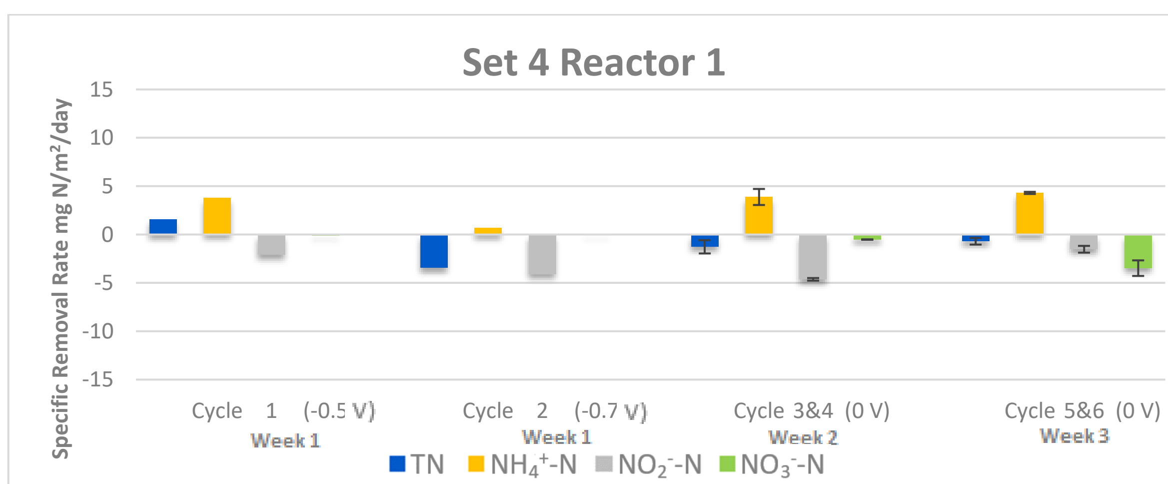


Figure 14. Set 4 Reactor 1 TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs.

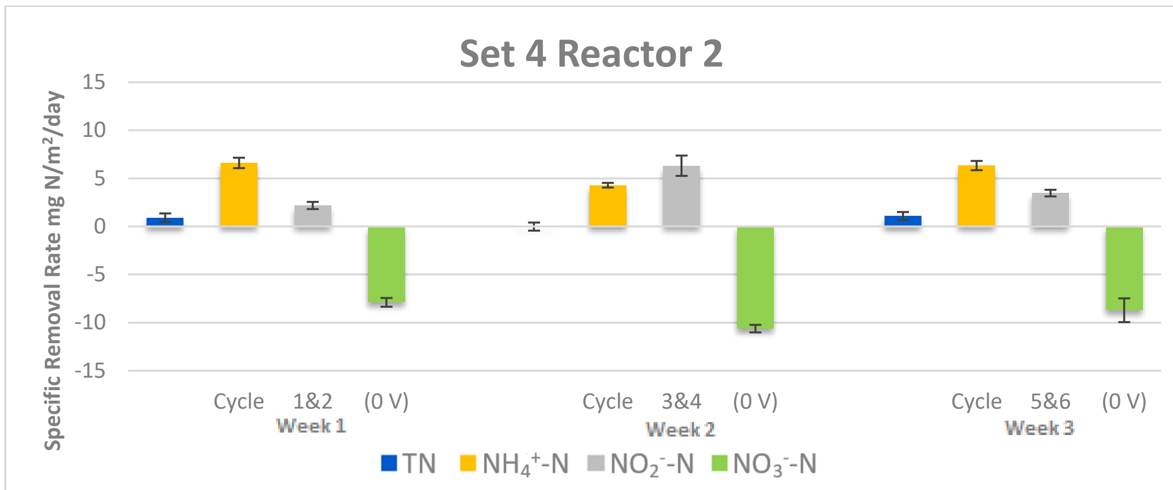


Figure 15. Set 4 Reactor 2 TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs.

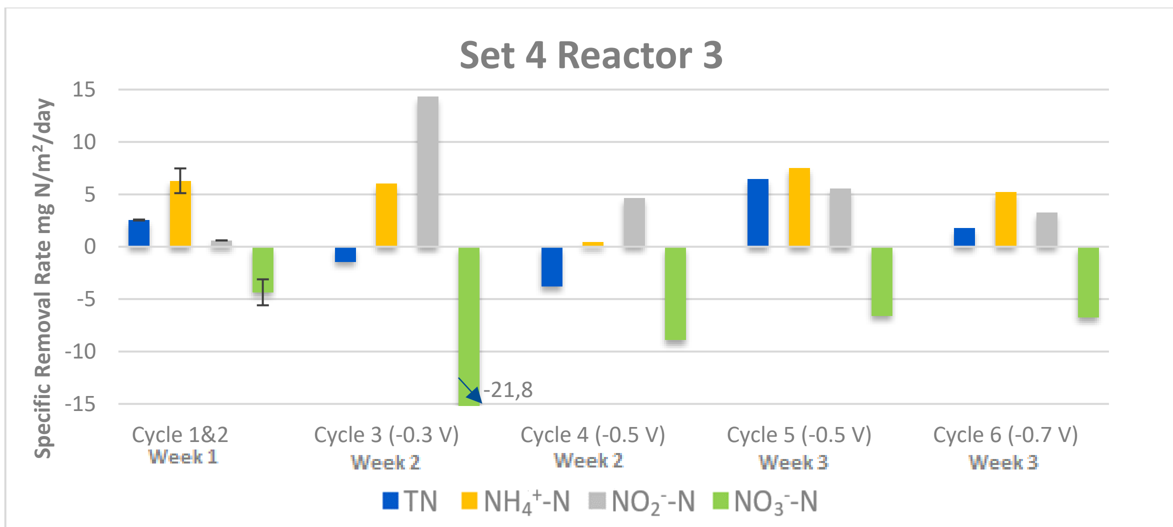


Figure 16. Set 4 Reactor 3 TN, NH_4^+-N, NO_2^--N, and NO_3^--N average RRs.

The electrical potentials of -300 mV and -500 mV seem to promote the NO_3^--N formation in the system and the potential of -700 mV appears to inhibit the NH_4^+-N removal process. From data of R3, nitrogen removal was higher (6.5 mg N/m^2/day) at cycle 5 at -500 mV than in any case of other reactors. Also, -500 mV promoted nitrogen removal in R1, being higher that in both of the other cases (no electrical potential and -700 mV), coinciding with the results of Gregoire et al., 2014, who found that the cytochrome activity peaks around -500 mV.

Ding et al., 2018, also reported that the best nitrogen removal rates for anammox occurred between -400 mV and -500 mV. This provided more evidence that an electrical

potential of -700 mV the electrical potential inhibited the nitrogen removal with compounds being oxidized and accumulated as NO_3^--N instead of being removed from the system.

However, a slow and gradual increase of the electrical potential from -300 mV to -500 mV allowed the R3 bioelectrode to increase over 30% of its nitrogen removal rate. This provided new knowledge that electrical potential could be beneficial to achieve better nitrogen removal rates after the biofilm had time to adapt to it were initially a negative effect is observed. Probably some stress occurs when electrical potential is increased, similar to the efficiency drop when transferring the bioelectrodes into the synthetic media, but after 2 weeks at the same electrical potential the bioelectrode seemed to stabilize and perform better in terms of nitrogen removal rate. Same stabilization period was attempted when increasing the potential to -700 mV, but nitrogen removal rate decreased due to this potential being too high for biomass inoculated for 13 weeks.

3.2 Comparison of nitrogen removal results

Achieved nitrogen removal efficiencies without electrical potential were found to be between 10-40% across sets 1, 3, and 4. This somewhat correlates with other studies done on small scale reactors or with different BES configurations since there are reports ranging between 30-70% (Malovanyy et al. 2015; Li et al. 2016; Ji et al. 2018) and in some cases, up to 90% (Kokabian et al. 2018). Total nitrogen removal rates without electrical potential (average of 4.5 mg N/m^2/day) were found around the lower end of what is commonly reported (Zekker et al. 2015; Regmi et al. 2016; Tomaszewski et al. 2018; Zhu et al. 2019). This was not considered problematic since the focus of this study was mainly on the electrical potential effect on nitrogen removal rates rather than achieving high removal rates.

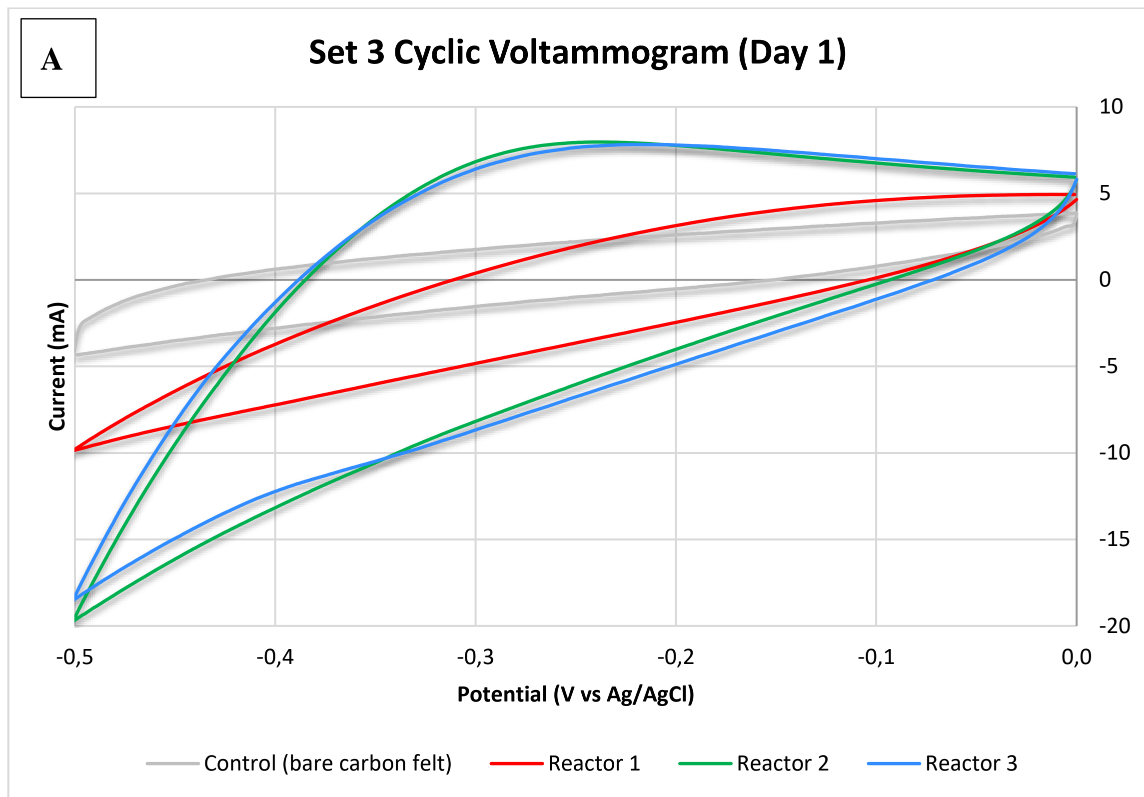
The removal rates achieved for the 1st set of experiments were significantly higher than the ones achieved on the 3rd or 4th set (21.7 mg N/m^2/day, 13.6 mg N/m^2/day, and 3 mg N/m^2/day respectively). Considering that the first set was performed with biocathodes being inoculated for more than 12 months and adapted with electrical potential, and because anammox bacteria are considered very slow growers, it is suggested that the inoculation periods for the 3rd and 4th set were not sufficient for annamox bacteria to develop high-rate nitrogen converting biofilm on the electrode.

Much longer time for inoculation is required for acquiring a high-efficient anammox bioelectrode. Even the longest inoculation period tested (8 weeks submersion in MBBR plus 5 weeks with MBBR recirculation biomass with biocathodes in the cathode chambers) achieved only 20% of the removal rate attained with the initial bioelectrodes. Also, results suggest that electrical potential should be increased gradually during a long period of time.

3.3 Electrochemical performance (Cyclic Voltammetry)

To calculate the specific capacitance and to compare the biocathodes, CV tests were done throughout the experiments conducted in set 3 and 4. It was observed that current values dropped initially when bioelectrodes were transferred from the MBBR inoculation into the MES reactors (Figure 17), probably because the organisms were submitted to stress when they were moved from real wastewater into the synthetic media. The anammox specific synthetic media also hinders the growth of other organisms present in MBBR and will most likely perish (Annex 2).

After 30 days, the biocathodes seemed to have stabilized and their current started increasing. To test the effect of the electrical potential on the biocathode, CV measurements were done after every time the applied electrical potential was changed (Figure 18).



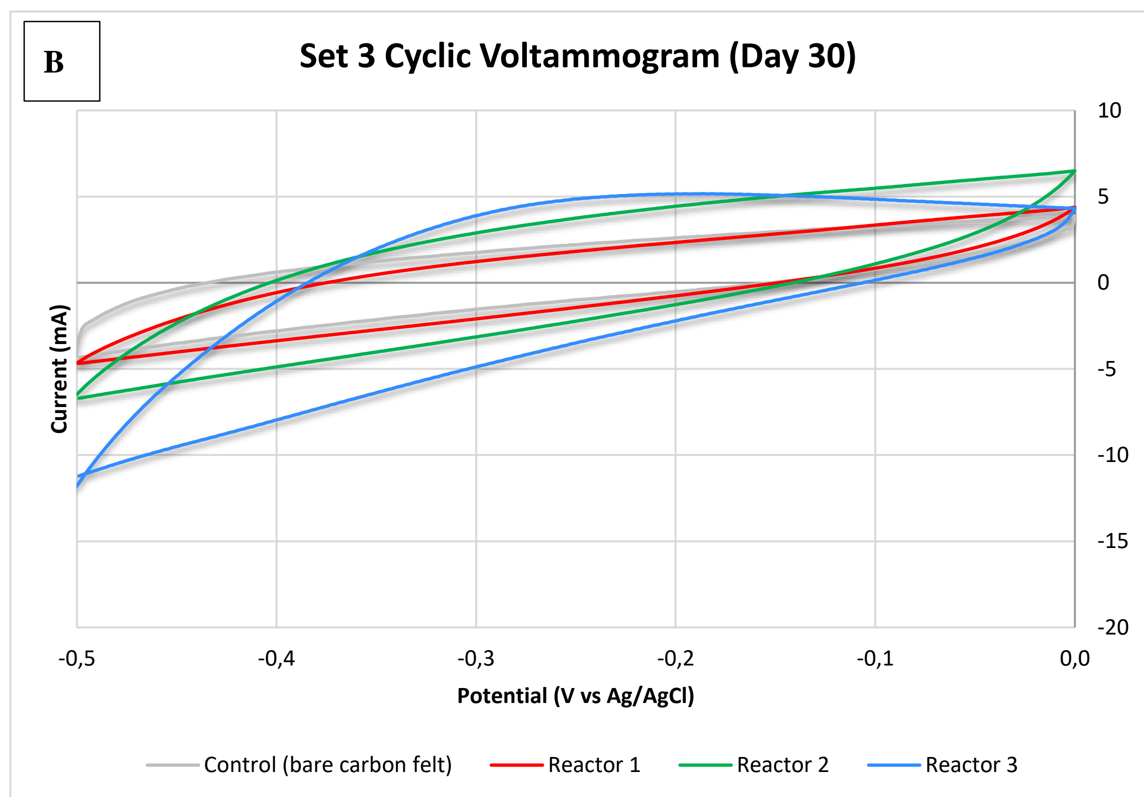
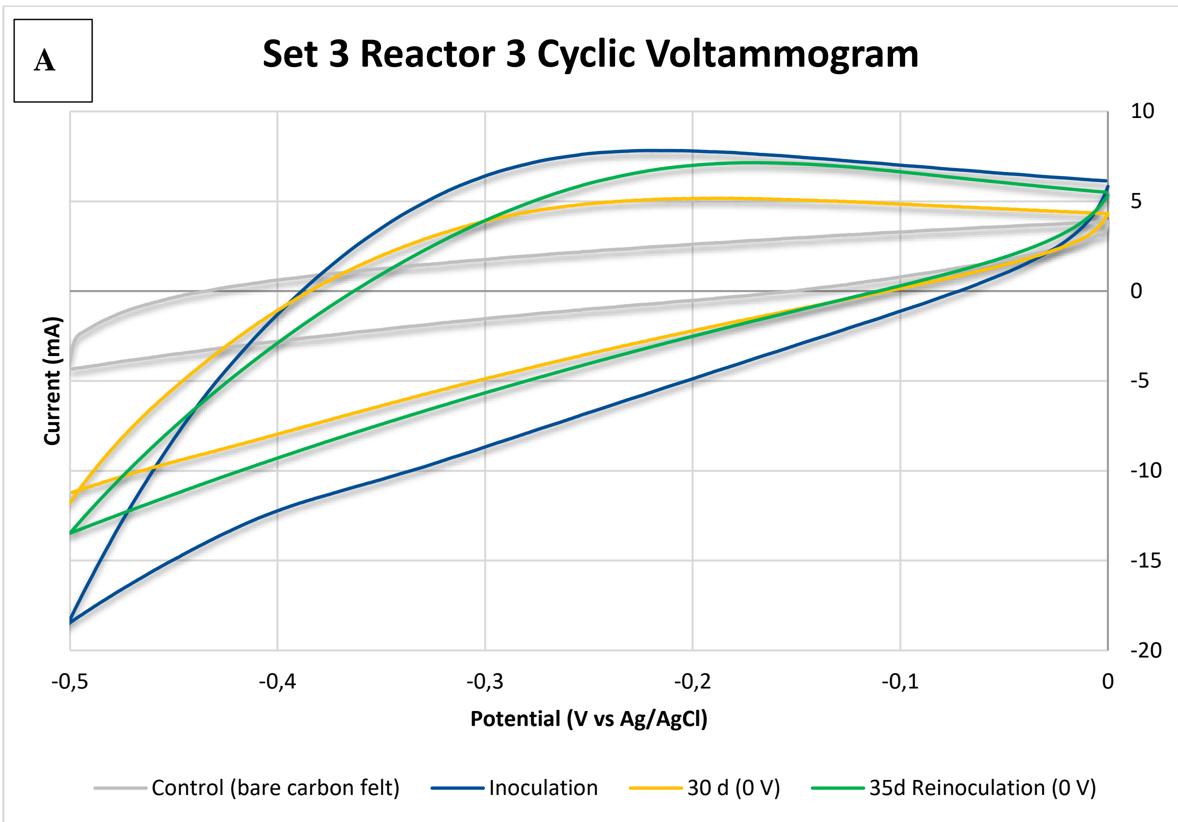


*Figure 17. Cyclic voltammograms of biocathodes of each reactor. (**A**) after 8 weeks inoculation in MBBR. (**B**) after 30 days in synthetic media.*



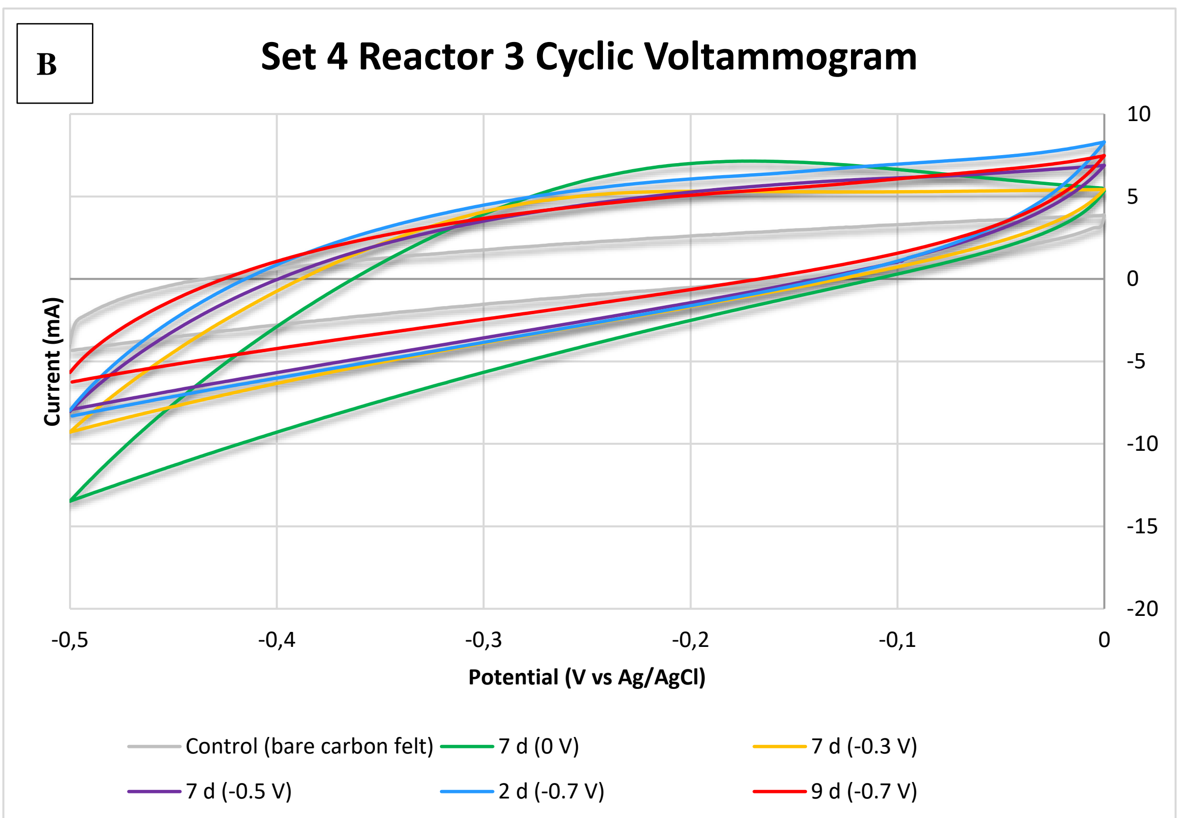


*Figure 18. Cyclic voltammograms of biocathodes on reactor 3. (**A**) Set 3. (**B**) Set 4.*

By observing the changes in current during CV measurements through the 3rd and 4th set, changes on the specific capacitance of R3 were estimated. After the initial drop due to switching to synthetic media, current dropped even further after applying electrical potential. This was probably because the electrical potential was increased too quickly and the bioelectrode did not have enough time to adjust to this condition, also probably not all microorganisms could tolerate the applied potential. To verify this, specific capacitance was calculated (Table 2).

Table 2 Specific capacitance (C_p) of the biocathodes at different stages of the experimental setups.

	Set	Setting	C_p (F/g)	C_p (%)
R1	3	After inoculation	2155,1	100
		30 d (0 V)	1362,7	63,2
	4	After 35 d reinoculation (-0,5 V)	888,1	41,2
		1 d (-0,7 V)	727,3	33,7
		7 d (0 V)	741,4	34,4
		14 d (0 V)	1180,8	54,8
R2	3	After inoculation	5167,9	100
		5 d (-0,5 V)	2471,7	47,8
	4	After 35 d reinoculation (0 V)	2378,2	46,0
		1 d (0 V)	2783,0	53,9
		7 d (0 V)	1880,3	36,4
		14 d (0 V)	1647,6	31,9
R3	3	After inoculation	5696,2	100
		30 d (0 V)	3342,3	58,7
	4	After 35 d reinoculation (0 V)	3171,9	55,7
		1 d (0 V)	3843,2	67,5
		7 d (0 V)	3029,9	53,2
		5 d (-0,3 V)	2248,2	39,5
		5 d (-0,5 V)	2976,8	52,3
		1 d (-0,7 V)	3525,6	61,9
		7 d (-0,7 V)	2693,7	47,3

With these values the drop in specific capacitance due to the exposure to the synthetic media seemed clear with an average reduction of ~57% throughout all 3 reactors.

The specific capacitance of R1 cathode dropped steadily through the reinoculation down to 1/3 of its initial specific capacitance, considering that it was also the only reactor with electrical potential during the reinoculation, it could be suggested that there was a

negative effect on the biofilm. This was proven by the fact that the specific capacitance recovered over 20% after removing the electrical potential from the biocathode.

Although for R2 is not clear how much of the drop was due to the synthetic media exposure or due to the electrical potential of -500 mV, the specific capacitance of R2 remained relatively stable with a 22% drop towards the end of the 4th set without any electrical potential applied, this was not reflected on the removal rates since at this point this biocathode performed its best in terms of nitrogen removal.

The reactor which specific capacitance values paired the best with its removal rates was R3. The specific capacitance was stable above 53% until electrical potential was applied. A drop of 13.7% first occurred after being exposed to a potential of -300 mV for 5 days, although this turned around when potential was increased to -500 mV for an additional 5 days where specific capacitance recovered back to 52.3%. Even when potential was further increased to -700 mV specific capacitance seemed to recover to 61.9%, even though it dropped back to 47.3% after one week of being exposed to this potential.

The bioelectrodes with the highest specific capacitance were in correspondence with the highest nitrogen removal rates and vice versa, proving that the specific capacitance (and therefore efficiency) of a bioelectrode is directly related to its nitrogen removal activity when no electrical potential is applied.

SUMMARY

Analytical methods used for measuring concentrations of nitrogen compounds proved to be suitable and consequently nitrogen removal rates could be determined. This study has shown that a bioelectrochemical system based on anaerobic ammonium oxidation process is a viable method for nitrogen removal in the wastewater treatment process achieving nitrogen removal rates up to 25.2 mg N/m^2/day with more than 64% of the nitrogen removal efficiency.

An 8-week inoculation period in wastewater seemed to be enough for the bioelectrodes to develop sufficient biomass for comparative tests. Nevertheless, bioelectrodes need to be adapted to test conditions after inoculation before achieving stable nitrogen removal rates since not only anammox bacteria develop on the available surface which increases the start-up time of a bioelectrode significantly.

Regarding the effect of an external electrical potential in the anammox process, it was found that -700 mV is too high of a potential for the anammox process and rather it seemed to have an inhibiting effect on the ammonium removal rate. No immediate positive effect was observed on the nitrogen removal rates just by applying electrical potential to a working bioelectrode. However, a potential of -500 mV appears to have a positive effect increasing more than 30% the nitrogen removal rate of a given bioelectrode as long as the electrode is exposed to the electrical potential gradually and through a relatively long period of time. A minimum training period of 3 weeks where changes are done gradually (1st week without electrical potential, second with -300 mV and 3rd with -500 mV) could be suggested, but more testing is required to validate this.

Even with slow growth rates, complex systems, and long setup times, anaerobic ammonium oxidation process-based bioelectrochemical systems appear to be an interesting new upcoming technology if not to replace at least to support the nitrogen removal in wastewater treatment process. This is a new approach that has been studied ~5 years and this work contributes important findings to this developing field. Much more research needs to be done but the future of this technology looks promising and could support a more resource-efficient process than the current conventional methods used today in most wastewater treatment plants.

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ANNEX

I. Annex 1: 16S rRNA sequencing analysis results

II. Annex 2: Tartu tap water composition

3. Indicators

Indicator	Unit	Permissible limit value	Content in the drinking water of the city of Tartu
Aluminium	µg / l	200	<0.5 - 0.93
Ammonium	mg / l	0.50	0.02 - 0.13
Electrical conductivity (at 20 0C)	µS cm-1	2500	602 - 917
Chlorides	mg / l	250	6.7 - 60.1
Manganese	µg / l	50	6 - 35
Sodium	mg / l	200	27 - 88.8
Oxidizability (PHT)	mg / l	5.0	0.50 - 0.72
Organic carbon (TOC)	mg / l	No unusual changes	0.57 - 1.1
Iron	mg / l	0.20	0.02 - 0.17
Sulfate	mg / l	250	3.2 - 38.2
pH	pH unit	≥6.5 and ≤9.5	7.2 - 8.0
Turbidity	NTU	No unusual changes	<0.18 - 0.33
The taste	degree of dilution	No unusual changes	1 - 2
Smell	degree of dilution	No unusual changes	1 - 2
Color	Pt / Co unit	No unusual changes	<2 - 3

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5. Characteristics for which no limit has been set

Indicator	Unit	Content in the drinking water of the city of Tartu
General hardness	mmol / l	1.7 - 4.5
	dH0	9.5 - 25.2
Dry residue	mg / l	370 - 607
Bromodichloromethane	µg / l	<0.2
Bromoform	µg / l	<0.2
Dibromochloromethane	µg / l	<0.2
Chloroform	µg / l	<1
Tetrachloroethylene	µg / l	<0.2
Trichlorethylene	µg / l	<0.3
Barium	µg / l	290-610
Beryllium	µg / l	<0.01
Potassium	mg / l	5.9-12.1
Calcium	mg / l	37-124
Magnesium	mg / l	28.8-37.5
Strontium	µg / l	127-825
Cobalt	µg / l	<0.02-0.06
Molybdenum	µg / l	0.43-1.8
Thallium	µg / l	<0.01
Thorium	µg / l	<0.02
Zinc	µg / l	2.7-94.2
Uranium	µg / l	0.21-6.1
Vanadium	µg / l	0.03-0.80
Monobasic phenols	µg / l	<0.5
Dibasic phenols	µg / l	<0.5

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