Sustainable Energy Technologies and Assessments 47 (2021) 101507

Contents lists available at ScienceDirect

Sustainable Energy Technologies and Assessments

journal homepage: www.elsevier.com/locate/seta

Enhanced bioelectrochemical nitrogen removal in flow through electrodes

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ARTICLE INFO

Keywords:
Anodic biofilm
3-electrode setup
COD
Total nitrogen
Flow through electrode

ABSTRACT

In the past decade, bioelectrochemical systems (BESs) have been studied extensively for the generation of power and maximizing power densities. In recent years, it was noticed that BESs applications can critically improve wastewater treatment. Most of the previous BESs work has used varied reactor geometry and configuration, wastewater composition, electrolyte solution, and constant electrode size to maximize power generation. However, there is limited research investigating the influence of increased electrode size on the wastewater treatment process. We investigated the effect of increased electrode surface area on wastewater treatment efficiency and studied the mechanism of nitrogen removal. In this study, we developed a flow-through electrode in a 3-electrode bioelectrochemical reactor. The anodic biofilms were enriched on electrodes for one week. Following the anodic enrichment period, the reactor was operated in a semi-continuous mode with raw domestic wastewater. To investigate the wastewater treatment efficiency, the chemical oxygen demand (COD), total nitrogen (TN), ammonia (NH_3-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) concentrations were measured. We found that increased surface area of anode did not significantly contribute to COD removal rate, most likely indicating the limits of BES. On the other hand, the TN removal rate increased proportionally to the surface area of the anode in the BES. We also found that outlet NO_3^--N and NO_2^--N concentrations were 1.2 ± 0.2 and 3.2 ± 0.9 mg/L, respectively. Our results indicated that it is possible to remove COD and TN simultaneously. Analysis of the microbial community structure showed that nitrogen removal was dominated by sulfidogenesis, anodic ammonia oxidation, autotrophic and heterotrophic denitrification as well as reducing NO_3^--N to NO_2^--N using *Geobacter* species in our system.

Introduction

According to the United Nations Environment Programme, the human population has continued to grow at a significant rate from 1.6 billion to 6.1 billion people over the last century and is expected to exceed 9 billion by 2050 [1–3]. As the world's population continues to grow, the demand for water also increases due in part to the increased burden on wastewater treatment plants [2,4]. Approximately 80 percent of domestic, industrial, and agricultural waste is discharged, untreated, into water bodies all over the world [5]. Contact with untreated waste is a health risk due to waterborne illnesses such as cholera, dysentery, and hepatitis. Additionally, there is an environmental risk due to the associated eutrophication caused by untreated wastewater. Finally, the uncontrolled decomposition of these wastewater streams contributes significantly to greenhouse gas emissions in the form of nitrous oxide and methane [2,6]. One of the most used wastewater processes is the activated sludge processes, which are highly energy-demanding [7], with public water treatment services accounting for 3–4% of the total energy consumption in the United States [8]. Rabaey et al. [9] reported that only wastewater aeration can be responsible up to 50% of total energy cost, which is approximately 1 kWh of energy is required for completely oxidation of 1 kg of organic matter in a wastewater treatment plant [9]. In addition, the conventional aerobic treatment process produces a large amount of sludge, which is also costly to treat and dispose of, and may count up to 35–60% of the total wastewater operation cost [10]. These facts show that it is critical to develop an energy-positive and environmentally sustainable wastewater treatment process.

Bioelectrochemical systems (BESs) have recently emerged as a promising technology that generates electricity, hydrogen or other useful chemicals by oxidizing biodegradable organic matters using electrochemically-active bacteria [11]. BESs such as microbial fuel cells (MFCs) are comprised of anode and cathode electrodes or sometimes built with only one working electrode (anode or cathode) in a 3-electrode system where the working electrode potential is controlled with

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https://doi.org/10.1016/j.seta.2021.101507
Received 25 October 2020; Received in revised form 23 June 2021; Accepted 22 July 2021
Available online 4 September 2021

a potentiostat against a reference electrode. For this research, we focused on anaerobic wastewater treatment using an anode as an electron acceptor. Various anode electrode materials, especially carbon-based materials (such as carbon fabric, carbon paper, graphite felt) have been widely used in BES applications due to their decreased costs and biocompatibility when microbial cultures are grown on them [12]. Recent approaches tend to switch from two-dimensional (2-D) flat electrodes to 3-dimensional (3-D) porous electrode materials such as felt and brush. It has been reported that 3-D electrodes have advantages over 2-D flat electrodes as 3-D electrodes have a higher surface area compared to 2-D electrodes, which gives rise to a higher surface-area-to-volume ratio. The higher surface area provides more space for microbial attachment which increases electron transfer rates [13]. In addition to high surface area characteristic of 3-D electrodes, a flow can be used to increase the availability of substrate inside the graphite felt [14].

Most prior BESs work has been conducted under laboratory-bench-scale [15] using different reactor configurations, electrode materials and sizes, and types of microorganisms with known electron donors with well-controlled conditions [16]. However, there is a critical need to investigate combined domestic wastewater treatment and nitrogen removal [7]. Nitrogen is one of the key contaminants found in wastewater, which exists in the reduced forms of ammonia (NH_3 or NH_4^+) and organic nitrogen [17]. The inappropriate discharge of wastewater may contain an excessive amount of nitrogen species into natural waters causes the excessive growth of algae. The excess algae growth promotes eutrophication of rivers, lakes, and coastal waters, impairing the quality of water resources [18]. Hence, there is an increase in research and development of wastewater treatment technologies due to strict discharge regulations on nitrogen [19]. Many conventional approaches to remove of organics and nitrogen species from domestic wastewater are based on highly energy-demanding activated sludge processes [7]. These processes consist of aerobic nitrification, which requires aeration for ammonium oxidation to nitrate using ammonia as the electron donor and oxygen as the electron acceptor, and anoxic denitrification, which requires external organic carbon additions for reducing nitrate (NO_3^--N) to dinitrogen (N_2) using the carbon source as the electron donor and nitrite (NO_2^--N) or nitrate (NO_3^--N) as the electron acceptor [20]. Therefore, conventional nitrogen removal is costly due to the requirements of extensive aeration during nitrification and exogenous organic carbon in denitrification [21]. Furthermore, conventional biological nitrogen removal processes produce nitrous oxide (N_2O) emissions during nitrogen removal, which contributes to global warming [20]. Avoiding the drawbacks of these processes (high energy requirement, exogenous organic carbon, N_2O emissions, etc.) to remove nitrogen from domestic wastewater, it is important to examine suitable methods to reduce or eliminate the drawbacks with process optimization or using more energy-positive and environmentally sustainable processes. Several studies demonstrated that nitrogen in domestic wastewater can be transformed and/or removed using BESs [22,23]. These studies were mostly based on air cathode microbial fuel cells (MFCs) in which nitrifying biofilm was enriched on the surface of an air cathode to oxidize ammonia to nitrate and volatile ammonia through pH increase at the air–cathode MFCs within different reactor configurations [7,19,24–28], electrode materials [21,28,29], different C/N ratios [30–32], and electrode sizes [19,23,24,26,28,33]). To the authors' knowledge, limited work have been done on the anaerobic 3-D flow through electron-accepting electrodes in a 3-electrode setup for nitrogen removal from domestic wastewater. The goal of this work is to investigate how chemical oxygen demand (COD) and total nitrogen (TN) removal rates correlate with the increased surface area of the anode and microbial community shiftin BESs.

In this study, we developed flow through a 3-electrode bioelectrochemical reactor and tested how the increased surface area could affect COD and TN removal rates, and the mechanisms of nitrogen removal. Along with BES experiments, we also performed control experiments using the same conditions, except the working electrode was not polarized. The efficiency of the wastewater treatment was investigated in terms of TN removal rate, COD removal rates, and outlet concentrations of ammonia (NH_3-N), nitrite (NO_2^--N), and nitrate (NO_3^--N). We expect the investigation of these factors help us to better understand the potential of using 3-electrode bioelectrochemical systems to treat wastewater and microbial community shifts in a smaller area compared to conventional aerobic treatment processes.

Materials and methods

Bioelectrochemical system setup and operation

The 123D© design software was used as a computer-aided design program with a stereolithography file of the object selected for printing. The acrylonitrile–butadienestyrene was used as a printing material, which is one of the most common thermoplastic polymers used in 3D printing [34]. We printed the reactors as a solid to ensure a low volume of pores in the printed reactor. The process took approximately 24 h for each reactor (WSU Frank Innovation Zone, Pullman, WA). Then, we used an acetone (Fisher Scientific, Catalog #A18-500, Hampton, NH, USA) vapor bath method to make the 3-D printed reactor waterproof and smooth the surface of each reactor.

The experimental setup used for the BES is shown in Fig. 1. The BES consists of a 3-electrode system: graphite felt working and counter electrode (HP Materials Solutions, Inc., Woodland Hills, CA, USA) and a Ag/AgCl reference electrode. The reference electrode is manufactured in-house according to previously published protocols [35]. The anodes consisted of cylindrical coupons of graphite felt (HP Materials Solutions, Inc., Woodland Hills, CA, USA) with a radius of 1.1 cm. A graphite felt anode connected via a screwed titanium wire (Malin Company, Inc., Cleveland, Ohio, USA) was used as the anode support (WE). Part of the titanium wire was inserted into the graphite felt and the remainder was protected by an insulating heat shrink sleeve filled with silicone (DAP Dynaflex 230, catalog #18357) and dried for 24 h. Finally marine sealant (3 M 05220 Marine Adhesive/Sealant 5200 Fast Cure, catalog # 06535) was applied at the two edges and dried for 24 h.

Electrodes with 3.8 cm^2, 15.2 cm^2, and 30.4 cm^2, projected surface areas, were compared to investigate domestic wastewater treatment efficiency in BES. Projected surface area is calculated as the surface area of the electrode, directly facing the medium (domestic wastewater). Counter electrodes made of graphite felt (HP Materials Solutions, Inc., Woodland Hills, CA, USA), with 32 cm^2 projected surface area, had a higher surface area than the working electrodes. The reactor was operated under anoxic conditions by continuously sparging nitrogen gas with a diffuser stone. The working electrode potential was controlled at 0 $V_{Ag/AgCl}$ using a previously developed custom potentiostat [36] and current was recorded as a function of time in the 3-electrode systems. We used 0 $V_{Ag/AgCl}$ because the lower overpotential allows for higher selectivity for electrochemically-active microorganisms, and is closer to the potential used for power generation in MFCs. Reactors had a working volume of 120 ml and were operated at room temperature (~25 °C). In the BES and control reactors, the fluid flowed perpendicular with 10 ml/min flow rate to the electrode surfaces. When we tested different flow rates (5, 10 ml/min) with the anode surface area of 30.4 cm^2 we found that there were no changes in COD removal rates (57.6 ± 7.0 and 58.2 ± 10.9 for 5 and 10 ml/min, respectively). Therefore, we have chosen a flow rate of 10 ml/min which did not cause clogged tubes or reactor overflow. The reactors and recycling bottles used were mixed by magnetic stirrer bars.

Municipal wastewater and mixed culture (as inoculum) were collected from the influent and anaerobic basin, respectively, at the Water Reclamation and Reuse Facility in Moscow, ID; it is a wastewater treatment plant that follows a biological nutrient removal wastewater treatment process. The first stage of the experiment, or the enrichment period, is the acclimatization and development of an electrochemically-active biofilm on the anode surface. The anodic biofilms were enriched

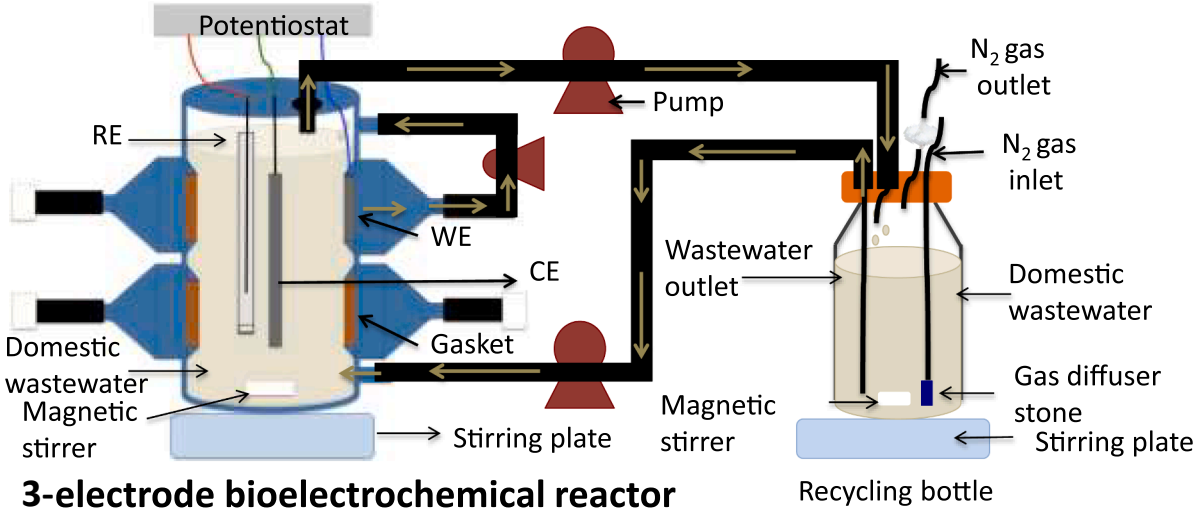

Fig. 1. Schematic diagram of the BES setup (left) including working electrode (WE), counter electrode (CE), reference electrode (RE), potentiostat, wastewater inlet and outlet with flow direction of wastewater; the recycling bottle setup (right) including N_2 gas inlet and outlet, wastewater inlet and outlet with flow. This setup shows only one single flow through electrode in BES (drawing is not to scale).

in wastewater with 20 mM acetate. We used mixed culture as a rich inoculum because we successfully grew anodic electrochemically-active biofilms in preliminary experiments using this inoculum. The enrichment period lasted until the current values reached a pseudo-steady-state. Once the enrichment period was done, the system was operated in batch mode with fresh domestic wastewater.

Along with BES experiments, control experiments were performed to further investigate the removal pathways of nitrogen. Control experiments were conducted using the same setup with BES experiments (Fig. 1) using anode projected surface areas of 3.8 cm^2, 15.2 cm^2, and 30.4 cm^2. Except, the working electrode in the control experiment was not polarized.

Analytical methods

To test the performance of anodic biofilms, samples from well-mixed bulk solution were collected and analyzed following standard protocols. The samples were centrifuged for 2 min at 10,000 rpm to separate suspended cells before filtering. Membrane filters (pore size 0.45 mm) (Tisch Scientific, Ohio, USA) were used to filter wastewater samples before measurements. The dissolved COD was determined using mercury-free potassium dichromate method (low range and high range COD 2 vials, Hach Company, Catalog # 2,565,025 and 2565115, respectively, Loveland, CO, USA).

The TN was determined using persulfate digestion method (low range and high range TNTplus^{TM} vials, Hach Company, Catalog # TNT 827 and TNT 828, respectively). The ammonia (NH_3-N) was determined using salicylate method (high range TNTplus^{TM} vials, Hach Company, Catalog # TNT 832). The NO_2^--N was determined using the diazotization method (high range TNTplus^{TM} vials, Hach Company, Catalog # TNT 840). The NO_3^--N was determined using dimethylphenol method (low range TNTplus^{TM} vials, Hach Company, Catalog # TNT 835). The TN, NH_3-N, NO_2^--N and NO_3^--N concentration were measured following HACH procedures with a spectrophotometer (DR 3900 HACH Company, CO). The total COD and TN removal efficiencies were calculated based on the difference between initial and final concentrations in the bulk solution for every sampling period, which is one day, divided by its initial concentration (Figs. 2B and 3B).

The medium pH was determined by collecting a 50 ml sample from the bulk medium at the beginning and end of each experiment and

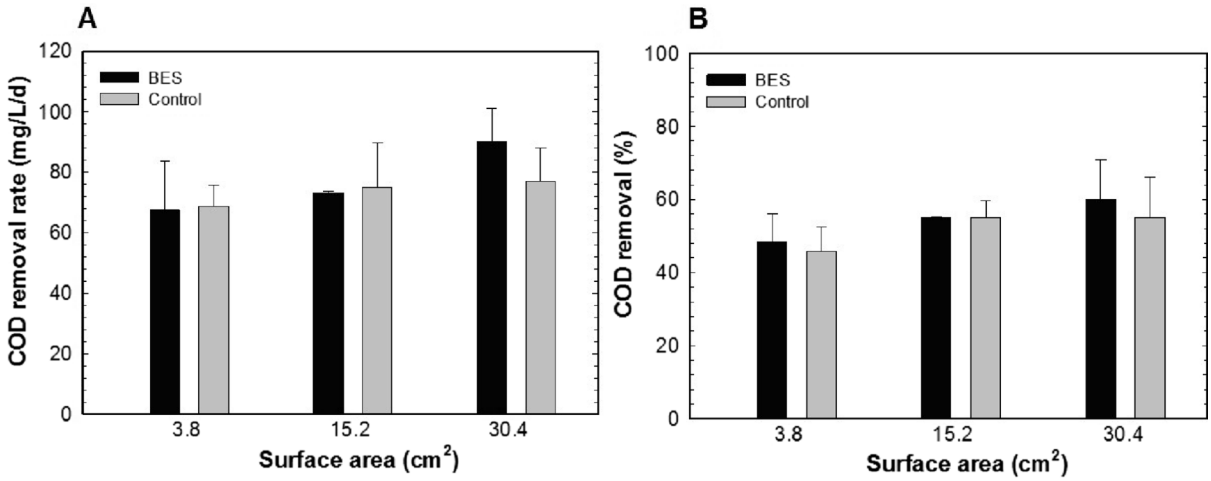

Fig. 2. (A) COD removal rate; (B) COD removal efficiency. The increased surface area did not significantly increase COD removal rate. There were no critical differences between BES and control indicating the use of BES did not contribute COD removal. The data are means, and the error bars represent the standard deviations of the means from two biological replicates.

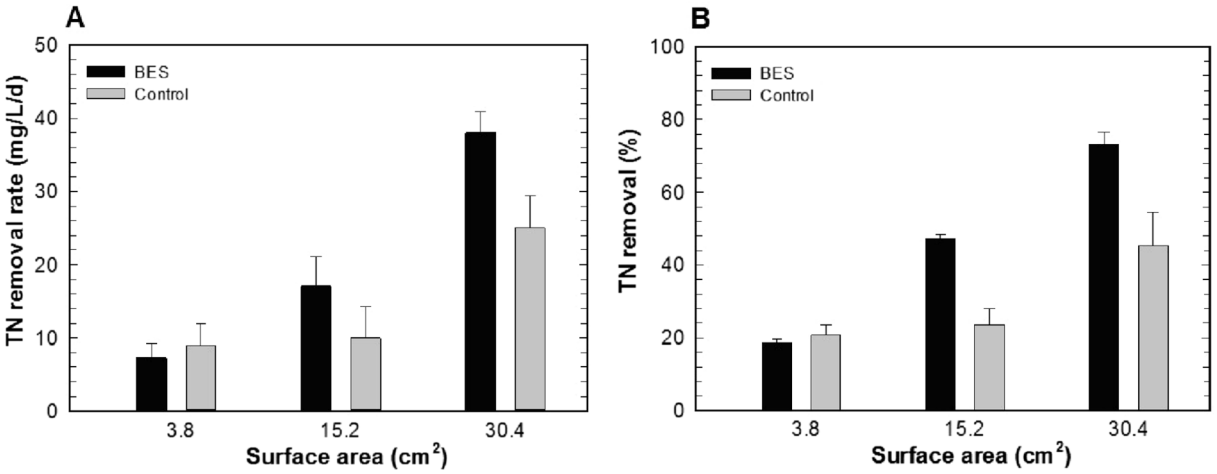

Fig. 3. (A) TN removal rate; (B) TN removal efficiency. Increased surface area improved TN removal. The BES improved ammonia removal rate. The data are means, and the error bars represent the standard deviations of the means from two biological replicates.

measured using a Denver Instruments UltraBasic pH Meter (Denver Instrument Company, Arvada, Colorado USA).

Results and discussion

COD removal and coulombic efficiency

The COD removal rates during a semi-continuous operation after one week of enrichment period were 67.5 ± 16.0, 73.0 ± 0.7, 90.0 ± 10.9 (mg/L/day) and COD removal efficiencies (%) were 48.3 ± 7.8, 55.0 ± 0.3, 60.0 ± 10.9 with anode projected surface areas of 3.8 cm^2, 15.2 cm^2, and 30.4 cm^2 respectively in BES reactors (Fig. 2). On the other hand, COD removal rates and COD removal efficiencies (%) with anode projected surface areas of 3.8 cm^2, 15.2 cm^2 and 30.4 cm^2 were found 68.8 ± 6.7, 75.0 ± 14.6, 77.0 ± 11.0 (mg/L/day) and 45.9 ± 6.7, 54.9 ± 4.7, 55.0 ± 11.0 respectively (Fig. 2B) in control reactors. The final effluent concentrations were between 59 and 73 mg/L and 62 and 80 mg/L with the surface area of the electrodes in BES reactors and control reactors, respectively. The findings from the literature indicate that the environmental regulations all around the world accept different COD effluent discharge limits for example, while some African countries such as Nigeria and Tanzania indicate maximum COD discharge limit is 60 mg/L, Asian countries determine like India set the limit with 250 mg/L [37]. Our results show that BES reactors can successfully meet even the most strict COD effluent discharge limits.

We also conducted the statistical ANOVA test to examine if there was a significant impact of electrode size COD removal rates. The p-values of BES and control experiments from the test were 0.281 and 0.768, respectively, suggesting that differences in electrode size had no significant impact on COD removal rates. These results also demonstrated that increased surface area did not significantly increase the COD removal rate. In other words, the use of BES did not critically contribute COD removal (Fig. 2).

The current densities were varied for the projected surface area of 3.8 cm^2, 15.2 cm^2, and 30.4 cm^2 biofilm anodes after the enrichment period. The domestic wastewater medium had low COD loading, (100–150 mg/L COD), and the smallest electrode 3.80 cm^2 generated higher average current density (=0.35 mA/cm^2) compared to largest electrode 30.4 cm^2 (=0.18 mA/cm^2) after the enrichment period. Our previous work confirmed that anodic biofilms were linearly scaled up at high COD loading (1500 mg/L), while current density decreased with increasing electrode size at lower COD loadings [38]. We calculated the coulombic efficiencies (CEs) based on the change in influent and effluent COD and current [39]. The CEs which used to evaluate the efficiency of BESs, are defined as the recovery of total electrons in wastewater as current. Thus, a higher value of CE means that more electrons are extracted in the substrate as electricity energy by electrochemically-active bacteria. The calculated CEs had maximum values of 2.0%, 3.2%, and 6.9% with anode projected surface area of 3.8 cm^2, 15.2 cm^2 and 30.4 cm^2, respectively. The maximum CE was about 7.0%, which demonstrated that substantial organic substrates are consumed without the current generation.

In the BES and control reactors, the fluid (domestic wastewater) flowed perpendicular to the electrode surfaces with 10 ml/min of flow rate. Reactors and recycling bottles for both BES and control setups were mixed continuously using magnetic stirrer bars (Fig. 1). This means that instead of external factors such as flow rate with mixing (the effects of advection), the biological factors, which is bacterial community, were the main method for removing organic matter by transferring the electrons from the donor to the acceptor. Hence, when we compared the COD removal rates of BES and control reactors, the polarized electrode with applied voltage improved bacterial metabolism in the BES reactor.

Nitrogen removal

The TN removal rates from the semi-continuous operation after one week of enrichment period were 7.2 ± 2.0, 18.1 ± 4.0, 38.0 ± 3.0 (mg/L/day) and TN removal efficiencies (%) were 18.5 ± 1.3, 47.3 ± 1.0, 78.2 ± 3.4 with anode projected surface area of 3.8 cm^2, 15.2 cm^2 and 30.4 cm^2 respectively in BES reactors (Fig. 3). On the other hand, TN removal rates and TN removal efficiencies (%) with anode projected surface area of 3.8 cm^2, 15.2 cm^2 and 30.4 cm^2 were found 9.0 ± 3.0, 10.0 ± 4.3, 25.0 ± 4.5 (mg/L/day) and 20.7 ± 3.0, 23.6 ± 4.3, 45.0 ± 9.2 respectively (Fig. 3) in control reactors. The statistical ANOVA showed that there was a significant impact of electrode size on TN removal rates. The p-values of BES and control experiments from the test were <0.001 and 0.049, respectively, which suggests that different electrode size had a significant impact on TN removal rates. These results also showed that when the projected surface area of the anode in BES reactors was increased, TN removal (%) increased proportionally to the surface area of the electrode ($R^2 = 0.99$). On the other hand, control reactors also showed increasing in TN removal (%), but it was not proportional to the surface area of the electrode ($R^2 = 0.45$). Hence, a critical conclusion of this study was that increased surface area improved TN removal in BES reactors.

For further investigation, the inlet and outlet concentrations of NH_3-N, NO_2^--N and NO_3^--N were tested every day. The outlet NH_3-N concentrations gradually decreased in the BES as the anode projected surface area was increased (Fig. 4A), illustrating that the BES improved ammonia removal rate. An ANOVA test was conducted to examine if

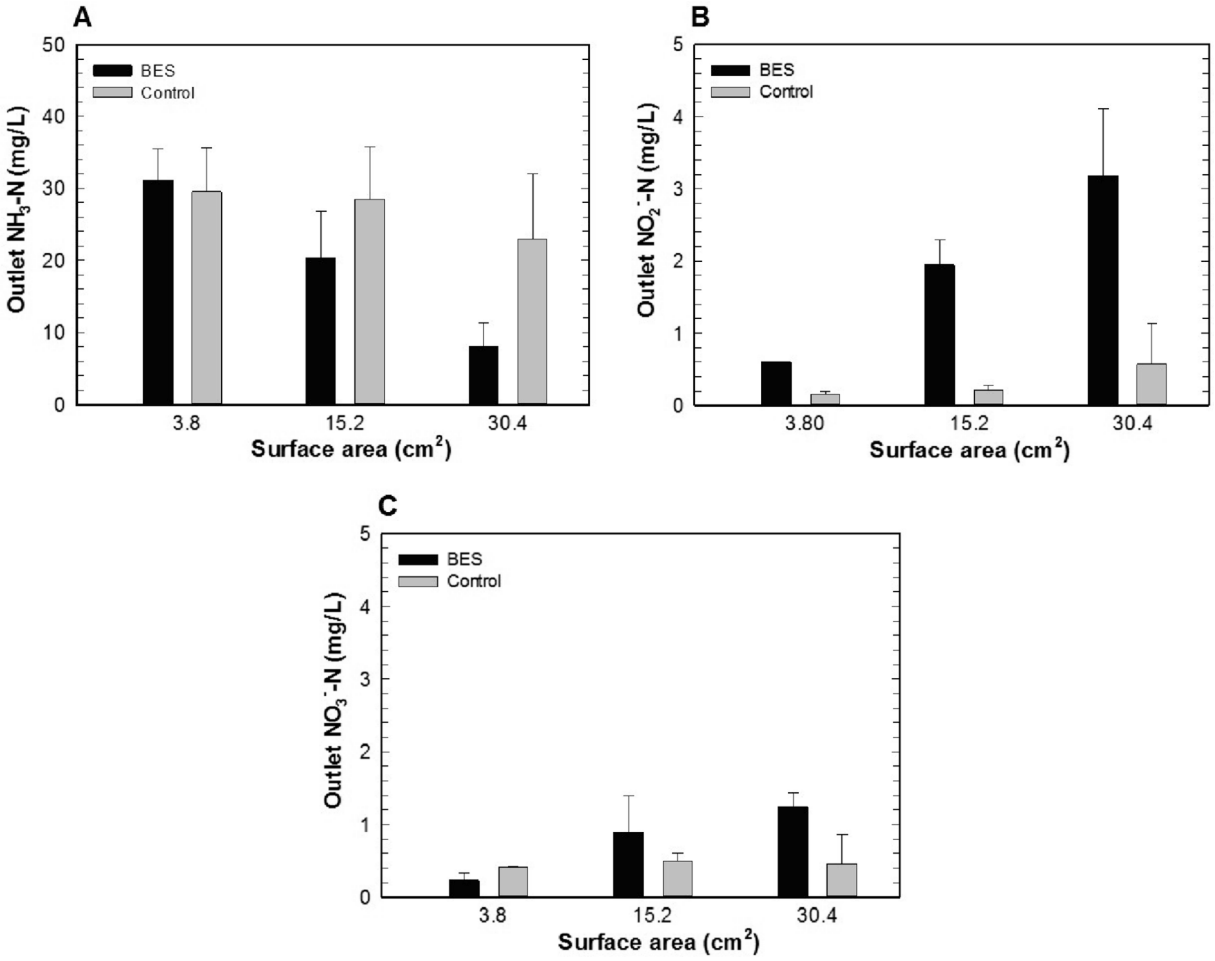

Fig. 4. (A) Outlet NH_3-N concentrations; (B) Outlet NO_2^--N concentrations; (C) Outlet NO_3^--N concentrations in the BES and control reactors during one day of operation period. The BES improved ammonia removal rate. However, outlet NO_2^--N and NO_3^--N concentrations were increased with increasing surface area. The data are means, and the error bars represent the standard deviations of the means from two biological replicates.

there was a significant impact of electrode size on the outlet concentration of NH_3-N. The BES p-value from the test was 0.042 which suggests that using different electrode size had a significant impact on outlet concentration of NH_3-N. In contrast, p-value from the test was 0.690 for the control experiment, which suggests that using different electrode size had no significant impact on the outlet concentration of NH_3-N. Inlet NO_2^--N concentrations of domestic wastewater were measured below the detection limit (BDL), of 0.6 mg/L NO_2^--N for Hach Company TNTplus^{TM} vials (catalog # TNT 840). Outlet NO_2^--N concentrations of both reactors were gradually increased with increased anode projected surface area. Outlet NO_2^--N concentrations reached a maximum concentration of 3.2 ± 0.9 mg/L with anode projected surface area of 30.4 cm^2 in BES reactors (Fig. 4B). The statistical ANOVA test showed that impact of electrode size on outlet concentration of NO_2^--N for BES experiments was significant (p-value from the test was 0.046). In contrast, p-value from the test was 0.975 for control experiment, which suggests that using different electrode size had no significant impact on outlet concentration of NO_2^--N. Inlet NO_3^--N concentrations of domestic wastewater were measured to be 0.59 ± 0.3, which was higher than the detection limit, of 0.23 mg/L NO_3^--N for Hach Company TNTplus^{TM} vials (catalog # TNT 835). Outlet NO_3^--N concentrations in both reactors were also measured slightly higher than inlet concentrations (Fig. 3D). Outlet NO_3^--N concentrations of both reactors were gradually increased when we increased the anode projected surface area. Outlet NO_3^--N concentrations reached a maximum concentration of 1.2 ± 0.2 mg/L with anode projected surface area of 30.4 cm^2 in BES reactors (Fig. 4C). The statistical ANOVA test showed that there was not a significant impact of electrode size on outlet NO_3^--N concentrations. The p-values of BES and control experiments from the test were 0.105 and 0.946, respectively. We aimed to use data of nitrogen species (NH_3-N, NO_2^--N, and NO_3^--N) to explain possible nitrogen removal mechanisms in the following sections; however, even though BES reactors with the anode projected surface area of 30.4 cm^2 meet the discharge limit for TN effluent for many countries, which is 10 mg/L, further studies on the removal of NH_3-N, NO_2^--N and NO_3^--N are needed to lower the TN level in the outlet.

Microbial community analysis

In order to interpret the nitrogen removal mechanisms between the BES and control reactors, the biofilms on the anode was investigated by a high-throughput sequencing technique. We compared the enriched microbial communities in raw wastewater supplemented with acetate of anodic biofilms while varying the size of the anodes in BES and control reactors, which were operated at the same conditions except the working electrode was not polarized in the control reactors. To examine the effect of electrode size on anode microbial community, we compared anodic biofilms from the 3.8-BES (3.8–cm^2 anode), 15.2-BES (15.2–cm^2 anode), 30.4-BES (30.4–cm^2 anode) reactors. In the microbial communities, the dominant groups at phylum level were similar in all of the bioelectrochemical reactors, but the the phyla were mainly classified into Bacteroidetes, Firmicutes, Proteobacteria, and Epsilonbacteraerota

groups. The remaining bacterial population present on anodes were at low percentages (only 0.05% to 4.90%) (Fig. 5A). When comparing the structures of microbial community on anodes of different sizes in 3.8-BES, 15.2-BES, 30.4-BES reactors, we observed slight differences in the portion of the total bacteria that belonged to Bacteroidetes (45.7%, 44.5%, and 40.2%, respectively); Firmicutes (25.2%, 25.6%, and 13.0%, respectively); Proteobacteria (13.6%, 13.4%, and 28.0%, respectively); and Epsilonbacteraerota (5.1%, 5.8%, and 5.3%, respectively). Members of Spirochaetes were only found in 30.4-BES (4.9%). *Bacteroidetes, Firmicutes,* and *Proteobacteria* were reported to be the dominant bacterial phylum in the anaerobic treatment plant with the ability to degrade organic pollutants and remove nutrients [40–43].

The dominant genus were *Geobacter* (6.7% in 3.8-BES, 7.4% in 15.2-BES, and 13.7% in 30.4-BES), *Rikenellaceae* (6.0% in 3.8-BES, 5.7% in 15.2-BES, and 2.3% in 30.4-BES), *Paludibacter* (5.6% in 3.8-BES, 5.7% in 15.2-BES, and 3.7% in 30.4-BES), *Arcobacter* (4.1% in 3.8-BES, 4.4% in 15.2-BES, and 2.3% in 30.4-BES), *Lentimicrobiaceae* (3.5% in 3.8-BES, 3.8% in 15.2-BES, and 5.1% in 30.4-BES), *Clostridiales* (3.0% in 3.8-BES, 3.2% in 15.2-BES, and 3.6% in 30.4-BES), *Macellibacteroides* (2.5% in 3.8-BES, 2.3% in 15.2-BES, and 1.3% in 30.4-BES), *A7P-90m* (2.3% in 3.8-BES, 2.3% in 15.2-BES, and 4.9% in 30.4-BES), *Desulfovibrio* (0.7% in 3.8-BES, 0.8% in 15.2-BES, and 6.2% in 30.4-BES), and *Sulfurospirillum* (1.0% in 3.8-BES, 1.1% in 15.2-BES, and 3.0% in 30.4-BES). *Geobacter* was the predominant genus in BES reactors that is able to facilitate both extracellular electron transfer on anodes and reduction of nitrate to nitrite [44–49]. Among the other dominant genera, *Arcobacter* and *Acinetobacter* are involved in denitrification [22,32,50,51]; *Desulfovibrio* and *Sulfurospirillum* are sulfate reducing bacteria [51–54], that are involved in ammonia oxidation by sulfate; *Clostridiales* and *Macellibacteroides* are anaerobic fermentative bacteria that may contribute to autotrophic denitrification [55,56] (Fig. 5B).

The similar bacterial community has been also observed at phylum level in control reactors ((3.8-C (3.8–cm^2 anode), 15.2-C (15.2–cm^2 anode), 30.4-C (30.4–cm^2 anode)), but there were differences in the portion of the total bacteria belonging to Bacteroidetes (25.4%, 29.4%, and 17.3%, respectively), Firmicutes (2.5%, 2.5%, and 1.5%,

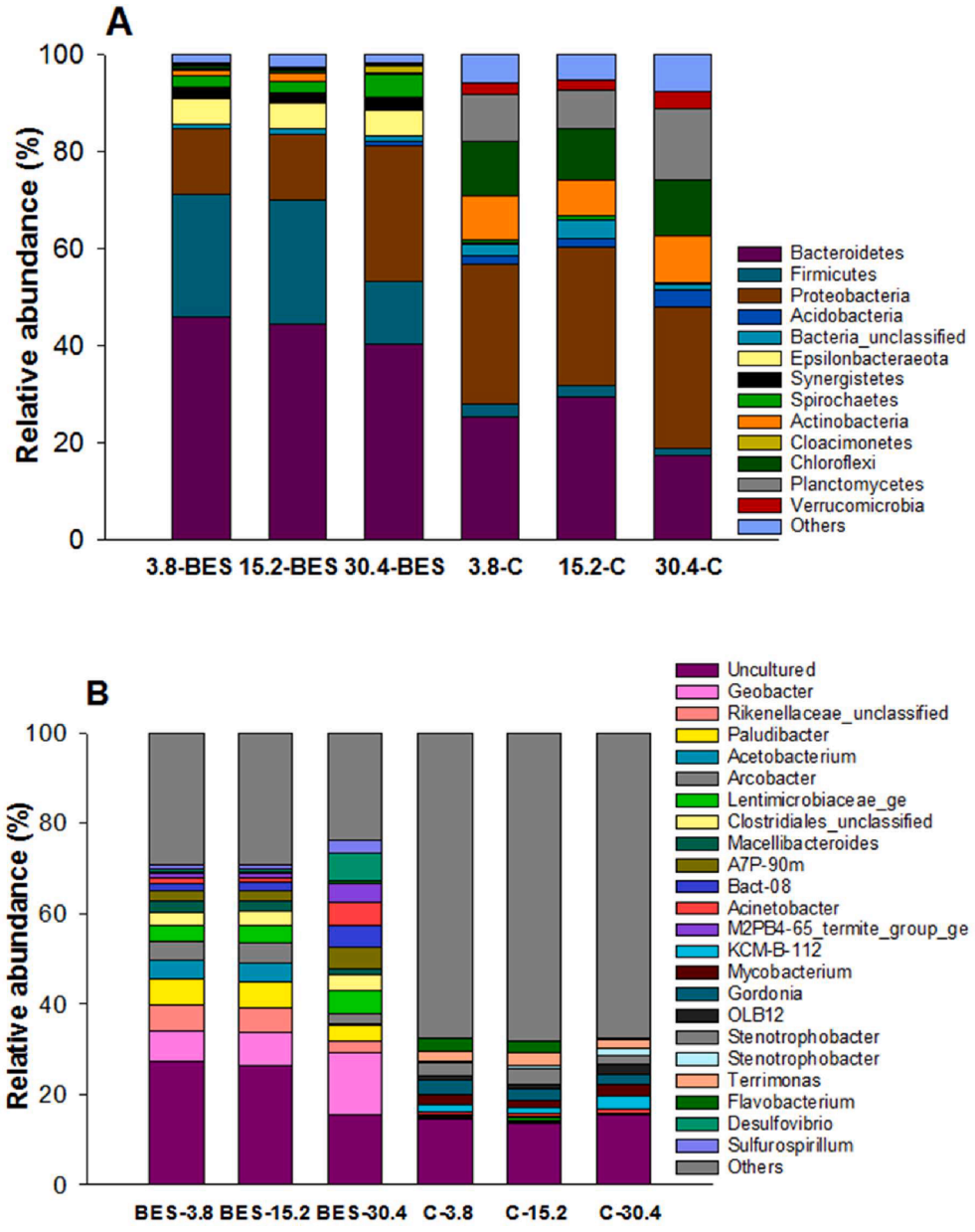

Fig. 5. (A) Relative abundances of major phylum-level bacterial groups estimated for the different reactors. (B) Relative abundances of major genus-level bacterial groups estimated for the different reactors.

respectively), Proteobacteria (28.8%, 28.5%, and 29.4%, respectively), and Epsilonbacteraerota (<0.1%).

On the other hand, members of Actinobacteria (9.2%, 7.5%, and 9.7%, respectively); Chloroflexi (10.9%, 10.6%, and 11.7%, respectively), and Planctomycetes (9.6%, 7.8%, and 14.5%, respectively) were abundantly found in control reactors as different from BES experiments. The remaining bacterial population present on anodes at low percentages (only 0.01% to 3.5%) (Fig. 5A). Actinobacteria was previously reported to have the ability of nitrate removal [57,58] and Chloroflexi and Planctomycetes were widely reported as anaerobic ammonia oxidation (ANAMMOX) bacteria [59–61].

In contrast to the dominant genera in the BES experiments, *KCM-B-112* (1.8% in 3.8-C, 1.4% in 15.2-C, and 2.9% in 30.4-C), *Mycobacterium* (2.1% in 1.6-C, 1.4% in 15.2-C, and 2.7% in 30.4-C), *OLB14_ge* (1.0% in 1.6-C, 0.9% in 15.2-C, and 2.5% in 30.4-C), *Gordonia* (3.1% in 1.6-C, 2.6% in 15.2-C, and 2.2% in 30.4-C), *SJA-28_ge* (2.7% in 1.6-C, 3.4% in 15.2-C, and 1.9% in 30.4-C), *Terrimonas* (2.3% in 1.6-C, 3.0% in 15.2-C, and 1.7% in 30.4-C), and *Flavobacterium* (2.9% in 1.6-C, 2.6% in 15.2-C, and 0.6% in 30.4-C) were observed in the control reactors as the dominant genera (Fig. 5B). In spite of high microbial diversity observed in the control reactors, there was a large fraction of uncultured bacteria as seen in Fig. 5B. Among the most dominant genera, *Gordonia* and *Mycobacterium* are actinobacteria that *Gordonia* can degrade of hydrocarbons that also simultaneously utilize nitrate as electron acceptors [58]. Also *Mycobacterium* has been shown to use nitrate reduction reactions (NAR) [62]. OLB14 is involved in ANAMMOX [63]. *SJA-28* (*Chlorobi* or *green sulfur bacteria*) can fix nitrogen [64] and *Flavobacterium* is involved in denitrification [65,66].

Nitrogen removal mechanisms

Table 1 summarized several nitrogen removal studies in BESs. We compared these studies based on medium type, anode material with projected surface area, TN removal efficiency (%), TN removal rates, which were normalized by anode surface area, outlet NO_2^--N and NO_3^--N concentrations. Most of these studies have an air–cathode configuration, in such a configuration one side of the cathode electrode faces air, and the other side faces the anodic compartment. Common configurations include stackable horizontal MFCs [26], flat-panel air–cathode MFC [23], and flat MFCs with a membrane bioreactor [28]. These studies reported that nitrification takes place on the surface of the air cathode to oxidize ammonia NH_3/ NH_4^+ and heterotrophic or/and autotrophic denitrification may contribute to the complete nitrogen removal. Interestingly, most of these studies did not report outlet NH_3-N, NO_2^--N and NO_3^--N concentrations (Table 1) although all nitrogen species should be considered because of their detrimental effects on the environment and human health. We normalized the TN removal rate by anode surface area of 30.4 cm^2 was calculated as 1.41×10^{-3} kg-N/m^3/d/cm^2, which was significantly higher than the previous nitrogen removal studies shown in Table 1.

In our BESs, different mechanisms can be involved in carbon and nitrogen removal (Fig. 6): (1) Hydrolysis and fermentation of complex organic matters in the anodic biofilm; (2) oxidation of simple organic matters and current generation by electrochemically active bacteria at the anode; (3) physicochemical ammonia removal process, such as ammonia volatilization; (4) the nitrogen assimilation; (5) anodic ammonia oxidation; (6) nitrification; (7) heterotrophic denitrification; (8, 9) ammonia removal with sulfate reduction; (10) *Geobacter* species were able to reduce NO_3^- to NO_2^-; (11) autotrophic denitrification. These proposed mechanisms are visualized in Fig. 6.

One of the proposed main mechanisms of nitrogen removal is ammonia volatilization (Eq.1), which is the conversion of ammonium ions into ammonia gas at high pH [70]. NH_4^+ has a $pK_a = 9.25$, so an increase in pH ($>pK_a = 9.25$), would result in a re-distribution of ammonium ions to the more volatile NH_3 form [71,72]. To examine the TN removal by ammonia volatilization in the BES reactors and control reactors, the pH of the media was measured as inlet and outlet pH every day and the outlet pH of the media was observed to be higher than the inlet (Fig. 7).

$$NH_4^+ \rightarrow NH_3 + H^+ \quad (1)$$

The average pH of the BES reactors rose from 7.4 ± 0.2 to 8.1 ± 0.0, 7.6 ± 0.2 to 7.9 ± 0.0, and 7.3 ± 0.2 to 8.0 ± 0.2 with anode projected surface area of 3.8 cm^2, 15.2 cm^2 and 30.4 cm^2, respectively (Fig. 7A).

Table 1
Nitrogen removal efficiency in studies of bioelectrochemical systems.

Reactor design	Medium type	Anode Material	Anode Projected Surface Area cm^2	Inlet TN (mg/L)	TN Removal Efficiency %	Outlet NO_2^- concentration (mg/L)	Outlet NO_3^- concentration (mg/L)	TN Removal Rate (kg-N/m^3/d/cm^2)
3-electrode BES (This study)	Domestic wastewater	Graphite felt	30.4	1.41×10^{-3}	52.5 ± 4.5	3.2 ± 0.9	78.2 ± 3.4	1.2 ± 0.2
Air-cathode MFC with membrane bioreactor [26]	Domestic wastewater	Graphite fiber brush	30	5.3×10^{-4}	32 ± 4.28[a]	N/A	97.3 ± 1.6[a]	N/A
Stackable horizontal MFC [24]	Domestic wastewater	Graphite fiber brush	1280[b]	7.8×10^{-7}	48 ± 7	N/A	71 ± 8.0	N/A
Flat-panel air–cathode MFC [23]	Domestic wastewater	Graphite felt	600	4.3×10^{-4}	28.6 ± 1.0	N/A	94 ± 0.3	5.0 ± 0.72
Three-chamber MFC with membrane [28]	Glucose as carbon source and NH_4Cl as nitrogen source	Graphite plate	55	4.0×10^{-4}	100[a]	N/A	90.2[a]	N/A
Rotation cathode MFC [25]	NH_4Cl, ammonium sulfate, ammonium phosphate	Graphite plate	150	3.1×10^{-4}	95	32 ± 4.8[c]	49.2 ± 5.9	6.84 ± 9.5[c]
Rotation cathode MFC with membrane [28]	Acetate as carbon source and NH_4Cl as nitrogen source	Carbon felt	200	3.3×10^{-4}	53.3 ± 3.5	N/A	91.5 ± 7.2	N/A
Up-flow BES reactor [67]	Acetate as carbon source and NH_3Cl, $NaNO_2$, KNO_3 as nitrogen source	Graphite fiber	300	6.8×10^{-4}	20	0.4 ± 1.6[c]	45 ± 12.3	3.6 ± 0.4[c]

[a] The ammonium removal results were reported.
[b] The anode surface area was calculated based on reference.
[c] Outlet NO_2^--N and NO_3^--N concentrations were calculated based on reference.

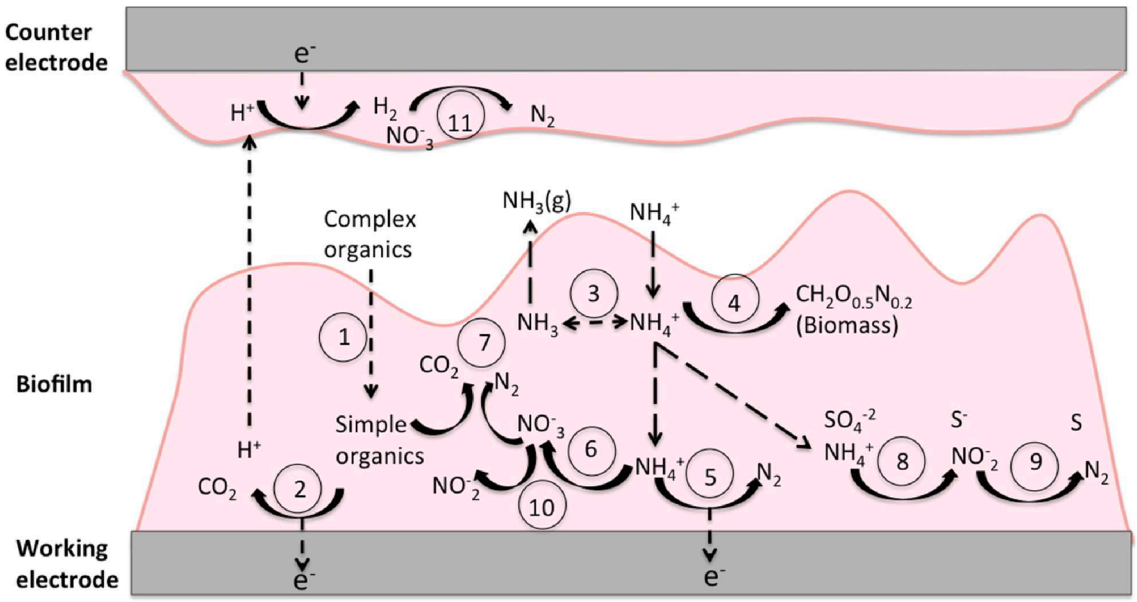

Fig. 6. Possible mechanisms of carbon and nitrogen removal in the BES reactor: (1) Hydrolysis and fermentation of complex organic matters in the anodic biofilm [68]; (2) oxidation of organic matters and electron generation by electrochemically active bacteria (such as *Geobacter* species) at the anode [69] ; (3) physicochemical removal of ammonia, such as ammonia volatilization [70–73]; (4) nitrogen assimilation [20,73]; (5) anodic ammonia oxidation [74]; (6) nitrification (such as *Nitrosomonas* cells) [75]; (7) heterotrophic denitrification (such as *Dechloromonas*, *Arcobacter* and *Acinetobacter* species) [28,32,51]; (8, 9) ammonia removal with sulfate reduction (such as *Desulfuromonas*, *Desulfovibrio,* and *Desulfromicrium* species) [51,52,54], (10) *Geobacter* species were able to reduce NO_3^- to NO_2^- with an electrode as the only electron donor [47,49]; (11) autotrophic denitrification (such as *Flavobacterium* and *Hydrogenophaga*) [50,65,76].

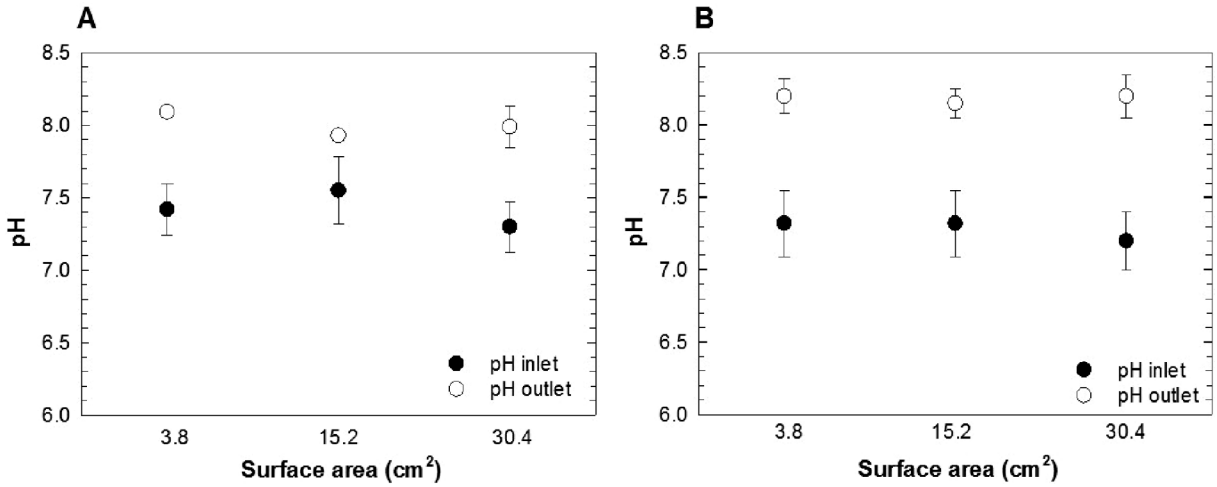

Fig. 7. Changes in mean pH in (A) the BES reactors and (B) the control reactors during one day of the operation period. In both reactors pH slightly increased to higher values. The error bars represent the standard deviations of the means from two biological replicates.

On the other hand, the average pH of the control reactors rose from 7.3 ± 0.2 to 8.2 ± 0.1, 7.3 ± 0.2 to 8.2 ± 0.1, and 7.3 ± 0.2 to 8.0 ± 0.2 with anode projected surface area of 3.8 cm^2, 15.2 cm^2 and 30.4 cm^2, respectively (Fig. 7B).

According to the results, BES and control experiments showed the same pH trend (Fig. 7). Since the pH increase is not highly elevated, ammonia volatilization may not be one of the primary nitrogen removal mechanisms. While the increased pH cannot be explained by the anodic current, an increase in pH could be related to different mechanisms including N_2 bubbling which drifts pH toward alkaline values [77] and denitrification [78].

Nitrogen is also utilized for the assimilation by microorganism [20] shown by the biomass synthesis reaction in Eq. (2). The growth rate of aerobic heterotrophic microorganisms in conventional biological treatment systems (0.6 g COD g COD^{-1}) is relatively higher than the growth rate of anaerobic microorganism (0.04–0.1 g COD g COD^{-1}) [79]. Hence, it is expected that the growth rate of electrochemically-active bacteria would be in similar range to anaerobic microorganisms. Therefore, the nitrogen removal related to assimilation would be considerably low in bioelectrochemical reactors treating domestic wastewater.

$$4CO_2 + HCO_3^- + NH_4^+ + H_2O \rightarrow C_5H_7O_2N + 5O_2 \quad (2)$$

Another nitrogen removal mechanism is the anodic ammonia oxidation reaction, which is reported to be one of the possible mechanisms in the literature [74]. The half reactions for anaerobic ammonia oxidation in the BES reactor are given by the following equations:

$$2NH_4^+ \rightarrow N_2 + 8H^+ + 6e^-,\ E^0 = -479\ \text{mV (vs Ag/AgCl)} \quad (3)$$

$$2NH_4^+ + 2H_2O \rightarrow 2NO^-_2 + 8H^+ + 4e^-,\ E^0 = -1089\ \text{mV (vs Ag/AgCl)} \quad (4)$$

$$2NH_4^+ + 3H_2O \rightarrow 2NO^-_3 + 14H^+ + 10e^-,\ E^0 = -1079\ \text{mV (vs Ag/AgCl)} \quad (5)$$

Since outlet concentrations of NO_3^--N and NO_2^--N are increased, it is possible to expect that this could be one of the dominant mechanisms in our BES reactors. The potentials for the half reactions are negative (Eqs. (3)–(5)), indicating that anaerobic ammonia oxidation reactions are thermodynamically non-spontaneous reactions. When 0 V vs Ag/AgCl is applied to the working electrode in a BES reactor, the working electrode can act as an electron acceptor for the electrons generated in Eqs. (3)–(5), therefore the anaerobic ammonia oxidation reactions would be possible our BESs as previously reported [51,80]. Reactions, 3–5 can be enhanced by the use of a flow through electrode, which improve NH_4^+ transfer through the biofilm.

Even though all of the reactors were operated anaerobically, ammonia oxidizing bacteria (*Nitrosomonas*) and nitrite oxidizing bacteria (*Nitrospira*) were observed in low abundance in both experiments (<1.0%). In addition, the inoculum can include NO_3^--N or NO_2^--N with concentrations below detection levels (0.23 and 0.6 mg/L, respectively), so it is possible that *Nitrosomonas* can use NH_4^+-N or H_2 as electron donors and NO_2^- and as electron acceptors under limited anaerobic conditions [75] (Eq. (6)).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (6)$$

In addition, NO_3^--N can accept electrons from organic compounds to be reduced to N_2 through some heterotrophic denitrifying bacteria under anaerobic conditions [74] (Eq. (7)). However, heterotrophic denitrification may not be a significant nitrogen removal mechanism since domestic wastewater contains low NO_3^--N concentrations and low organic matter, which limits the application of heterotrophic denitrification unless supplemented by external organic carbon sources. Microbial community analysis showed that heterotrophic denitrifying bacteria such as *Dechloromonas*, *Arcobacter*, and *Acinetobacter* were present. *Arcobacter* and *Acinetobacter* are the dominant denitrifying genera in BES reactors (4.1% and 1.2% in 3.8-BES, 4.4% and 1.1% in 15.2-BES, 2.3% and 4.9% in 30.4-BES, respectively). *Dechloromonas* was present on anode surface even though it is not the dominant genus in the reactors (<1.0%).

$$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O,\ E^0 = 1041\ \text{mV (vs Ag/AgCl)} \quad (7)$$

Unlike the heterotrophic denitrifying bacteria, autotrophic denitrifying bacteria utilize inorganic carbon sources, such as CO_2, and use electron donors, such as hydrogen, which is generated at the cathode to reduce NO_3^--N into N_2 and net reaction on cathode [22] as shown Eqs. (8)–(10).

$$2NO_3^- + 2H_2 \rightarrow 2NO_2^- + 2H_2O \quad (8)$$

$$2NO_2^- + 2H_2 \rightarrow N_2O + H_2O + 2OH^- \quad (9)$$

$$N_2O + H_2 \rightarrow N_2 + H_2O \quad (10)$$

Combining of Eqs. (8)–(10) gives the following reaction:

$$2NO_3^- + 6H_2O + 10e^- \rightarrow N_2 + 12OH^- \quad (11)$$

Microbial community analysis showed that *Flavobacterium* and *Hydrogenophaga* were present on the anode surface in BES and control reactors. However, the compositions of these denitrifying bacteria were also different among the two systems. *Flavobacterium* is the dominant denitrifying genus in the control reactors: 2.9% in 3.8-C, 2.6% in 15.2-C, 0.6% in 30.4-C. The remaining autotrophic denitrifying bacterial populations belonged to phyla present on the anodes at low percentages (<0.5%). On the other hand, *Clostridiales* and *Macellibacteroides* are other dominant genera in BES reactors: 3.02% and 2.5% in 3.8-BES, 3.17% and 2.27% in 15.2-BES, 3.59% and 1.30% in 30.4-BES, respectively. They are both known as anaerobic fermentative bacteria that utilize a broad range of carbon sources into volatile fatty acids, hydrogen, and carbon dioxide. Thus, these bacteria also may contribute to autotrophic denitrification.

It has also been demonstrated that *Geobacter* species such as *Geobacter metallireducens* is able to reduce NO_3^--N to NO_2^--N with an electrode as the only electron donor [46,47]. Microbial community analysis showed that *Geobacter* is one of the dominant genus in the BES reactor (6.69% in BES-3.8, 7.38% in BES-15.2, 13.73% in BES-30.4) confirming that one of the main mechanisms was the nitrate reduction in the BES reactor. This also explains why NO_2^--N concentrations higher in BES reactors than in control reactors.

If we consider that the sulfate concentration of domestic wastewater influent is in the range of 20–50 mg/L [81], one of the possible nitrogen removal mechanisms could be sulfidogenesis in which ammonia is oxidized by sulfate (SO_4^{-2}) with sulfate-reducing bacteria (SRB) [51,54], that acts as terminal electron acceptor, producing sulfide (S^{-2}) and nitrite (NO^-_2) as follows:

$$4NH_4^+ + 2SO_4^{-2} \rightarrow 3S^- + 4NO_2^- + 4H_2O + 8H^+ \quad (12)$$

$$3S^- + 2NO^-_2 + 8H^+ \rightarrow N_2 + 3S + 4H_2O \quad (13)$$

$$2NO_2^- + 2NH_4^+ \rightarrow 2N_2 + 4H_2O \quad (14)$$

Combining of Eqs. (12)–(14) gives the following reaction:

$$SO_4^{-2} + 2NH_4^+ \rightarrow S + N_2 + 4H_2O \quad (15)$$

Liu et al. [53] reported that SRB such as *Desulfuromonas*, *Desulfovibrio,* and *Desulfromicrium* are the microbial communities responsible for nitrogen removal of wastewater [53]. The compositions of these SRB were observed at low percentages (<1.0%) in both systems except *Desulfovibrio* was 6.12% in BES-30.4 (and 0.7% in 3.8-BES, 0.8% in 15.2-BES). Therefore, the genus related to desulfurization also participates in the reaction of NH_4^+-N and sulfate for nitrogen removal.

Limitations, further work and final remarks

BESs have emerged as a remarkable bio-based technology over the past few decades. Especially, while the research area of BESs has been expanded, the performance has been improved significantly. Even though the initial concern was increasing power output using various MFCs configurations, most recent studies have been also focused on wastewater treatment since it offers a sustainable pattern of wastewater treatment. Significant advances have since been made especially in MFC technology thanks to improved design and operational parameters including reactor configurations, electrode materials, electrode surface areas, and types of substrates used as electron donors [82–84]. Among all the parameters, electrode materials are one of the key components of these systems since they dictate the performance of the BESs. Various materials are available for both the anodes and cathodes, but currently it has been reported that 3-D electrodes have advantages over 2-D flat electrodes since 3-D electrodes provide higher surface-area-to-volume ratios [85]. In this context, we designed flow-through a 3-electrode bioelectrochemical reactor to investigate that how domestic wastewater treatment efficiency changes with the electrode size as well as microbial community analysis. The results obtained in this study serve as an initial reference for TN removal rate which is increased proportionally to the surface area of the electrodes in the BES. However, the BES reactor did not critically contribute to COD removal, which is one of the limitations of this study. This is mainly because BESs still suffer from scale-up problems. Our previous work reported that current density linearly scaled up at high COD concentrations, while it decreased with increasing electrode size at lower COD concentrations [38]. Also, another limitation is low CE related to COD removal rate which is

mainly because of overgrowth of non-electrogens (such as methanogens and fermentation bacteria) on anodic biofilms [86]. Additionally, the presence of alternative electron acceptors such as sulfate is also responsible for low CE [87]. To investigate these limitations, further research is needed with higher-strength wastewaters and continuous mode operation. Although this work focused on investigating nitrogen removal with a flow-through electrode in a 3-electrode bioelectrochemical reactor, a more detailed study needs to be carried out including phosphorus removal in future studies since the main objective of wastewater treatment is to remove organic matter and nutrients simultaneously. Besides the limitations and proposed further works, this current study has clearly shown that the developed system could be successfully applied to domestic wastewater with simultaneous removal of carbon and nitrogen. Furthermore, this study provides knowledge on microbial communities at anodes, which provides us an opportunity to understand the nitrogen removal pathways.

Conclusions

In this study, we developed a flow through 3-electrode bioelectrochemical reactor, tested how increased electrode surface area could affect COD and TN removal rates, and studied the mechanism of nitrogen removal. We reached the following conclusions:

- There were no statistical differences between BES and control reactors indicating that the use of BES reactor did not critically contribute to COD removal. Increased surface area improved TN removal in the BES and the control reactors. The results showed that TN removal rate increased proportionally to the surface area of the electrodes in the BES, and reached 78.2 $\pm$ 3.4 TN removal rate with anode projected surface area of 30.4 cm^2 while the control reactors reached only 45.0 $\pm$ 9.2 TN removal rate at the same surface area. On the other hand, concentrations of and NO_3^--N and NO_2^--N in the outlet increased (1.2 $\pm$ 0.2 and 3.2 $\pm$ 0.9 mg/L, respectively). Our results indicated that it is possible to anaerobically remove COD while removing TN.
- Since the pH increase is not highly elevated, ammonia volatilization may not be one of the dominant nitrogen removal mechanisms in BES and control reactors.
- The potential mechanisms for nitrogen removal in flow through bioelectrochemical reactors are the nitrogen removal associated with sulfidogenesis, anodic ammonia oxidation, autotrophic and heterotrophic denitrification, and the reduction of NO_3^--N to NO_2^--N by *Geobacter* species.

CRediT authorship contribution statement

Secil Tutar Oksuz: Visualization, Data curation, Investigation, Writing – original draft, Writing - review & editing, Formal analysis, Validation, Software, Methodology, Conceptualization. **Haluk Beyenal:** Methodology, Conceptualization, Writing - review & editing, Resources, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by a grant from Office of Naval Research award number N000141512471 and US National Science Foundation award CBET #1706889. The authors acknowledge the staff at the Moscow Water Reclamation and Reuse Facility (Moscow, ID) for providing wastewater and mixed culture inoculum. We are grateful for the help of Phuc Ha for the part of microbial community analysis. Secil Tutar Oksuz also acknowledges support from Turkish Ministry of National Education Scholarships.

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