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Effect of Electrostatic Field Strength on Bioelectrochemical Nitrogen Removal from Nitrogen-Rich Wastewater

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Abstract: The effect of electrostatic fields on the bioelectrochemical removal of ammonium and nitrite from nitrogen-rich wastewater was investigated at strengths ranging from 0.2 to 0.67 V/cm in bioelectrochemical anaerobic batch reactors. The electrostatic field enriched the bulk solution with electroactive bacteria, including ammonium oxidizing exoelectrogens (AOE) and denitrifying electrotrophs (DNE). The electroactive bacteria removed ammonium and nitrite simultaneously with alkalinity consumption through biological direct interspecies electron transfer (DIET) in the bulk solution. However, the total nitrogen (ammonium and nitrite) removal rate increased from 106.1 to 166.3 mg N/g volatile suspended solids (VSS).d as the electrostatic field strength increased from 0.2 to 0.67 V/cm. In the cyclic voltammogram, the redox peaks corresponding to the activities of AOE and DNE increased as the strength of the electrostatic field increased. Based on the microbial taxonomic profiling, the dominant genera involved in the bioelectrochemical nitrogen removal were identified as *Pseudomonas*, *Petrimonas*, *DQ677001_g*, *Thiopseudomonas*, *Lentimicrobium*, and *Porphyromonadaceae_uc*. This suggests that the electrostatic field of 0.67 V/cm significantly improves the bioelectrochemical nitrogen removal by enriching the bulk solution with AOE and DNE and promoting the biological DIET between them.

Keywords: bioelectrochemical; nitrogen removal; electrostatic field; direct interspecies electron transfer; electroactive bacteria

1. Introduction

Nitrogen compounds in the water environment can over-stimulate the growth of aquatic life, thus significantly reducing the value of water use [1,2]. Nitrogen in the water environment is primarily associated with the discharge of nitrogen-rich wastewater that is incompletely treated [3]. Treatment of nitrogen-rich wastewater has been a concern in the management of the water environment. Nitrogen in wastewater is usually removed by a conventional biological nitrogen removal (BNR) process consisting of aerobic autotrophic nitrification and anoxic heterotrophic denitrification [1,4]. However, aerobic nitrification requires enriching autotrophic nitrifiers selectively by removing the organic matter first

and supplying a large amount of oxygen and alkalinity [1,5–7]. Heterotrophic denitrification requires an organic carbon source as an electron donor [1,7,8]. Thus, conventional BNR is an expensive process for the treatment of nitrogen-rich wastewater. Partial nitrification and denitrification technologies have also been studied to improve the economics of the conventional BNR process [5–7]. However, several stringent operational conditions including a high temperature, a short retention time, and a low level of dissolved oxygen required for partial nitrification and denitrification have limited their field applications [1,5,6]. Recently, the Anammox process that uses ammonium as an electron donor and nitrite as an electron acceptor under anaerobic conditions has received great attention [2,9,10]. However, Anammox microorganisms grow slowly, and the Anammox process requires strict partial nitrification and produces nitrate as a by-product [4–7].

Interestingly, it has been found that the polarized potential of the electrode in an anaerobic bioelectrochemical reactor can enrich the surfaces of an anode and cathode with electroactive bacteria, including ammonium oxidizing exoelectrogens (AOE) and denitrifying electroautotrophs (DNE) [11–15]. The combined activities of AOE and DNE can simultaneously remove ammonium and nitrite nitrogen under anaerobic conditions, as in the Anammox process, by oxidizing the ammonium on the anode and reducing the nitrite on the cathode, respectively [11,16–18]. This indicates that the polarized anode and cathode can mediate direct interspecies electron transfer (DIET) between AOE and DNE for nitrogen removal. However, nitrogen removal through the electrode-mediated DIET depends on the surface area of the electrode. The use of an electrode with a sufficient surface area is not cost effective in field scale facilities [19,20]. In addition, the electrode-mediated DIET can be limited by activation, ohmic, and polarization overpotentials for electron transfer [21–24]. Interestingly, it has been found that electroactive species can be enriched in the bulk solution exposed to the electrostatic field in bioelectrochemical reactors [18,22,24]. In the bulk solution, exoelectrogens can transfer electrons directly to electroautotrophs that are electrically connected to each other by physical contact called biological DIET [21]. The biological DIET between AOE and DNE could be promoted by the electrostatic field [17,18]. The biological DIET has advantages in thermodynamics and kinetics in electron transfer compared to the electrode-mediated DIET [21,24]. The biological DIET for nitrogen removal could be selectively promoted in the bulk solution by insulating the electrode surface with a dielectric polymer. The bioelectrochemical nitrogen removal via the biological DIET in the bulk solution exposed to electrostatic fields is a novel nitrogen removal mechanism with great potential but is in its infancy.

In the present work, the bioelectrochemical nitrogen removal in the bulk solution was studied at electrostatic fields ranging from 0.2 to 0.67 V/cm. Based on the electrochemical analysis, the activities of electroactive bacteria, depending on the strength of the electrostatic field, were investigated to describe the role of biological DIET in bioelectrochemical nitrogen removal. In the microbial communities, the electroactive bacteria involved in nitrogen removal were identified. Results demonstrated that the biological DIET between electroactive bacteria for bioelectrochemical nitrogen removal depends on the strength of the electrostatic field.

2. Materials and Methods

2.1. Bioelectrochemical Reactor Set Up and Operation

A bioelectrochemical nitrogen removal reactor (BENR, 3 sets, cylindrical type, effective volume of 0.5 L, diameter of 8.5 cm, and height of 10 cm) was prepared using acrylic resin as described in a previous study [22] (Figure 1). In brief, the top of the reactor was flanged with a cover plate for sealing. A DC (direct current) motor was mounted on the cover plate and a mixing blade connected to a metal shaft, placed inside the reactor. Moreover, on the cover plate, a gas sampling port, a liquid sampling port, and an off-gas valve were placed. The gas and the liquid sampling ports were covered with n-butyl rubber stoppers. Acrylic sealing tubes were attached to the holes in the bottom of the cover plate for the liquid sampling port and metal shaft hole, that extended into the liquid phase. Two copper

foils (0.3 T, copper 99.9%, KDI Co.) of large (26 cm × 9 cm) and small size (5.5 cm × 7 cm) were prepared and surfaces of the foils were coated with a dielectric polymer (Alkydenamel, VOC 470 g/L, Noroo Paint & Coatings co., Ltd., Anyang, Gyeonggi, Busan, Korea) to obtain surface-insulated electrodes. Electrodes were bent in annular shapes and placed on the inner wall of the BENR reactor and the outer wall of the metal shaft, respectively. The spatial distance between the two electrodes was about 3 cm. These electrodes were bounded to terminals of the external DC voltage source (ODA Technologies, Co., Incheon, Korea) with titanium wires.

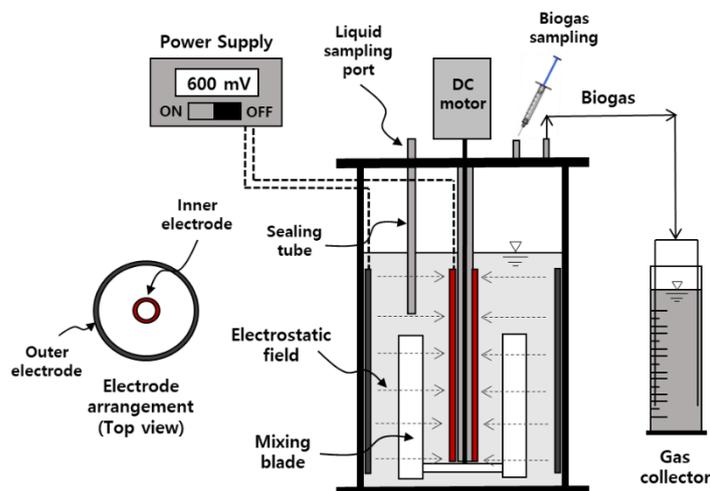


Figure 1. Schematic of the bioelectrochemical nitrogen removal reactor exposed to an electrostatic field.

Artificial nitrogen-rich wastewater containing 0.3 g/L KH_2PO_4 , 1.0 g/L $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.5 g/L NaCl, 2.0 g/L NaHCO_3 , 0.1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L CaCl_2 , 1.91 g/L NH_4Cl , and 1.4 g/L NaNO_2 was prepared as described in previous studies [17,24]. The initial concentration of $\text{NH}_4\text{-N}$ was 500 mg/L and $\text{NO}_2\text{-N}$ was 300 mg/L. The activated sludge was obtained from a sewage treatment plant (Busan, Korea), and was screened and precipitated for a day to obtain a concentrated inoculum. The initial VSS (volatile suspended solids) and pH of the inoculum were noted as 14,000 mg/L and 7.89, respectively. For the batch experiments, the nitrogen-rich wastewater (250 mL) and inoculum (250 mL) were added into three bioelectrochemical reactors, and the insulated electrodes were polarized by voltage from 0.6 V to 2.0 V to form different electrostatic fields (0.20 V/cm, 0.33 V/cm, and 0.67 V/cm) in the bulk solution. The prepared anaerobic reactors were referred to as BENR20, BENR33, and BENR67, respectively, depending on the strength of the electrostatic field. BENRs were operated at room temperature in sequential batch mode by replacing the wastewater when the $\text{NH}_4\text{-N}$ was depleted in the reactor by settling suspended sludge for 30 min and the fresh artificial wastewater was added later. During the experiment, the $\text{NO}_2\text{-N}$ concentration was intermittently monitored and supplemented when decreased to a small quantity.

2.2. Analytical Techniques and Calculation

During the experiment, pH was monitored day-to-day by a pH meter (YSI pH1200 laboratory pH meter 115–230V (T1)). Physicochemical properties, including alkalinity, $\text{NH}_4\text{-N}$, $\text{NO}_2\text{-N}$, and VSS, were also estimated in triplicates every day according to the Standard Methods [25]. Biogas production was observed using a floating type gas collector, and biogas composition was examined using gas chromatography (Gow-Mac Instrument Co., Bethlehem, PA, USA) with a Porapak-Q column (6 ft × 1/8", SS) and thermal conductivity detector. Based on the measurements of biogas volume, composition and individual biogas production, including nitrogen, methane, and carbon dioxide, were estimated. Biogas production was quantified at standard temperature and pressure (STP), as described in the previous study [17]. In electron balance, the total number of electrons released from the electron donor was estimated by the number of moles of $\text{NH}_4\text{-N}$ removal. Hence, during the last batch cycle,

the electrons reduced to nitrogen and methane were calculated based on their yields as gaseous forms, respectively. Cyclic voltammograms (CVs) for the bulk solution of the BENRs were obtained at the end of the experiment in a voltage range from -1.0 V to 1.0 V (vs. Ag/AgCl) with a scan rate of 10 mV s^{-1} using a potentiostat (ZIVE SP1, WonA Tech, Seoul, Korea). Stainless mesh ($1 \text{ cm} \times 1 \text{ cm}$) was used as working and counter electrodes to identify the activities of electroactive bacteria. Redox peak potentials and heights were obtained using software ("SMART Manager" ZIVE BP2 Series, WonATech Co., Seoul, Korea). The electrochemical impedance spectrum (EIS) was also obtained using the potentiostat (ZIVE SP1, WonA Tech, Seoul, Korea), as described in the previous study [26]. The ohmic resistance was estimated from the left intersection with the real axis in the Nyquist plot, and the charge transfer resistance was attained from the distance between two intersections of the semicircle with the real axis.

2.3. Bacterial Community Analysis

Taxonomic profiling of microbial communities was performed by investigating 16S rRNA in the BENRs at the end of the experiment. Using an MO BIO Power soil DNA kit, the DNA was extracted from the suspended sludge in the bulk solution according to the kit protocol. For taxonomic profiling, the V1 to V3 region of the bacterial 16S rRNA gene was targeted and therefore the amplification, construction of the sequencing library, and bioinformatics analysis were performed as described in a previous study [27] and completed by Chunlab Inc., (Seoul, Korea). Based on 16S rRNA sequence data, the sequence reads were identified using an extended EzTaxon-e database (<http://eztaxon-e.ezbiocloud.net/>), as described in the previous study [22].

3. Results and Discussion

3.1. Bioelectrochemical Removal of Ammonium and Nitrite

Ammonium and nitrite began to decrease from BENRs as soon as the bulk solution was exposed to electrostatic fields during the first batch cycle (Figure 2). It seems that the biological DIET involved in the ammonium oxidation and nitrite reduction to form nitrogen gas was quickly activated in BENRs by electrostatic fields. However, when the nitrite was reduced to a low level, the ammonium removal abruptly stopped, and when the nitrite was supplemented to a high level, it resumed. This indicates that the removal of ammonium and nitrite under the electrostatic field is intensively interdependent. Anammox is a well-known process for the simultaneous removal of ammonium and nitrite in anaerobic conditions [2,9,10]. However, Anammox bacteria are not usually abundant in the inoculum that was the activated sludge collected from a sewage treatment plant. It seems that electrostatic fields in BENRs contribute to enriching the bulk solution with electroactive bacteria, including AOE and DNE, and converting ammonium and nitrite into nitrogen gas via the biological DIET [17,18,22].

In BENRs, the removal of ammonium and nitrite was significantly dependent on the strength of the electrostatic field, as well as the nitrite concentration. The time for the first batch cycle required for the removal of ammonium in BENR20 was about 18 days, reduced to 16 and 14 days for BENR33 and BENR67, respectively. The cycle time in BENRs was gradually improved with the operating time of BENRs and then stabilized from the fourth cycle. The operating time required for stabilization of the removal of ammonium and nitrite took around 40 days for BENR20, which was reduced to 38 and 34 days for BENR33 and BENR67, respectively. The stabilized cycle time of BENR20 was 9 days, BENR33 was 8 days, and BENR67 was 6 days. After the stabilization, the specific ammonium removal rate was $59.6 \text{ mg NH}_4\text{-N/g VSS.d}$ in BENR20. However, it increased to $64.0 \text{ NH}_4\text{-N/g VSS.d}$ and $81.5 \text{ NH}_4\text{-N/g VSS.d}$ in BENR33 and BENR67, respectively (Table 1). It means that the biological DIET for the removal of ammonium and nitrite is promoted as the strength of the electrostatic field increases. In the Anammox process, the specific removal rate of $\text{NH}_4\text{-N}$ was varied, and ranged from 43.1 to 193 mg/L g VSS/d [17,28,29]. It is believed that electrostatic field-promoted bioelectrochemical nitrogen removal might be able to compete with the Anammox process for nitrogen removal, although it requires further studies for optimization. The specific total nitrogen removal rate was 107.3 mg N/g

VSS.d in BENR20, which was increased to 137.6 mg N/g VSS.d and 166.3 mg N/g VSS.d in BENR33 and BENR67, respectively (Table 1).

However, the principle that the electrostatic field promotes the biological DIET for the removal of ammonium and nitrite is still veiled. One of the hypotheses based on quantum physics can be proposed to thermodynamically describe the biological DIET for the removal of ammonium and nitrite, which depends on electrostatic fields. In general, the bioelectrochemical redox reaction requires overpotentials, including activation, ohmic, and polarization overpotentials, to transfer electrons [22,24]. Among the overpotentials, the activation energy is directly dependent on the potential energy barrier of the molecule in quantum physics.

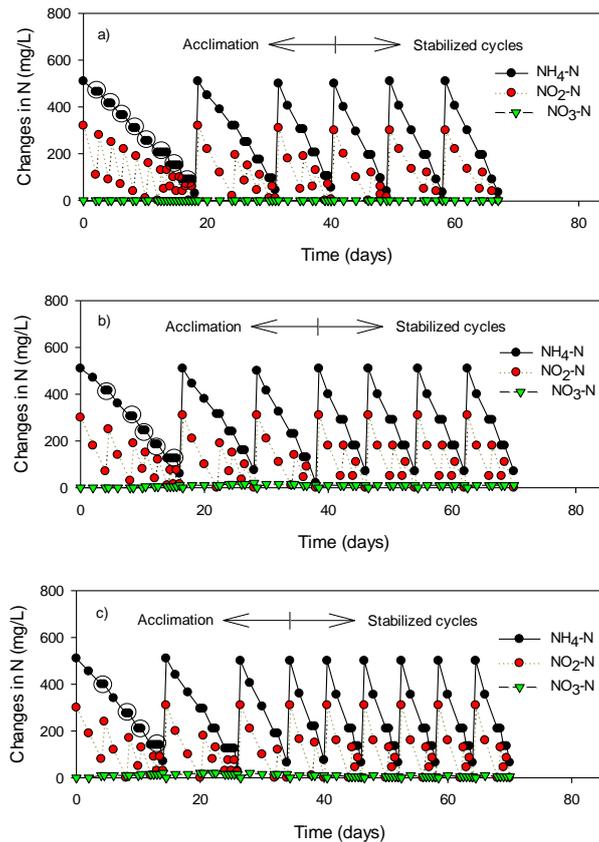


Figure 2. Changes in ammonium and nitrite concentrations depending on the electrostatic field strength in bioelectrochemical nitrogen removal reactors: (a) BENR20, (b) BENR33, and (c) BENR67.

Table 1. Performance of bioelectrochemical nitrogen removal depending on the electrostatic field.

| Contents | | BENR20 | BENR33 | BENR67 |
|---|-----------------------------|---------------|---------------|---------------|
| Electrostatic field (V/cm) | | 0.20 | 0.33 | 0.67 |
| Specific NH ₄ -N removal rate (mg/g VSS.d) | | 59.6 | 64.0 | 81.5 |
| NO ₂ -N/NH ₄ -N (mg/mg) | | 0.80 ± 0.12 | 1.15 ± 0.13 | 1.04 ± 0.03 |
| Alkalinity/NH ₄ -N (mg/L as CaCO ₃ /mg) | | 1.42 ± 0.13 | 1.11 ± 0.06 | 1.41 ± 0.06 |
| VSS (mg/L) | | 920 ± 0.00 | 860 ± 9.57 | 890 ± 9.65 |
| Biogas (N ₂ /CH ₄) (mL) | | 561.0/14.7 | 602.2/15.1 | 651.0/5.1 |
| Specific total N removal rate (mg/g VSS.d) | | 107.28 ± 3.58 | 137.60 ± 3.20 | 166.26 ± 3.26 |
| Electron balance | N ₂ (%) | 73.8 | 85.5 | 93.5 |
| | CH ₄ (%) | 5.2 | 5.7 | 5.9 |
| | Others (biomass, losses, %) | 21.0 | 8.8 | 0.6 |

However, external electric fields increase the energy levels of molecules with the motions of rotation, vibration, and translation [30,31]. The energy level increased in the molecule increases the probability of electrons jumping over the potential energy barrier or passing it by the tunneling effect [32], which indicates that the electric field enhances electron transfer involved in the redox reaction of molecules. It suggests that the electrostatic field enriches electroactive bacteria, including AOE and DNE, in the bulk solution and promotes the biological DIET between them by reducing the activation energy [33–36].

3.2. Nitrite and Alkalinity Requirements

In BENRs, the amounts of nitrite and alkalinity required for ammonium oxidation were gradually stabilized with the operating time (Figure 3). In BENR20, the nitrite required for ammonium oxidation ($\text{mg NO}_2\text{-N/mg NH}_4\text{-N}$) was approximately 0.80, and slightly increased to 1.15 and 1.04 in BENR33 and BENR67, respectively (Table 1).

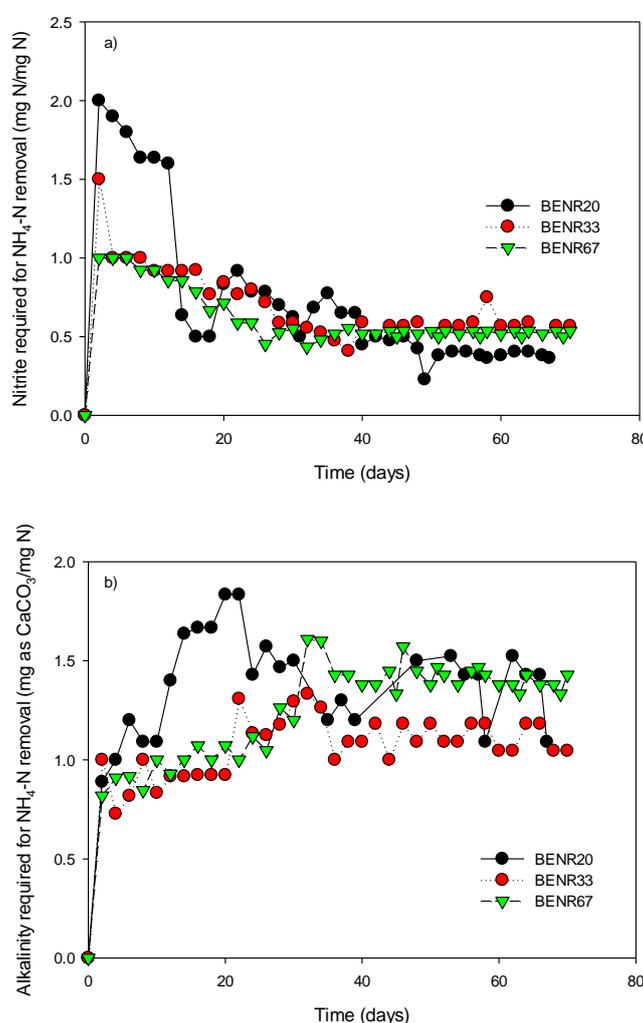


Figure 3. Consumptions of nitrite and alkalinity for ammonium removal in bioelectrochemical nitrogen removal reactors: (a) nitrite and (b) alkalinity.

However, the nitrite consumptions used for ammonium oxidation in BENRs were always less than the Anammox process of 1.32 [4,5]. In BENRs, biogas production was observed from the ammonium and nitrite removal under the electrostatic field. The main component of the biogas was nitrogen gas, and methane and carbon dioxide were minor components (Figure 4a). This indicates that the electrons released from ammonium oxidation are mainly used to form nitrogen by reducing nitrite.

However, some electrons were also used in the side reactions to form other products like methane or to synthesis biomass. Based on electron balance, the percentage of electrons converted into nitrogen gas was 73.8% in BENR20, which increased to 85.5% and 93.5% in BENR33 and BENR67, respectively (Table 1). The electron equivalent of nitrite required to form nitrogen gas is equal to the electron equivalent of oxidized ammonium. It seems that the percentage of the electrons used to reduce nitrite increases as the strength of the electrostatic field increases, while the percentage used for the side reactions decreases.

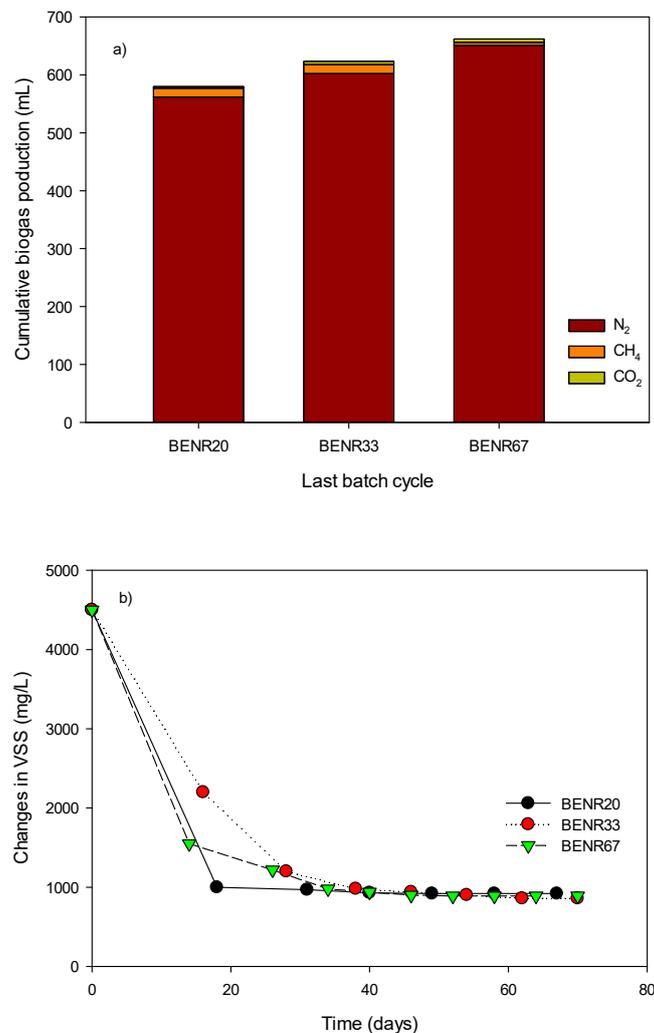


Figure 4. Biogas production and volatile suspended solids in bioelectrochemical nitrogen removal reactors (a) Cumulative biogas production during the last batch cycle, and (b) changes in volatile suspended solids (VSS) over time.

The initial VSS in BENRs was as high as 4500 mg/L, but it decreased exponentially with the operating time (Figure 4b). BENRs were anaerobic reactors exposed to electrostatic fields, and the only carbon source for microbial growth in the bulk solution was bicarbonate, the main alkalinity component at a neutral pH range. It seems that aerobic heterotrophs initially inoculated might have been decayed over time due to the depletion of the oxygen and organic carbon source, while anaerobic autotrophs, including AOE and DNE, were selectively enriched [17,18,22]. The steady value of VSS in BENR20 was 920 mg/L, higher than 860 mg/L in BENR33 or 890 mg/L in BENR67. In BENRs, the alkalinity required for the ammonium oxidation (mg as CaCO₃/mg NH₄-N) ranged from 1.11 to 1.42, which was significantly higher than 0.24–0.47 in the Anammox process [4,5]. The dependence of alkalinity requirement on the strength of the electrostatic field was not clearly explained, but it was in

agreement with the order of the final VSS concentration. It seems that the alkalinity is mainly used for the carbon source for microbial growth, although the bicarbonate can be partially converted to carbon dioxide. This suggests that the high alkalinity requirement for the ammonium oxidation under the electrostatic field would be beneficial for the stable operation of BENRs for nitrogen removal.

3.3. Electrochemical Properties in the Bulk Solution

The redox peaks in the CV provide the information on the electroactive bacteria, including AOE and DNE, in bioelectrochemical reactors [17,18]. In BENRs, one oxidation peak and two reduction peaks at the non-turnover condition were observed from the voltammogram in BENRs (Figure 5a). The peak potentials and heights were affected by the strength of the electrostatic field exposed to the bulk solution.

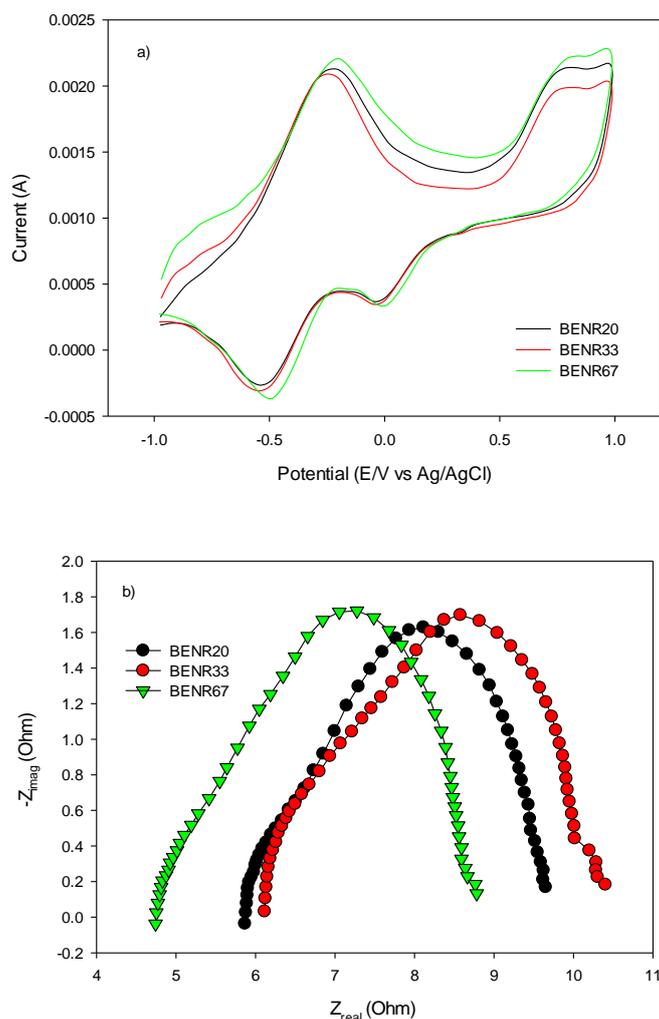


Figure 5. Electrochemical characteristics of the bulk solution depending on the electrostatic field: (a) Cyclic voltammogram and (b) Nyquist plot of the electrochemical impedance spectrum (EIS).

In BENR67, the oxidation peak was 0.80 mA at -0.20 V vs. Ag/AgCl, and the reduction peaks were 0.51 mA at -0.01 V vs. Ag/AgCl and 0.90 mA at -0.49 V vs. Ag/AgCl (Table 2). In BENR20 and BENR33, the redox peak potentials shifted slightly in the negative direction, compared to BENR67. However, the redox peak heights tended to decrease as the strength of the electrostatic field decreased. The redox peak height is in good agreement with the removals of ammonium and nitrite, which depend on the strength of the electrostatic field (Figure 2). For bioelectrochemical reactors, the substances contributing to the redox peak in the voltammogram can be abiotic redox compounds and electroactive

bacterial species such as AOE and DNE [37,38]. There are several known abiotic redox compounds that are endogenously secreted from microorganisms or supplied from outside of the bioreactor, including flavin, quinone, sulfur-based compounds, phenazines, and humic substances [38–40]. However, the raw wastewater for BENRs did not have any redox compounds. In addition, the nitrogen removal in BENRs was not significantly affected by replacing the medium with the raw wastewater for a new batch cycle (Figure 2). This indicates that electroactive bacteria rather than redox compounds contributed mainly to the redox peak in the voltammogram. This suggests that electroactive bacteria were enriched in the bulk solution by the electrostatic field and contributed to the bioelectrochemical nitrogen removal.

Table 2. Electrochemical characteristics of the bulk solution depending on the electrostatic field strength in the bioelectrochemical nitrogen removal reactor.

| BENR | Electrostatic Field (V/cm) | Redox | E_p (V) | I_p (mA) | R_s/R_{ct} (Ω/Ω) |
|--------|----------------------------|-----------|-----------|------------|----------------------------------|
| BENR20 | 0.20 | Oxidation | −0.22 | 0.58 | 5.86/3.88 |
| | | Reduction | −0.04 | 0.45 | |
| BENR33 | 0.33 | Oxidation | −0.54 | 0.72 | 6.12/4.35 |
| | | Reduction | −0.24 | 0.81 | |
| BENR67 | 0.67 | Oxidation | −0.04 | 0.47 | 4.72/4.09 |
| | | Reduction | −0.55 | 0.75 | |
| | | | −0.20 | 0.80 | |
| | | | −0.01 | 0.51 | |
| | | | −0.49 | 0.90 | |

The electrochemical impedance spectra for BENRs obtained from at the end of the experiment were semi-circular in Nyquist plots (Figure 5b). The solution ohmic resistance in BENR67 was 4.72 Ω , which was lower than the others, and the charge transfer resistance was lower in BENR20 at 3.88 Ω (Table 2). In either case, the ohmic and charge transfer resistances were higher in BENR33 than BENR20 and BENR67. The solution and charge transfer resistances were somewhat matched with total biomass estimated from the VSS concentration, but not perfect (Table 1). The conductive protein of electroactive bacteria increases the electrical conductivity in the bulk solution [41,42]. However, the abundant species of electroactive bacteria in the bulk solution may depend on the strength of the electrostatic field. It seems that the activation energy for the electron transfer of the electroactive bacteria is different depending on the bacterial species.

3.4. Microbial Community Analysis

Based on the taxonomic profiling, the dominant microbial groups in the bulk solution were similar for all BENRs (Figure 6). However, their abundances were varied, depending on the strength of the electrostatic field. At the phylum level, three dominant groups in BENRs were Proteobacteria, Bacteroidetes, and Firmicutes. In BENR67, the first dominant phylum was Proteobacteria (44.4%), while Bacteroidetes were the most dominant phylum in BENR33 (42.6%) and BENR20 (38.8%) (Figure 6a). Firmicutes ranged from 10.4% to 12.0%, similar in all BENRs. In a previous study, the first dominant phylum in a BENR exposed to an electrostatic field of 0.2 V/cm was Bacteroidetes [17,22]. It seems that bacterial abundance in the bioelectrochemical reactor for nitrogen removal depends on the strength of the electrostatic field.

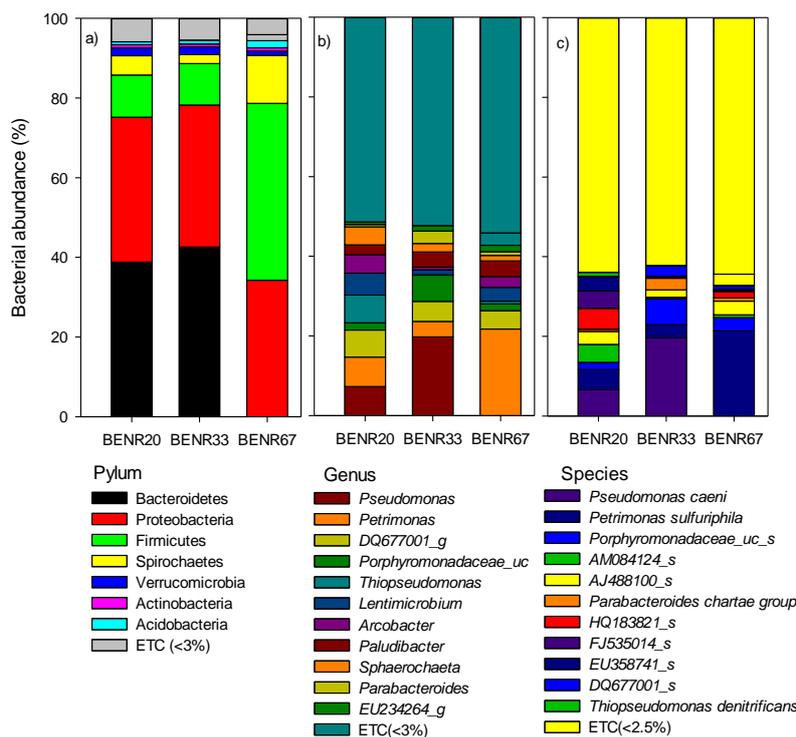


Figure 6. Relative abundance of bacterial communities depending on the electrostatic field strength: (a) phylum, (b) genus, and (c) species.

At the genus level, bacterial abundance differed more clearly depending on the strength of the electrostatic field (Figure 6b). In BENR67, the most dominant genus was *Pseudomonas* (21.7%), followed by *Petrimonas* (4.6%), *Arcobacter* (3.9%), and *Thiopseudomonas* (3.4%). In BENR33, the abundance of *Pseudomonas* was slightly less, at 19.7%, than BENR67, but uncultured genera *Porphyromonadaceae_uc* (6.6%) and *DQ677001_g* (5.0%) were more abundant than in BENR67. In BENR20, the abundance of *Pseudomonas* was significantly lower at 7.2% than that of BENR33 or BENR67, while *Petrimonas* (7.4%), *Thiopseudomonas* (6.9%), *DQ677001_g* (6.8%), and *Lentimicrobium* (5.5%) were abundant in BENR20. It seems that the electroactive species belong to the genera *Pseudomonas*, *DQ677001_g*, *Petrimonas*, and *Thiopseudomonas*, which are directly involved in bioelectrochemical ammonium oxidation or nitrite reduction. These genera were also abundant in a bioelectrochemical reactor for nitrogen removal in previous studies [17,22]. However, the genera *Nitrosomonas* and *Nitrobacter* known to oxidize ammonium and nitrite and genera such as *Brocadia*, *Kuenenia*, *Scalindua*, *Jettenia*, and *Anammoxoglobus*, known as Anammox bacteria, were not observed in any BENRs. This suggests that the mechanism of electrostatic field-driven nitrogen removal in the bioelectrochemical reactor is distinctly different from the Anammox process.

At the species level, *Pseudomonas caeni* was the most abundant species in BENR67 (21%) and BENR33 (19.6%), but its abundance in BENR20 was significantly lower at 6.6% (Figure 6c). It seems that the metabolic activity of *P. caeni* is largely dependent on the strength of the electrostatic field. *P. caeni* is known as a denitrifying species that can reduce nitrite and nitrate [43]. Recently, *P. caeni* has also been identified not only in a bioelectrochemical reactor with a polarized electrode for nitrogen removal, but also in microbial fuel cells [22,23]. *Arcobacter AM084124_s* was abundant at 3.4% in BENR67 and 4.5% in BENR20. *A. AM084124_s* is denitrifying bacteria belonging to Epsilon proteobacteria. It has been shown that the genus *Arcobacter* can contribute to denitrification in the activated sludge system [44]. It is likely that *P. caeni* and *A. AM084124_s* are the denitrifying electro-trophs (DNE).

Petrimonas sulfuriphila was also an abundant species in BENR67 (3.4%), as well as BENR33 (3.3%) and BENR20 (5.1%), which is an anaerobic fermenter that uses the element sulphur and nitrate as electron acceptors [45]. This species was also observed in a bioelectrochemical reactor with a polarized

electrode for nitrogen removal in a previous study [22]. The genus *Petrimonas* are known as fermenters of carbohydrates and have been observed in microbial fuel cells [45,46]. *Thiopseudomonas denitrificans* was another abundant species (2.8%) in BENR67, which is known as a sulphide oxidizer anaerobically with nitrate as an electron acceptor [47]. The abundance of *T. denitrificans* was less than 1.0% in BENR33 or BENR20, indicating that its activity depends on the strength of the electrostatic field. It is likely that *Petrimonas sp.* and *Thiopseudomonas sp.* are the AOE species that can bioelectrochemically oxidize ammonium. The uncultured species *Porphyromonadaceae_uc_s* and AJ488100_s were abundant in BENR33, and HQ183821_s and FJ535014_s in BENR20. It is likely that these species were also involved in bioelectrochemical nitrogen removal, but their functions have not been identified yet.

4. Conclusions

The electrostatic field enriched the bulk solution with electroactive bacteria, including AOE and DNE, and simultaneously removed ammonium and nitrite through the biological DIET. The biological DIET for ammonium and nitrite removal was enhanced as the strength of the electrostatic field increased in the range of 0.2 V/cm to 0.67 V/cm. In the bioelectrochemical nitrogen removal, nitrite and alkalinity were used as an electron acceptor and a carbon source for microbial growth, respectively. Under the electrostatic field, the electroactive bacterial species involved in the bioelectrochemical nitrogen removal belong to the genera *Pseudomonas*, *Petrimonas*, *DQ677001_g*, *Thiopseudomonas*, *Lentimicrobium*, and *Porphyromonadaceae_uc*, and their relative abundances depended on the strength of the electrostatic field. The electrostatic field-promoting biological DIET is a novel anaerobic nitrogen removal approach that is competitive to Anammox for the treatment of nitrogen-rich wastewater.

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Abbreviations

| | |
|------|--|
| AOE | Ammonium oxidizing exoelectrogens |
| DNE | Denitrifying electrotrophs |
| BENR | Bioelectrochemical nitrogen removal reactors |
| BNR | Biological nitrogen removal |
| CV | Cyclic voltammogram |
| DIET | Direct interspecies electron transfer |
| EIS | Electrochemical impedance spectroscopy |
| VSS | Volatile suspended solids |

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