工學博士 學位論文

Bioelectrochemical nitrogen removal for nitrogen rich wastewater

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2019年 8月

韓國海洋大學校 大學院

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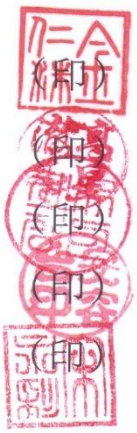
本 論文을 Anna Joicy의 工學博士 學位論文으로
認准함.



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Bioelectrochemical nitrogen removal for nitrogen rich wastewater

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Abstract

하폐수에 함유된 질소는 호기성 질산화와 무산소 탈질로 이루어진 생물학적 질소제거 공정을 이용하여 처리하여 왔다. 그러나, 질산화를 위해서는 하폐수에 함유된 유기오염물을 우선적으로 제거하고 많은 양의 산소와 알카리도를 공급하여 독립영양 질산화균을 우점성장시켜야 하며, 탈질을 위해서는 전자공여체인 외부탄소원이 필요하다. 따라서, 질소제거에 필요한 산소와 알카리도 및 외부탄소원 요구량을 감소시키기 위하여 암모니아성 질소를 아질산성 질소로 산화시켜 탈질시키는 단축질소제거공정이 연구되어 왔다. 최근에는 혐기성상태에서 암모니아성 질소를 전자공여체로 그리고 아질산성 질소를 전자수용체로 사용하여 질소를 제거하는 아나목스공정이 고농도질소의 경제적인 처리기술로 크게 관심을 받고 있다. 그러나, 단축질산화공정이나 아나목스공정은 선택적

아질산화를 포함한 여러 가지 한계들로 인하여 폭넓은 현장적용에 어려움을 겪고 있다. 최근 생물전기화학반응조에서 암모니아성 질소는 산화전극표면의 체외전자방출균 (ammonium oxidizing exoelectrogens, AOE)에 의해서 산화될 수 있으며, 환원전극에서는 전자영양탈질균 (denitrifying electrotrophs, DNE)에 의해 질산이온이나 아질산이온이 질소가스로 환원될 수 있음이 밝혀졌다. 따라서, 생물전기화학반응조에서 AOE와 DNE를 이용한 질소제거는 고농도 질소 폐수의 경제적인 처리를 가능하게 할 수 있는 큰 잠재력을 가지고 있다. 따라서, 본 논문에서는 생물전기화학반응조에서 암모니아성 질소와 아질산성 질소를 각각 전자공여체 및 전자수용체로 사용하는 아나목스반응과 유사한 반응기작에 의해 질소를 제거하기 위한 연구를 수행하였다.

생물전기화학반응조에 혐기성소화 슬러지를 식종하고 분극 전극쌍을 설치하여 외부 전압원으로 0.6V를 인가하였다. 이때 생물전기화학반응조에서는 아질산성 질소 및 알칼리도의 소모를 동반하는 암모니아성 질소제거 현상이 관측되었다. 이것은 혐기성슬러지로부터 우점 배양된 질소제거에 관여하는 AOE 및 DNE가 독립영양균이며, 암모니아성 질소가 전자공여체로 사용되며 아질산성질소가 전자수용체로 사용되었음을 의미한다. 이때 암모니아성 질소 제거에 필요한 아질산성 질소 및 알칼리도는 각각 0.58 mg NO_2-N/mg NH_4-N과 2.0 mg as $CaCO_3$/mg NH_4-N이었다. 또한, 생물전기화학반응조에서 암모니아성 질소의 산화과정은 아나목스반응과는 달리 부산물로서 질산성 질소를 생성하지 않았다.

활성 슬러지를 접종한 뒤 전압을 0.6V로 인가하여 생물전기화학반응조에서 발견된 질소제거에 식종균의 영향을 조사하였다. 폐수는 암모니아성 질소, 아질산성 질소, 알칼리도 및 미량 미네랄이 함유되도록 준비하였으며, 생물전기화학반응조를 연속 회분식으

로 수회 반복운전하였다. 이때 순환전압전류곡선의 산화환원피크로부터 벌크 용액 내에 존재하는 AOE와 DNE의 존재를 확인하였다. 생물전기화학 질소제거 반응조에서 발생한 바이오 가스는 대부분이 질소 가스였으며, 메탄과 이산화탄소도 미량 관측되었다. 암모니아성 질소산화를 위해 필요한 아질산성 질소는 약 0.72 mg NO_2-N/mg NH_4-N로서 혐기성식종슬러지를 사ㅣ용한 경우보다 약간 높았으나 와 알칼리도 요구량은 1.73 mg $CaCO_3$/mg NH_4-N으로서 약간 낮았다. 또한, 혐기성 조건에서 암모니아성 질소 및 아질산성 질소의 제거는 생물전기화학반응조 내의 벌크 용액에서 AOE와 DNE 간의 직접 종간 전자 전달 (direct interspecies electron transfer, DIET)에 의해 이루짐을 확인하였다.

생물전기화학반응조에서 정전기장을 이용하여 전기활성균을 우점성장 시키고 암모니아성 질소 및 아질산성 질소의 제거를 촉진시키기 위한 연구를 수행하였다. 암모니아성 질소의 제거율은 정전기장이 0.2 V/cm이었을 때 약 52.5 mg N/g VSS.d이었으나, 0.67 V/cm에서는 78.7mg N/g VSS.d 로 증가하였다. 생물전기화학반응조의 주요 미생물종은 전기활성 미생물로 알려진 *Pseudomonas*와 *Petrimonas* 및 *Thiopseudomonas* 이었다. 벌크용액에 대한 순환전압전류곡선에서 AOE 및 DNE의 피크가 확인되었으며, 전기화학임피던스분광법으로 조사한 전자 전달을 위한 과전위는 정전기장의 세기가 증가함에 따라 감소하였다. 생물전기화학적 질소 제거 반응에 있어서 벌크 용액을 정전기장에 노출시키는 것은 질소 제거에 효과적이었으며, 정전기장의 세기를 증가하였을 때 질소 제거 효율도 증가하였다.

분극전극을 설치한 상향류식 생물전기화학반응조를 이용하여 고농도의 암모니아성 질소 폐수의 부분질산화를 위한 연구를 수행하였다. 상향류식 반응조의 벌크용액에 노

출된 정전기장은 0.24, 0.40, 0.80 및 1.96 V/cm이었으며, HRT는 1 일에서 4 일까지 변화시켰다. 유입폐수의 암모니아성 질소농도는 500 mg/L의 NH_4-N이었다. 정전기장 0.4 V/cm에서 아질산성 질소는 HRT가 1 일에서 4 일로 증가할 때 100 mg NO_2-N/L에서 270 mg NO_2-N/L까지 증가하였다. 그러나, 0.24 V/cm의 정전기장에서는 0.4 V/cm에서보다 아질산성 질소의 축적이 적었다. 질산성 질소는 0.4 V/cm의 정전기장에서 HRT가 1일에서 4일까지 증가하였을 때 약 35 mg NO_3-N/L에서 50 mg NO_3-N/L로 증가하였다. 이는 생물전기화학 질산화가 HRT 뿐만 아니라 정전기장의 강도에 따라 달라진다는 것을 의미한다. 0.80 V/cm 및 1.96 V/cm의 높은 정전기장에서 아질산성 질소는 약 320 mg NO_2-N/L까지 증가하였으며, 질산성 질소는 85 mg NO_3-N/L로 증가하였다. 그러나, 1.96 V/cm에서는 아질산성 질소의 농도는 155 mg NO_2-N/L로 감소한 반면, 질산성 질소는 135 mg NO_3-N/L로 크게 증가하여 정전기장 0.8 V/cm 이하에서 암모늄 산화 효율이 더 높은 것으로 평가되었다.

생물전기화학반응조를 이용한 질소제거는 혐기성상태에서 암모니아성 질소를 전자공여체로 그리고 아질산성 질소 및 질산성 질소를 전자수용체로 이용하여 질소를 제거하는 새로운 기술이다. 향후 반응에 영향을 미치는 다양한 영향인자에 대한 연구가 수행되고 최적화된다면 아나목스를 대체할 수 있는 새로운 경제적인 기술로 발전할 수 있을 것으로 평가된다.

키워드(keyword): 생물전기화학(bioelectrochmical), 아질산화(nitritation), 고농도 암모니아 폐수(ammonium rich wastewater), 아질산성 질소(nitrite), 질산성 질소(nitrate), 질소 제거(nitrogen removal), 정전기장(electrostatic field), 직접 종간 전

자 전달(direct interspecies electron transfer), 전기활성 미생물(electroactive bacteria), 생물전기화학 혐기성 암모늄 산화 (bioelectrochemical anaerobic ammonium oxidation)

Chapter 1: Introduction

1.1 Background

Overabundance of nitrogen in the natural water system from industrial and domestic discharge causes serious water environmental problems such as eutrophication in lakes, river, estuaries and surface water reservoirs where waste water is discharged (Angenent et al., 2004; Ghafari et al., 2008) and also over-stimulate the growth of aquatic life, significantly reducing the value of water use (Ahn 2006; Du et al., 2015). The nitrogen in wastewater has been removed by physiochemical and biological methods. Several physiochemical methods such as chemical precipitation (Chen et al., 2013), ion exchange (Sica et al., 2014; Alshameri et al., 2014), adsorption (Zheng et al., 2008; Babou Kammoe et al., 2014), nanofiltration, reverse osmosis (Košutić et al., 2014) and forward osmosis (Zhang et al., 2014) for nitrogen rich wastewater.

In conventional biological nitrogen removal (BNR) process the nitrogen rich wastewater treatment consisting of autotrophic nitrification and heterotrophic denitrification. However, the nitrification of ammonia N requires a large amount of oxygen and alkalinity, and the organic matter in the wastewater is removed so that the autotrophic nitrifying bacteria grows dominantly (Ahn 2006; Ge at al., 2015; Ma et al., 2016; Nancharaiah et al., 2016). The carbon source as electron donor is needed for the heterotrophic denitrification (Ahn 2006; Nancharaiah et al., 2016; Komorowska-Kaufman et al., 2006). In addition, the nitrifying bacteria grow slowly, and sensitive to environmental conditions. In particular, the nitrification is a step to limit kinetically the nitrogen removal when the wastewater temperature is less than 15℃ in winter (Komorowska-Kaufman et al., 2006; Shalini and Joseph 2012). The short-cut nitrogen removal technologies such as Sharon process have introduced to mitigate the excess burden of

oxygen, alkalinity and carbon source in the conventional BNR process (Ahn 2006; Shalini and Joseph 2012; Ge at al., 2015; Nancharaiah et al., 2016). The shortcut nitrogen removal is a process oxidizing ammonium into nitrite and then reducing the nitrite into nitrogen gas. The oxygen and carbon source needed to remove the nitrogen from wastewater could be significantly reduced by the shortcut nitrogen removal process compared to the conventional BNR (Shalini and Joseph 2012; Ge at al., 2015; Nancharaiah et al., 2016). The nitrite required for the shortcut nitrogen removal is obtained by the nitritation of ammonia. Ammonia is generally oxidized into nitrite by ammonia oxidation bacteria (AOB) such as *Nitrosomonas* and the nitrite oxidized into nitrate by the nitrite oxidizing bacteria (NOB) such as *Nitrobacter*. Increasing temperature facilitate AOB to out compete NOB, and the optimum temperature for AOB and NOB are 35°C and 38°C, respectively (Ahn 2006; Ge at al., 2015; Shalini and Joseph 2012). Recently, Anammox process that removes nitrogen under anaerobic condition has been attracted much attention as a sustainable and energy neutral technology (Jin et al., 2012; Du et al., 2015; Ma et al., 2016). Anammox bacteria use nitrite as an electron acceptor to oxidize ammonia. However, the challenges of Anammox process are to resolve the limitations involved in the nitrite requirement, slow growth rate of Anammox bacteria and nitrate production from the Anammox reaction (Shalini and Joseph 2012; Ma et al., 2016). The nitrite for the Anammox reaction is generally obtained by oxidizing about half of the ammonium contained in the wastewater, but sometimes, the nitrate is partially denitrified into nitrite (Du et al., 2015; Ma et al., 2016).

In recent, bioelectrochemical technology that activates direct electron transfer between bacterial species with the help of electrical energy has been attracted much attention (Feng et al., 2016; Blasco-Gómez et al., 2017). When the electron acceptor is outside the cells under anaerobic condition, some bacteria, called exoelectrogens, can transfer electrons to the external electron acceptor through c-type cytochrome that is over-expressed up to the outer membrane

(Kiely et al., 2011; Lovely 2011; Feng et al., 2017). Another type bacterium, called as electrotrophs, can accept the electrons directly from the exoelectrogens or electron donors outside the cell (Lovely 2011; Schröder and Harnisch 2017). Ammonium can be bioelectrochemically oxidized under anaerobic condition by exoelectrogens to generate the electrons (He et al., 2009; Qu et al., 2014; Zhan et al., 2014; Zhu et al., 2016). In addition, some of electrotrophs use the oxidized forms of nitrogen as the electron acceptors, and reduce the electrons generated from the ammonium oxidation by exoelectrogens into nitrogen gas (Zhan et al., 2012; Huang et al., 2013; Kondaveeti et al., 2014).

Summing up the above, implies that nitrogen removal can be expected through the direct interspecies electron transfer between the exoelectrogens and the electrotrophs in bioelectrochemical reactor through Direct Interspecies Electron Transfer (DIET). The electroactive bacteria can be enriched selectively by the control of the polarized electrode potential that allows a great possibility to enrich the ammonium oxidizing exoelectrogens (AOE) and the denitrifying electrotrophs (DNE) in bioelectrochemical reactor. Hence, in the bioelectrochemical reactor, it is expected that the nitrogen could be efficiently removed by the DIET pathway between the AOE and DNE.

1.2 Objective

In this study, the optimal design and the operational parameters for the bioelectrochemical nitrogen removal was investigated. Bioelectrochemical nitrogen removal was demonstrated in a bioelectrochemical reactor with a pair of polarized bioelectrodes that produces the electrostatic field, which enriches the electroactive bacteria, ammonium oxidizing exoelectrogens (AOE) and the denitrifying electrotrophs in the anaerobic reactor and facilitates the nitrogen removal via the biological DIET between the electroactive bacteria. To enrich the electrochemically active microorganisms, experiments were conducted in batch reactors with activated and anaerobic sludge separately and their performance is noted. Other parameters

including applied voltages, nitrate and nitrite as electron acceptor for bioelectrochemical ammonium oxidation were also studied in separate batch reactors. Moreover, continuous reactor for ammonium oxidation and nitritation experiments were also performed based on the batch experiment results.

1.3 Scope and Content

The scope and the content of this study consists of five parts for achieving the thesis purpose, and they are as follows:

Chapter 3 – Bioelectrochemical nitrogen removal through Direct Interspecies Electron Transfer in the bulk solution

A bioelectrochemical anaerobic ammonium oxidation via direct interspecies electron transfer in bulk solution was demonstrated in an anaerobic reactor equipped with a pair of bioelectrode polarized at 0.6V. The AOE and DNE in the bulk solution were enriched from anaerobic sludge by a pair of polarized bioelectrode, and the nitrogen was mainly removed by the DIET between the AOE and DNE in the bulk solution. The amounts of nitrite and alkalinity required for the bioelectrochemical ammonium removal were estimated, and the microbial communities were also identified.

Chapter 4 – Electroactive microorganisms enriched from activated sludge for bioelectrochemical nitrogen removal

The bioelectrochemical anaerobic nitrogen removal was demonstrated in an anaerobic batch reactor equipped with a pair of polarized bioelectrodes. The bioelectrochemical reactor was operated in sequential batch mode after inoculating activated sludge and polarizing the electrode to 0.6V. The medium contains ammonium, nitrite, alkalinity and trace minerals, but no organic carbon source. By the repetitive sequential operation, simultaneous removals of ammonium, nitrite and alkalinity were improved, and the electrochemical activity of the bulk sludge was confirmed from the redox peaks of the cyclic voltammogram. This indicates that

ammonia oxidizing exoelectrogens (AOE) and denitritating electrotrophs (DNE) were enriched more in the bulk solution. Biogas production that mainly consisted of nitrogen was observed from the bioelectrochemical reactor, and the minor components in the biogas were methane and carbon dioxide. This demonstrates that AOE use nitrite as an electron acceptor to oxidize ammonia.

Chapter 5 – Nitrite and nitrate as electron acceptors in bioelectrochemical ammonium oxidation through the electrostatic field

Nitrate, as well as nitrite are used as electron acceptors for ammonium oxidation in a bioelectrochemical anaerobic reactor. In the bioelectrochemical reactor, the electroactive nitrogen removing bacteria, including ammonium oxidizing exoelectrogens (AOE) and denitrifying electrotrophs (DNE), were enriched by the electric field of 0.2 V/cm in the bulk solution containing nitrite, nitrate, and ammonium. The ammonium was oxidized simultaneously with the decrease in the nitrite and nitrate as the electron acceptors by direct interspecies electron transfer between AOE and DNE. Nitrate is a less effective electron acceptor than nitrite for bioelectrochemical ammonium oxidation. It is essential to be able to use nitrate as an electron acceptor for bioelectrochemical ammonia oxidation to overcome the disadvantage in anammox process. This finding provides an advantage that the strict nitritation to selectively produce only nitrite from ammonium can be avoided when treating ammonium-rich wastewater in a bioelectrochemical reactor.

Chapter 6 - Electrostatic field facilitates direct interspecies electron transfer for bioelectrochemical anaerobic nitrogen removal

The electrostatic field facilitates direct interspecies electron transfer (DIET) to remove ammonium and nitrite in anaerobic condition. When the suspended microorganisms in bulk solution exposed to the electrostatic field, it was observed to remove ammonium in an anaerobic batch reactor with the consumption of nitrite and alkalinity. This study is to

demonstrate that ammonium and nitrite nitrogen could be bioelectrochemically removed by the electrostatic field-driven DIET, a novel approach for the treatment of nitrogen-rich wastewater.

Chapter 7 – Bioelectrochemical partial nitritation of ammonium rich wastewater in upflow reactors: electrostatic field and HRT effects

The partial nitration of ammonium is an essential step to economically remove nitrogen from ammonium-rich wastewater in biological nitrogen removal processes including Anammox and bioelectrochemical processes. The bioelectrochemical partial nitritation of ammonium rich wastewater depending on electrostatic field and HRT was investigated in an upflow reactor. This study is to suggest that the bioelectrochemical nitritation and nitrification are dependent on the intensity of electrostatic field as well as HRT.

Chapter 2 – Literature review

Industrial waste water is one of major sources of pollution that harms our environment badly. Rising population and industrial growth in India are contributing towards escalated demand for water treatment products across the country. The release of untreated wastewater has resulted in increased pollution and depletion of clean water resources. The most polluting of them are the city sewage and industrial waste discharged into the rivers. The facilities to treat waste water are not adequate in any city in India. Presently, only about 10% of the waste water generated is treated; the rest is discharged as it is into our water bodies (Fig. 2.1).

Nitrogen is an essential component of all living things, but excessive concentrations of nitrogen lead to significant environmental problems. As nitrogen was used in number of industrial facilities, high levels of nitrogen content were monitored in industrial wastewaters that leads to serious water environmental problems. To overcome this outburst wastewater management is the best option.

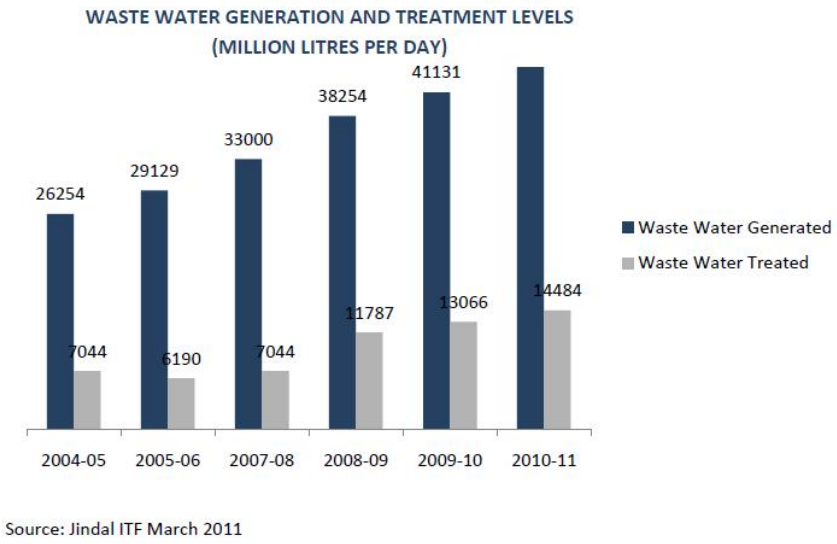


Fig. 2.1. Wastewater generation and treatment levels

Since the process of reducing contaminants will takes decades of dedicated effort, the better solution would be to manage the wastewater. Fortunately, many technologies are developed to overcome this crisis to treat wastewater. There were physiochemical methods which was surpassed later by biological methods to treat the nitrogen rich wastewater.

2.1 Biological nitrogen removal

The removal of NH_4-N from wastewater has become a worldwide emerging concern because NH_4-N is toxic to aquatic species and causes eutrophication in natural water environments (Tchobanoglous et al., 2003). Nitrogenous compounds in wastewater can only be effectively removed by biological approaches (EPA 1993; Zhu et al., 2007a, b). Based on the microbial nitrogen cycle and the metabolism of inorganic nitrogen compounds (Fig. 2.2), many biological technologies and processes have been developed and implemented for nitrogen removal from wastewater.

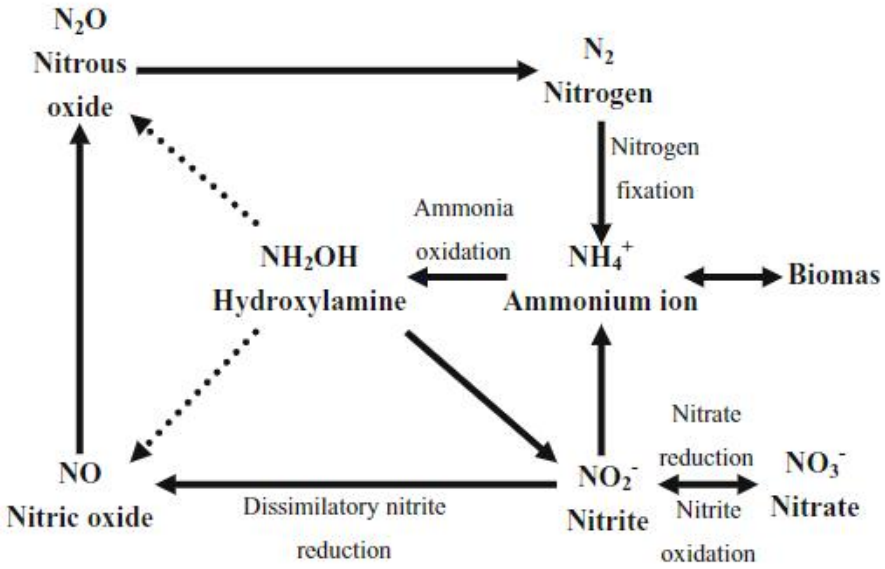


Fig. 2.2. Microbial nitrogen cycle. (From Rick and Stuart 2001)

2.1.1 Conventional nitrification and denitrification processes

Wastewater treatment facilities ammonium through biological nitrification and denitrification treatment that converts ammonium into nitrogen gas.

The first step of the treatment process is the nitrification. It takes place in an aerated basin containing nitrifying bacteria and inorganic carbon source. Each oxidation step is a distinct process involving separate groups of bacteria. The ammonia oxidizing bacteria (AOB) convert ammonia to nitrite, and then the nitrite oxidizing bacteria (NOB) convert nitrite to nitrate. The process of ammonium oxidation to nitrite is called nitritation and can be described by equation 2.1. Further oxidization of nitrite to nitrate is referred to as nitratation and proceeds according to equation 2.2 (Cuidad et al., 2005).

$$NH_4^+ + {}^3/_2\, O_2 \xrightarrow{\text{Ammonia oxidizers}} NO_2^- + 2H^+ + H_2O \qquad \Delta G^\circ = -278\ \text{kJ/mol} \qquad 2.1$$

$$NO_2^- + {}^1/_2\, O_2 \xrightarrow{\text{Nitrite oxidizers}} NO_3^- \qquad \Delta G^\circ = -73\ \text{kJ/mol} \qquad 2.2$$

The most common types of AOBs found in wastewater treatment plants (WWTP) are *Nitrosomonas europaea/ eutropha*, *Nitrosomonas oligotropha*, *Nitrosomonas communis*, and *Nitrosospira* lineages, while the common NOBs include *Nitrobacter* and *Nitrospira* species (Siripong & Rittmann, 2007). The second stage of ammonia treatment is denitrification. Denitrification takes place in an anaerobic or oxygen limited basin. Denitrifiers are common among the Gram-negative alpha and beta classes of the Proteobacteria, such as *Pseudomonas, Alcaligenes, Paracoccus, and Thiobacillus*. Some Gram-positive bacteria (such as *Bacillus*) and a few halophilic Archaea (such as *Halobacterium*) are able to denitrify (Zumft 1992). This step reduces nitrate back to nitrite which is further converted to nitrogen gas and released into the atmosphere according to equations 2.3, 2.4, 2.5, and 2.6 (Clauwaert et al., 2007).

$$2NO_3^- + 4H^+ + 4e^- \xrightarrow{\text{denitrifying bacteria}} 2NO_2^- + 2H_2O \quad (2.3)$$

$$2NO_2^- + 4H^+ + 2e^- \xrightarrow{\text{denitrifying bacteria}} 2NO + 2H_2O \quad (2.4)$$

$$2NO + 2H^+ + 2e^- \xrightarrow{\text{denitrifying bacteria}} N_2O + H_2O \quad (2.5)$$

$$N_2O + 2H^+ + 2e^- \xrightarrow{\text{denitrifying bacteria}} N_2 + H_2O \quad (2.6)$$

But the disadvantages and the limitations of this process is that nitrification and denitrification are carried out by different micro-organisms under different conditions, they should be designed and operated in separate time sequences or spaces (Lee et al., 2001). Consequently, a long retention time or a large volume is required to accomplish complete nitrogen removal. Moreover, a high level of oxygen, set as 4.2 g O_2/g NH_4^+-N, is required for nitrification (Bruce and Perry 2001), and a sufficient organic carbon source (2.86 g chemical oxygen demand (COD)/g NO_3-N) is necessary for denitrification (Gradly and Lim 1980). A high level of external carbon sources (methanol, acetate, etc.) is normally added in the denitrification process when treating wastewater with high nitrogen concentration or low C/N ratio (Tam et al., 1992), which increases the operational cost for conventional biological processes. The limitations of low removal efficiency, high oxygen requirement, long retention time, and an external carbon source are the driving forces for developing new low-cost biological treatment processes for complete nitrogen removal (Jetten et al., 2002).

2.1.2 Alternatives to Conventional Nitrification and Denitrification

Much of the recent research on nitrification has concentrated on adjusting the traditional process in order to enhance the treatment capability or decrease the energy and costs associated with it. Many of the proposed variations on the conventional nitrification and denitrification processes address the issues of aeration costs and supplemental organic carbon demand. The nitrification process requires large amounts of air and the denitrification process often needs

additional organic carbon such as methanol, acetate, and glucose as an energy source and electron donor (Ahn 2006). Review of biological nitrogen removal technologies by various researchers has identified alternative nitrogen removal processes that are novel and cost effective. These include simultaneous nitrification and denitrification, partial nitrification, anaerobic ammonium oxidation, aerobic deammonitrification, completely autotrophic nitrogen removal over nitrite, and oxygen limited autotrophic nitrification denitrification (Ahn 2006; Parades et al., 2007; and Zhu et al., 2008).

a) Simultaneous Nitrification and Denitrification (SND)

The conventional nitrification and denitrification process need to be operated in separate vessels sequentially. However, through simultaneous nitrification and denitrification (SND), the two distinct processes can be unified. The mechanism of SND can be either physical or biological. The physical mechanism takes advantage of the natural oxygen concentration gradient that form inside the activated sludge flocks (Fig. 2.3). The outer region of the flock would be exposed to higher level of oxygen so it supports nitrification reaction and the center of the flock, the region exposed to less than 0.5 mg DO/L, support denitrification reaction (Zhu et al., 2008).

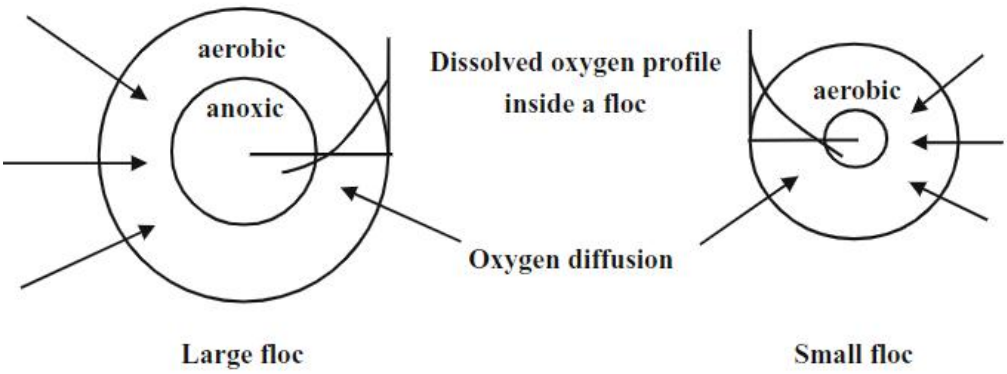


Fig. 2.3 Schematic of oxygen concentration profile within a microbial floc

The biological mechanism utilizes heterotrophic nitrifiers such as *Alcaligenes sp.*, *Corynebacterium sp.*, *Acinetobacter sp.*, *Xanthomonas sp.*, *Bacillus sp.* and aerobic denitrifiers

such as *Paracoccus denitrificans*, *Microvirgula aerodenitrificans*, and *Thaurea mechernichensis* (Zhu et al., 2008). The SND process has the benefit of integrating the nitrogen removal processes and saving the costs of an extra reactor. The influential parameters controlling SND process are the carbon and DO concentrations, and the floc size. There needs to be adequate amount of organic carbon for the complete denitrification. The BOD level which is typically present in wastewater (100-150 mg/L) is sufficient for this purpose (Zhu et al., 2008). The DO concentration needs to be low enough to provide the anoxic condition that enables both nitrification on the surface and denitrification inside the floc. The size of the floc needs to be larger than 125 μm in diameter in order to provide the aerobic and anaerobic zones within the particle (Zhu et al., 2008).

b) Partial Nitrification (Shortcut Nitrification and Denitrification)

Shortcut nitrification and denitrification, namely partial nitrification-denitrification, is the process in which nitrification and denitrification are correlated by NO_2^- instead of NO_3^-. As an intermediate product, NO_2^- is produced in nitrification and reduced to N_2 in the following NO_2^- denitrification (Fdz-Polanco et al., 1996; van Dongen et al., 2001; Peng et al., 2006). Compared with traditional nitrification and denitrification via NO_3^-, short-cut nitrification and denitrification has the following advantages (Beccari et al., 1983; Turk and Mavinic 1989; Peng and Zhu 2006):

1. 25% lower oxygen consumption in the aerobic phase implies 60% energy saving in the entire process.
2. The requirement for electron donors is as much as 40% lower in the anoxic phase.
3. $NO2^-$ denitrification rate is 1.5 to 2 times higher than NO_3^- denitrification rate.

Partial nitrification via NO_2^- is reported to be technically feasible and economically favorable, especially when treating wastewater with high ammonia concentration or low C: N ratio (Turk and Mavinic 1989; Villaverde et al., 1997).

The Single reactor system for High Ammonia Removal Over Nitrite (SHARON) process, the first full-scale process with NO_2^- as the intermediate product, is a cost-effective treatment system for total nitrogen removal from wastewater with high nitrogen concentrations (>550 mg/L) (Ahn 2006). SHARON process utilizes temperature, aeration pattern, and SRT as control parameters to accumulate nitrite and start denitrification in the same reactor. In the SHARON process, both nitrification and denitrification steps occur in the same reactor through aeration control triggering oxidation or reduction, the high temperature favors the AOB, while the short SRT washes out the NOBs. One of the main functions of the denitrification step in the SHARON process is to control the pH during acidification caused by nitrification (Ahn 2006). In their partial nitrification study with a SHARON reactor, Mosquera-Corral et al., 2005 used the faster growth rate of ammonia oxidizers at 30^{o}C to wash out the nitrite oxidizers with a short sludge retention time (SRT) of 1 day. A full-scale SHARON reactor in the Netherlands used sludge digestion effluent as feed with ammonia concentration around 0.5 – 1.5 g N/L and ended up with over 90% conversion to nitrite (Van Kempen et al., 2001).

2.1.3 ANAMMOX

The anaerobic ammonium oxidation (ANAMMOX) (Fig. 2.4) is carried out by lithoautotrophic bacteria of order *Planctomycetales* (Strous et al., 1998) such as *Procadia anammoxidans*, *Kuenenia stuttgartiensis*, *Candidatus Scanlindua brodae*, and *Candidatus Scalindua wagneri* (Zhu et al., 2008). The stoichiometric mass balance of the ANAMMOX reaction, seen in equation 2.7 (Strous et al., 1998), highlights nitrite as an important reactant. In fact, a ratio of 1:1.3 ammonia to nitrite concentration in the influent is needed for optimum reaction. However, a level above 50-150 mg N/L (Van der Star et al., 2007) is toxic to ANAMMOX organisms.

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

Since an inorganic carbon source is used in ANAMMOX, doubling time of bacteria is very long, more than 11 days (Paredes et al., 2007), which has the benefit of reducing the need for sludge treatment. However, it also means that the SRT needs to be kept long to maintain adequate biomass, or retention mechanisms such as biofilm must be used. Dissolved oxygen concentration, temperature, and biofilm thickness are the important factors for this process. The optimum pH for the system is 7.7 – 8.3 and the optimum temperature is between 26 – 28°C (Zhu et al., 2008). Overall, the process could reduce the operating cost by 90% by reducing aeration and eliminating organic carbon.

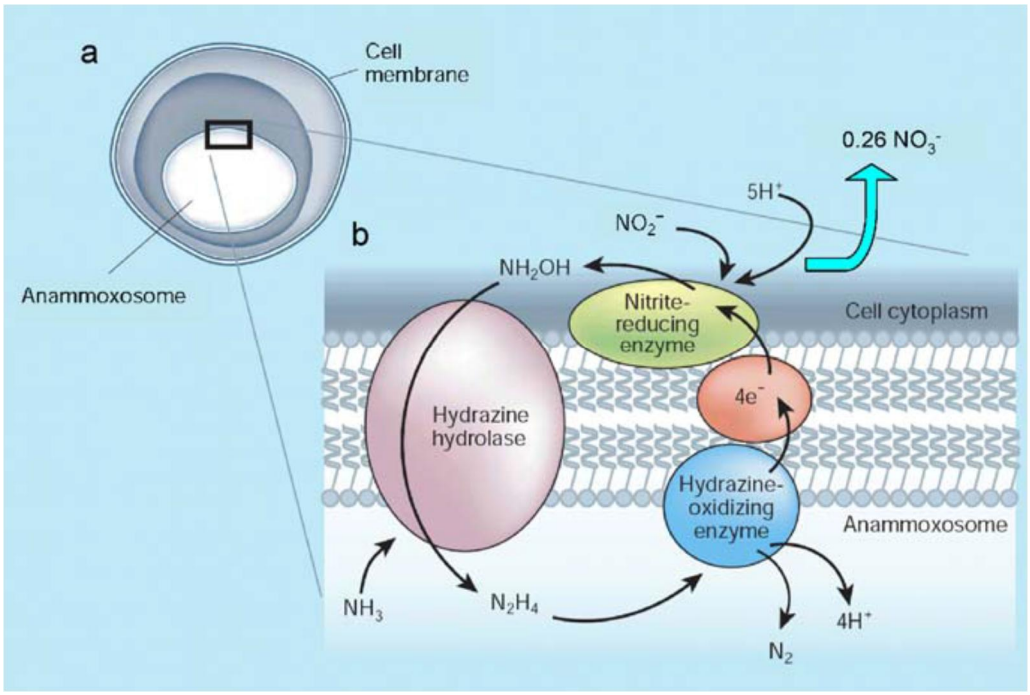


Fig. 2.4 Mechanism of Anammox process

There are two pathways for ANAMMOX reaction to proceed. First, is to utilize the SND process inside a single reactor. This requires the upper layer of biofilm exposed to higher DO to carry out partial oxidation of ammonia (nitrite accumulation), and the anaerobic zone underneath to carry out the full ANAMMOX reaction. There are multiple variations of the single reactor ANAMMOX process that have been discussed by different research groups.

These variations include aerobic deammonification, OLAND, CANON, and denitrifying ammonium oxidation (DEAMOX). One should note that from a biological point of view the processes themselves are similar and the name variations reflect the laboratories in which they were developed.

A single reactor system is preferred but it is also much more complex than the two-reactor system. In the two-reactor system, the first reactor would carry out partial nitrification, converting about 55-60% of the ammonia to nitrite (Ahn 2006). The second reactor would use the effluent of the first to carry out the ANAMMOX reaction. In the Netherlands, several full scale ANAMMOX WWTP reactors are operational (Van der Star et al., 2007). They used reject from sludge dewatering with 750 kg –N/d ammonia loading in a two-reactor partial nitrification and ANAMMOX system.

a) Aerobic Deammonification

The aerobic deammonification process is a version of the one reactor ANAMMOX system. The outer layer of biofilm exposed to oxygen carries out nitrification, while the deeper anoxic layer converts ammonia and the intermediate nitrification product such as nitrite, to nitrogen gas in one step (Zhu et al., 2008). Another pathway for this process is for nitrite to be converted to nitrogen gas in the inner layer of the biofilm with $NADH_2$ as an electron donor (Zhu et al., 2008). This system is best suited for low nitrogen load water such as municipal wastewater. This reaction has been typically observed in conventional nitrification reactors so there has not been a concerted effort to isolate it and optimize it in full scale reactors (Zhu et al., 2008).

b) CANON

The completely autotrophic nitrogen removal over nitrite (CANON) utilizes two types of bacteria, *Nitrosomonas* and ANAMMOX like bacteria (Ahn 2006), within the same reactor. The main difference between CANON and ANAMMOX process is that in CANON, nitrite acts as an electron acceptor, as opposed to an electron donor in ANAMMOX, so nitrite does

not need to be present in the influent wastewater. The entire process occurs in an aerated but oxygen limited environment (DO around 0.5 mg/L) where the oxygen requirements of the first bacteria and the anoxic conditions needed for the second bacteria are met simultaneously. Equations 2.8 and 2.9 demonstrate the two steps in the CANON reaction (Zhu et al., 2008).

$$NH_4^+ + 1.5O_2 \xrightarrow{\text{Aerobic AOB}} NO_2^- + 2H^+ + H_2O \quad (2.8)$$

$$NH_4^+ + 1.3NO_2^- \xrightarrow{\text{Anaerobic AOB}} 1.02N_2 + 0.26NO_3^- + 2H_2O \quad (2.9)$$

The controlling parameters for these reactions are DO, ammonia concentration, and AOB concentration (Zhu et al., 2008). In order to inhibit NOB, either DO or nitrite concentrations need to be kept low. Also, the feed ammonium concentration needs to be around 14 mg/(L*hr) in order to ensure nearly 100% of the nitrite generated goes through the anaerobic ammonia oxidation step (Zhu et al., 2008). The CANON process reduces aeration by 63%, eliminates the need for organic carbon, and works best for high strength or low C:N ratio wastewaters. The conflicting needs of the CANON process make it very sensitive to changes in DO, temperature, nitrogen loading, and biofilm thickness so further research needs to improve CANON's resistance to shock (Ahn 2006).

c) OLAND

The oxygen limited autotrophic nitrification – denitrification (OLAND closely resembles CANON). In OLAND, only aerobic AOB is used, which is slightly different than CANON. Aerobic AOB being the sole biomass makes this process very sensitive to DO concentration, with dramatic AOB inhibition taking place at DO < 0.1 mg/L. The main advantage of the OLAND process is its tolerance for fluctuation in ammonium and nitrite concentration better than CANON but it has lower nitrogen removal efficiency (40%) than CANON (Zhu et al., 2008).

2.1.4 Bioelectrochemical system (BES)

Bioelectrochemical wastewater treatment has emerged as a potentially interesting technology for the treatment of wastewater. Two configurations of BESs are used for wastewater treatment purposes: microbial fuel cell (MFC) or microbial electrolysis cell (MEC) (Fig. 2.5). Microbial fuel cells (MFCs) convert an organic substrate into electricity using micro-organisms as a biocatalyst, whereas in microbial electrolysis cell (MEC), with the help of the ‘‘electro-active’’ microorganisms and the external voltage is invested to prepare enough energy for accomplishment of reactions in the anode and the cathode to produce the desired product (Rabaey and Rozendal, 2010; Pant et al., 2012; Nam et al., 2011).

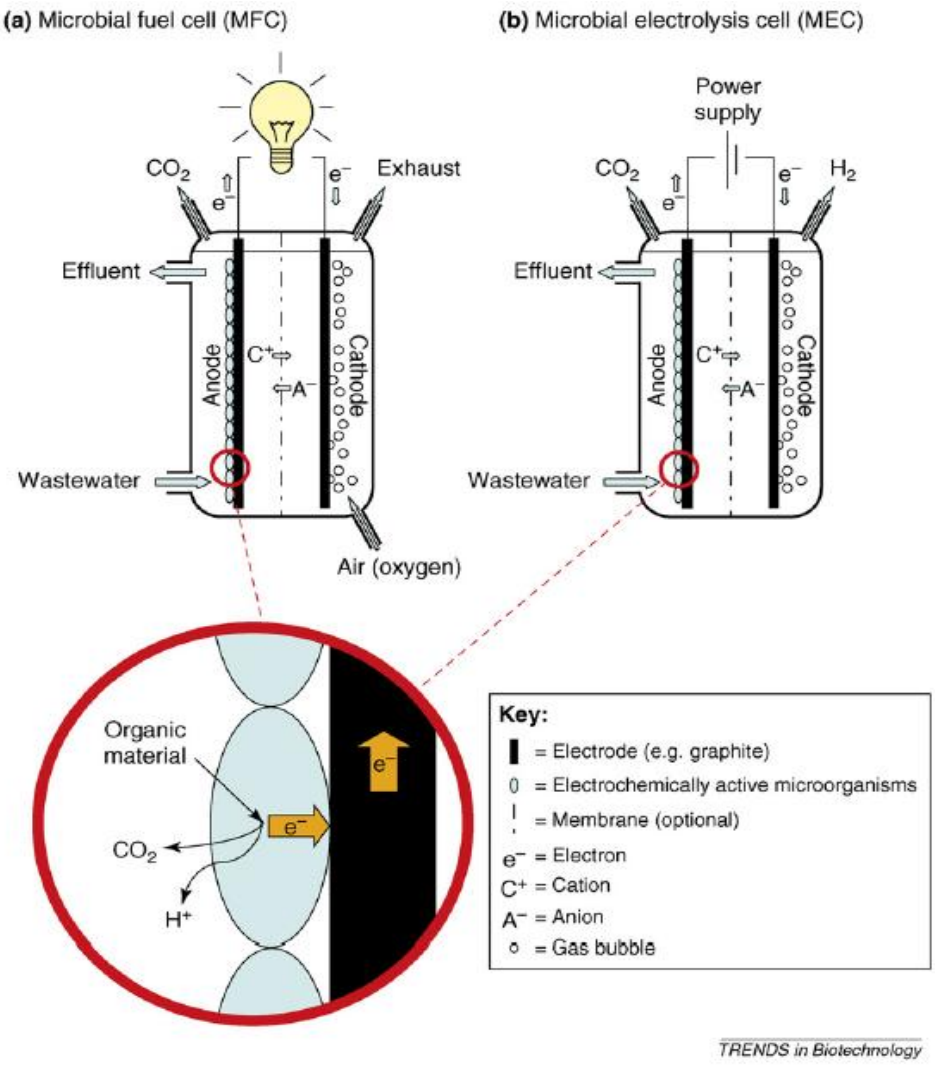


Fig. 2.5 Bioelectrochemical system a) Microbial fuel cell b) Microbial electrolysis cell

a) Bioelectrochemical system for nitrogen removal

BES is the advanced technique that gained popularity for removal of nitrogen from the wastewater (Venkata Mohan et al., 2014; Kelly and He, 2014; Iskander et al., 2016). Bioelectrochemical wastewater treatment is based on the use electrochemically active microorganisms in the reactor by polarizing the electrodes which are capable of extracellular electron transfer (Rabaey et al, 2007). It has been revealed in past few years that ammonium can be bioelectrochemically oxidized on the polarized anode surface by electroactive bacterial species and the oxidized forms of nitrogen including nitrite and nitrate can be reduced into nitrogen gas on the cathode surface by another species of electroactive bacteria (Qu et al., 2014; Zhan et al., 2012; Mook et al., 2013; Hussain et al., 2017). So, these microorganisms that are capable of transferring electron from the inside of the cell to the electrodes which are referred as extracellular electron transfer. These extracellular electron transfer have two mechanisms. The first one is the indirect mechanism in which the redox cycling of the electron shuttling compounds between the microorganisms and the electrode. The electron shuttling compounds are redox active organic or inorganic compounds such as humic acid and sulfur species (Stams et al, 2006), or through microorganisms such as quinones (Newman and Kolter 2000) or phenazines (Rabey et al, 2005). The second is a direct mechanism where the electroactive microorganisms and the electrode have a direct contact which was established by some microorganisms which utilize the cytochromes that conduct the electrons from inside of the cell to the electrode (Lovely 2006). Moreover, many electrochemically active species also capable of producing electrically conductive pili (Gorby et al, 2006; Reguera et al, 2005) or nanowires outside of the cells. Nanowires stretch over tens of microns which allow the multi-layered biofilms of the microorganisms, which depends on the direct interspecies electron transfer through contact and to grow on the electrode surfaces (Logan and Regan 2006; Reguera et al, 2005).

Chapter 3 - Bioelectrochemical nitrogen removal through Direct Interspecies Electron Transfer in the bulk solution

3.1 Introduction

Generally, the ammonium-rich wastewater has been treated by conventional biological nitrogen removal (BNR) process, which includes autotrophic nitrification, and heterotrophic denitrification. However, for autotrophic nitrification, organic matter should first be removed from the wastewater so that nitrifying bacteria can grow dominantly, and the large amounts of oxygen and alkalinity are required (Ahn 2006). In particular, the nitrifying bacteria grow slowly and are susceptible to environmental stresses. Nitrification is generally considered to be a rate-limiting step in nitrogen removal when the wastewater temperature is less than 15℃ in temperate or cold regions. In addition, the heterotrophic denitrification requires a carbon source as the electron donor.

The shortcut nitrogen removal process was developed to alleviate the excess burden of oxygen, alkalinity and carbon source in a conventional BNR process (Ahn 2006; Ge et al., 2015; Nancharaiah et al., 2016; Shalini and Joseph 2012). In the shortcut process, ammonium is oxidized into nitrite, which is then reduced into nitrogen gas. However, shortcut nitrogen removal requires a selective growth of ammonia-oxidizing bacteria (AOB) for the nitritation of ammonium than the nitrite oxidizing bacteria (NOB) (Wang et al., 2015). Favorable conditions for the growth of AOB over NOB are high free ammonia or nitrous oxide concentrations, high temperature, low solid retention time, and low dissolved oxygen concentration. This indicate that the advantages of shortcut process for the treatment of nitrogen-rich wastewater depends on the type of wastewater. Anammox process that removes nitrogen under the anaerobic condition is also considered to be a more sustainable, energy

neutral technology for the treatment of ammonium rich wastewater (Ma et al., 2016; Jin et al., 2012; Du et al., 2015). Anammox bacteria use nitrite as an electron acceptor to oxidize ammonium. However, there are still several challenges to Anammox process, including nitrite requirement, Anammox bacteria enrichment, and nitrate production as a by-product (Ma et al., 2016; Nancharaiah et al., 2016; Shalini and Joseph 2012).

Over past few years, it has been revealed that ammonium can be bioelectrochemically oxidized on the polarized anode surface by electroactive bacterial species (He et al., 2009; Qu et al., 2014; Zhan et al., 2014; Zhan et al., 2012). In addition, the oxidized forms of nitrogen including nitrite and nitrate can be reduced into nitrogen gas on the cathode surface by another species of electroactive bacteria (Huang et al., 2013; Mook et al., 2013; Hussain et al., 2017). This implies that bioelectrochemical technology has a potential in the removal of nitrogen from the wastewater. The electroactive species oxidizing ammonium are the exoelectrogens that can transfer electrons through c-type cytochrome or conductive pili that is over-expressed up to the outer membrane of the cell (Kiley et al., 2011; Lovley 2011). The other electroactive species reducing the nitrite and nitrate are the electrotrophs that can accept electrons directly from electron donors that are present outside the cells (Lovely 2011; Schröder and Harnisch 2017). It is well known that the exoelectrogens and electrotrophs can be enriched on the surfaces of the polarized anode and cathode in a bioelectrochemical reactor. This bioelectrochemical nitrogen removal based on the surface reaction of the polarized bioelectrode is an effective process when the surface area of the electrode is sufficiently provided in the reactor. However, the bioelectrochemical reactor installed with the electrodes with a sufficient surface area requires a large capital cost. Intriguingly, it has been found that the exoelectrogens and electrotrophs in close contact with each other in bulk solution can be electrically connected through conductive pili, and can exchange the electrons directly (Rotaru et al., 2014; Feng et al., 2018). It has been observed that the direct interspecies electron transfer (DIET) is

considered to be more thermodynamically favorable than other routes for electron transfer during anaerobic digestion (Kiley et al., 2011; Lovley 2011; Feng et al., 2017; Feng et al., 2016). The DIET between the ammonia-oxidizing exoelectrogens (AOE) and the denitrifying electrotrophs (DNE) in the bulk solution is also a possible electron transfer route in the bioelectrochemical reactor for nitrogen removal, but it has not been investigated yet.

In this study, we demonstrated for the first time that the AOE and DNE in the bulk solution are enriched from anaerobic sludge by a pair of polarized bioelectrode, and the nitrogen is mainly removed by the DIET between the AOE and DNE in the bulk solution. The amounts of nitrite and alkalinity required for the bioelectrochemical ammonium removal were estimated, and the microbial communities were also identified.

3.2 Experimental methods and analysis

3.2.1 Bioelectrochemical anaerobic reactor

A bioelectrochemical anaerobic reactor (diameter 10 cm, height 16 cm, effective volume 1L) was prepared with acrylic resin. An anode (7cm × 10 cm) and a cathode (7cm × 10cm) facing each other were installed at an interval of 5cm inside the reactor. The anode and cathode were fabricated by modifying the surface of a GFF (Graphite Fabric Fiber, Samjung C&D Co., South Korea) with MWCNT (Multiwall Carbon Nanotube, Carbon Nano-material technology Co., Ltd., South Korea), as described in previous studies (Feng et al., 2018; Feng et al., 2016; Feng et al., 2017). In brief, the GFF and MWCNT were treated in concentrated nitric acid solution for 24 hours to improve hydrophilicity and to remove impurities and then washed under running tap water until the pH was neutral. An electrolyte solution was prepared by dispersing 1g MWCNT, 0.25g nickel chloride, and 0.5g polyethyleneimine into the 1L distilled water. The MWCNT and Ni were electrophoretically deposited on the GFF surface by applying 30 V for 30 min using a direct current (DC) power source (OPM series, ODA technologies Co. Ltd,

Incheon, South Korea). For the electrophoretic deposition, the GFF sheet and a stainless-steel mesh (STS, 316L) were used as the working and counter electrodes, respectively. Each electrode inside the bioelectrochemical reactor was connected to a DC power supply (OPM series, ODA (Technologies Co. Ltd, Incheon, Korea) using conductive wire. The top of the bioelectrochemical reactor was covered with an acrylic resin plate to seal the reactor. A biogas sampling port for experimental studies was covered with a butyl rubber stopper was provided on the acrylic resin plate. The biogas sampling port was connected to a floating type gas collector that was filled with water - acidified with sulphuric acid and saturated with salt to prevent the resolution of biogas (Feng et al., 2017). For liquid sample collection, another port was provided on the acrylic resin plate and the tube was extended into the reactor until it was submerged inside the waste wastewater.

For the experiment, an artificial wastewater (0.460 L) and inoculum (0.540 L) was added to the reactor. The artificial wastewater contained 0.3 g/L KH_2PO_4, 1.0 g/L $Na_2HPO_412H_2O$, 0.5 g/L NaCl, 2.0 g/L $NaHCO_3$, 0.1 g/L $MgSO_47H_2O$, 0.01 g/L $CaCl_2$, and 1.91 g/L NH_4Cl, as in previous study (Zhan et al., 2012). Anaerobic sludge was collected from S Sewage Treatment Plant (B-city, South Korea), screened to remove impurities and then the inoculum was obtained by thickening with the gravitational force for a day. The initial VSS and pH of the inoculum was about 13,000mg/L and 7.67, respectively. A voltage of 0.6V was applied between the anode and cathode and the bioelectrochemical reactor for nitrogen removal was start to operate at room temperature with constant stirring using a magnetic bar (500 rpm). The initial concentration of NH_4-N was 500 mg/L and NO_2-N was adjusted to 300 mg/L by adding $NaNO_2$ respectively. NO_2-N was supplemented when the concentration decreased to a smaller level. When the concentration of NH_4-N was depleted, the artificial wastewater was then replaced with a fresh one after settling suspended sludge for 30 minutes. In the final cycle of the batch

experiment, the circuit between the anode and cathode was opened to investigate the role of the polarized electrode.

3.2.2 Analysis and calculation

During the operation, the pH and alkalinity of the bioelectrochemical reactor were monitored everyday using a pH meter (Orion Model 370), and titration method, respectively. NH_4 -N, NO_2 -N, and VSS were measured every day, in accordance to the Standard methods for the examination of water and wastewater (ALPHA 2005). The potentials of the anode and cathode were intermittently measured against an Ag/AgCl reference electrode (RE-1B, ALS Co., Ltd, Japan) using a portable digital multimeter (DM-1010, Dong Hwa Electronics, Co., Korea). The current in the external circuit was monitored using a digital multimeter (DMM, Ni cDAQ-9174, National Instruments), installed between the electrodes and the DC power source. The percentage of NH_4-N oxidized by the AOB on the anode surface $= \frac{\int idt}{nF(\Delta NH_4 - N)V} \times 100$ was, estimated from the total amount of NH_4-N removed in the bioelectrochemical reactor and the current in the external circuit. Where, i is the electric current (A), n is the number of electrons for NH_4-N oxidation to NO_2-N, F is the Faraday constant (96,485C/e^- mole), ΔNH_4-N is the concentration of NH_4-N removed, and V is the liquid volume (1L) of the batch reactor. The biogas production was monitored over time using the floating type gas collector, and the biogas composition was analyzed using a GC (Gaw-Mac Instrument Co., PA, USA) with Porapak-Q column (6 ft×8th" SS) and thermal conductivity detector. The production of the biogas including nitrogen, methane and carbon dioxide at each monitoring time interval was estimated from the measurement of the biogas volume and the biogas composition and converted to the volume at standard temperature and pressure (STP) using Eq. (3.1) (Feng et al., 2017).

$$V_{Biogas\ at\ STP}(L) = V_{Biogas\ at\ T} \times \frac{273}{273+T} \times \frac{760-W}{760} \qquad (3.1)$$

Where, T is the experimental temperature (25°C), and W is the water vapor pressure at that temperature (23.76 mm Hg). During the last three batch cycle of the closed-circuit experiment, the average changes of the NH_4-N and NO_2-N in the liquid phase and the N_2 in biogas were converted into the mole number and then used in the nitrogen balance. In the electron balance, the total electrons released from the electron donor was obtained from the mole number of the NH_4-N removal. The electrons converted into nitrogen gas via the biological DIET in the bulk solution was estimated by subtracting the electrons transferred via the electrode (eDIET) from the total electrons consumed for nitrogen gas production. The difference in the electron moles contained in between the NH_4-N removed and nitrogen gas produced was considered to be the electrons converted into biomass or lost during the transferring process.

Cyclic voltammogram (CV) for the bulk solution was also obtained in the potential range of -1.0 V to 1.0 V (vs. Ag/AgCl) with a 10mV s^{-1} scan rate using the electrochemical instrument (ZIVE SP1, WonA Tech, South Korea). The pieces of stainless meshes (1cm × 1cm) were used as the working and counter electrodes for the cyclic voltammetry test. The peak currents and potential values for oxidation and reduction were obtained from cyclic voltammetry data using the analysis software, 'SMART Manager' (Zive Lap Wonatech, co., Korea).

3.2.3 Pyrosequencing analysis of microbial communities

16S rRNA gene-based pyrosequencing was used to investigate bacterial communities in the bioelectrochemical reactor by collecting the samples from the bottom of the reactor after it is allowed to settle at the end of the close circuit experiment. The variable region (V1-V3) of the bacterial 16S rRNA gene was amplified from the genomic DNA of each sample of the full-scale system using a fusion primer (Hur et al., 2011). The amplification conditions, sequencing library construction, sequencing, and analysis were performed using a previously applied (Chun et al., 2010) 454 GS FLX Junior Sequencing System (Roche, Brandford, CT, USA). In the filtering process, we read all the different samples with unique barcodes containing two or

more ambiguous nucleotides (average quality score <25 or short reading> 300 bp). Chimera checks and taxonomic assignments of these readings were done using the extended EzTaxon database (http://eztaxon-e.ezbiocloud.net/).

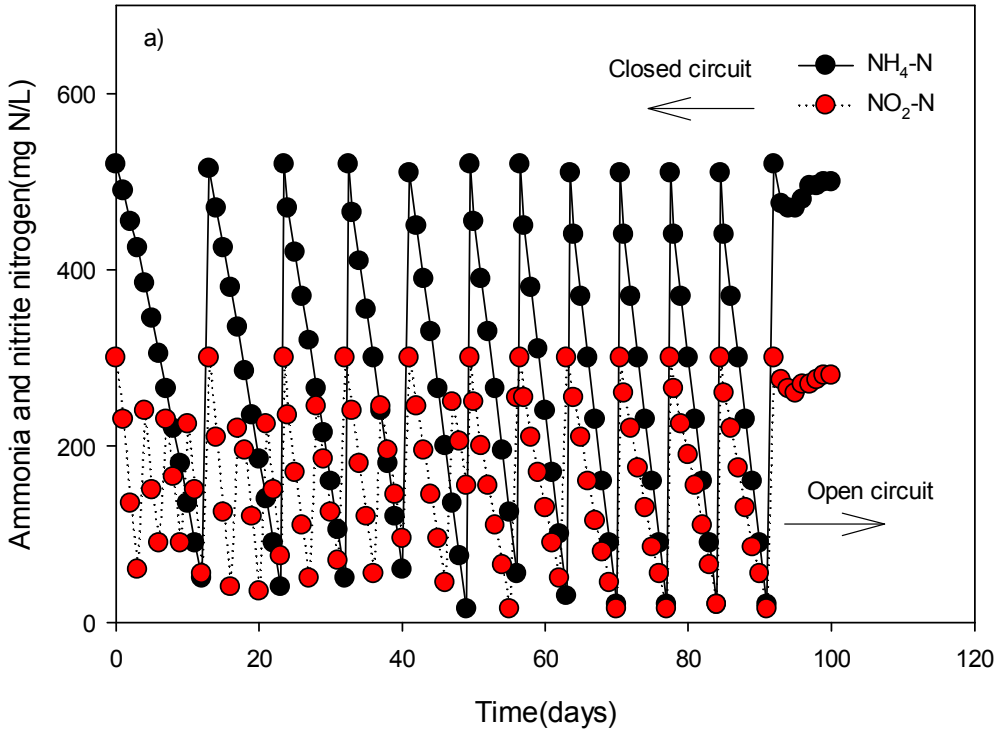
Statistical analyses of microbial communities were performed with the Mothur program, using a 3% difference cut-off value (Schloss et al., 2009). To compare samples with different read numbers, the sizes of different samples were normalized, where applicable, by random subtraction. Principal coordinate analysis (PCoA) and fast Unifrac analysis were conducted with CLcommunity software (Chunlab, Inc., Seoul, Republic of Korea).

3.3 Results and discussion

3.3.1 Bioelectrochemical anaerobic nitrogen removal

Simultaneous removal of ammonium and nitrite under anaerobic condition, which was different from Anammox process, was demonstrated in a bioelectrochemical reactor. The concentrations in NH_4-N and NO_2-N began to decrease by electrically polarizing the electrodes in the bioelectrochemical reactor inoculated with anaerobic sludge (Fig. 3.1a). There was no organic carbon source in the artificial wastewater supplied into the bioelectrochemical reactor. This indicates that the heterotrophic microorganisms contained in the anaerobic sludge inoculum starved, and then the organic matter was eventually released into the bulk solution by the cell lysis. During the initial several batch cycles, it is likely that a large portion of the NO_2-N removed in the bioelectrochemical reactor was used as the electron acceptor for the oxidation of the organic matter. However, the biological removal of ammonium is generally not possible under anaerobic condition although a small amount of ammonium can be synthesized into the cells. A potential mechanism that can explain the removals of ammonium and nitrite in the bioelectrochemical reactor is that the ammonium is oxidized under anaerobic condition by AOE and the nitrite is reduced as an electron acceptor by DNE (He et al., 2009;

Qu et al., 2014; Zhan et al 2014; Zhan et al., 2012; Mook et al., 2013; Hussain et al., 2017; Xie et al., 2013). The removal rates of NH_4-N and NO_2-N increased further with the repetition of the sequential batch operation of the reactor (Fig. 3.1a). It seems that the AOE and DNE involved in the bioelectrochemical nitrogen removal were enriched more over time. Intriguingly, the pH and alkalinity were decreased along with the decreases in NH_4-N and NO_2-N (Fig. 3.1b). This suggests that the AOE and DNE involved in the anaerobic nitrogen removal are autotrophs which use the bicarbonate as the carbon source (Zhan et al., 2012).



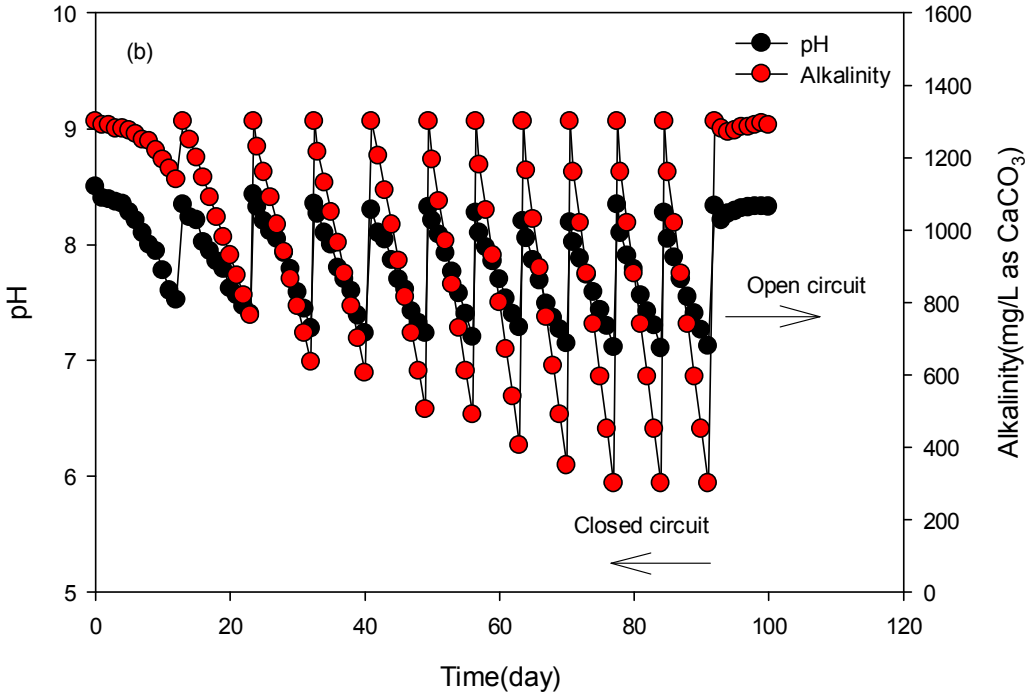


Fig.3.1 Changes of (a) NH_4-N and NO_2-N and (b) pH and alkalinity in bioelectrochemical reactor during batch operation

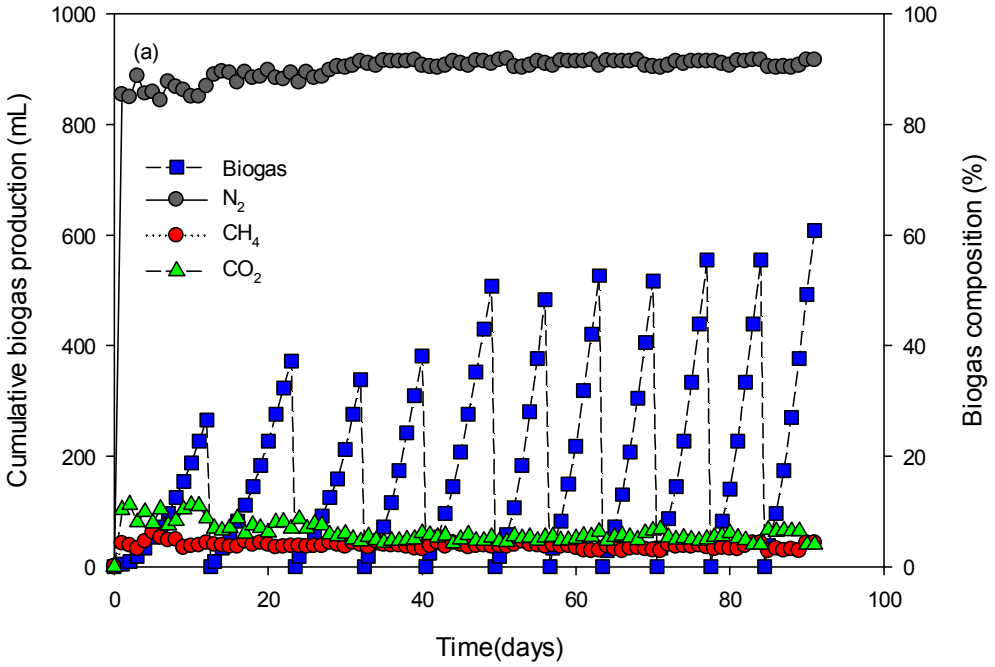
The ammonium oxidation under anaerobic condition is normally explained (Ma et al., 2016; Zhang et al., 2008; Jetten et al., 2009):

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+$$
$$\rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.5} + 2.03H_2O \qquad (3.2)$$

In the bioelectrochemical reactor, the removal of ammonium accompanies the decrease in nitrite and alkalinity, which means that the bioelectrochemical nitrogen removal is a process similar to the Anammox. However, the concentrations of NH_4-N and NO_2-N decreased with the start-up of the bioelectrochemical reactor (Fig. 3.1b). The inoculum was the anaerobic sludge from a sewage treatment plant that was not rich in Anammox bacteria. Anammox bacteria grow slowly, and the biomass yield is also very low. In addition, nitrate as a by-product was not observed during the whole experimental period. This indicates that the nitrogen

removal in the bioelectrochemical reactor is clearly different from Anammox process. During the operation of the bioelectrochemical reactor, biogas production was observed as a product of nitrogen removal (Fig. 3.2a). The cumulative biogas production increased from 265.7 mL up to 608.8 mL with the repetition of the sequential batch operation. The main component of the biogas was nitrogen, but methane (3-4%) and carbon dioxide (5-6%) were also included in the biogas (Fig. 3.2a). It seems that the carbon dioxide was produced by the conversion of bicarbonate as the pH decreases. However, the methane was possibly derived from the carbon dioxide reduced by electrotrophic methanogens. The ammonium was the only electron donor in the bioelectrochemical reactor. It is likely that the electrons released from the ammonium oxidation were mainly used for nitrite reduction, and the remaining electrons were used to reduce carbon dioxide for methane production. In the bioelectrochemical methane production, it is well known that exoelectrogens generate electrons from the fermentation of organic matter as the electron donor, and directly transfer the electrons to electrotrophs to reduce carbon dioxide for methane production (Zhan et al., 2012; Feng et al., 2018; Feng et al., 2017). In the bioelectrochemical reactor for nitrogen removal, the methane production indicates that carbon dioxide can be used as an electron acceptor instead of nitrite, suggesting that the nitrite requirement as the electron acceptor for ammonium removal can be less than the theoretical value. In the bioelectrochemical reactor, it has been described that the ammonium and nitrite can be removed by the direct electron transfer between AOE and DNE via the anode and cathode (eDIET) (Zhan et al., 2012). The number of electrons via the eDIET can be estimated based on the electric current monitored in the external circuit (Zhan et al 2012; Feng et al., 2018, Feng et al., 2017). In this study, the electric current was monitored in the external circuit for the 9^{th} sequential batch cycle (Fig. 3.2b). The theoretical value of the nitrogen gas estimated from the current in the cycle was 121.7mL, indicating that the nitrogen removal via the eDIET was only 23.3%. In the cyclic voltammogram for the suspended microorganism in the bulk

solution, two distinct redox peaks were observed at 0.13 V vs Ag/AgCl for the oxidation and -0.21 V vs Ag/AgCl for the reduction (Fig. 3.2b). These oxidation and reduction peaks reflect the activities of the exoelectrogens and electrotrophs, including AOE and DNE, respectively. In the bioelectrochemical methane production, it is well known that the electrons can be transferred by the direct contact biologically between interspecies in the bulk solution, which is called as biological DIET (bDIET) (Lovley 2011; Feng et al., 2016; Feng et al., 2017; Shen et al., 2016). In a similar manner, this suggests that the bDIET between AOE and DNE in the bulk solution played an important role in the bioelectrochemical nitrogen removal.



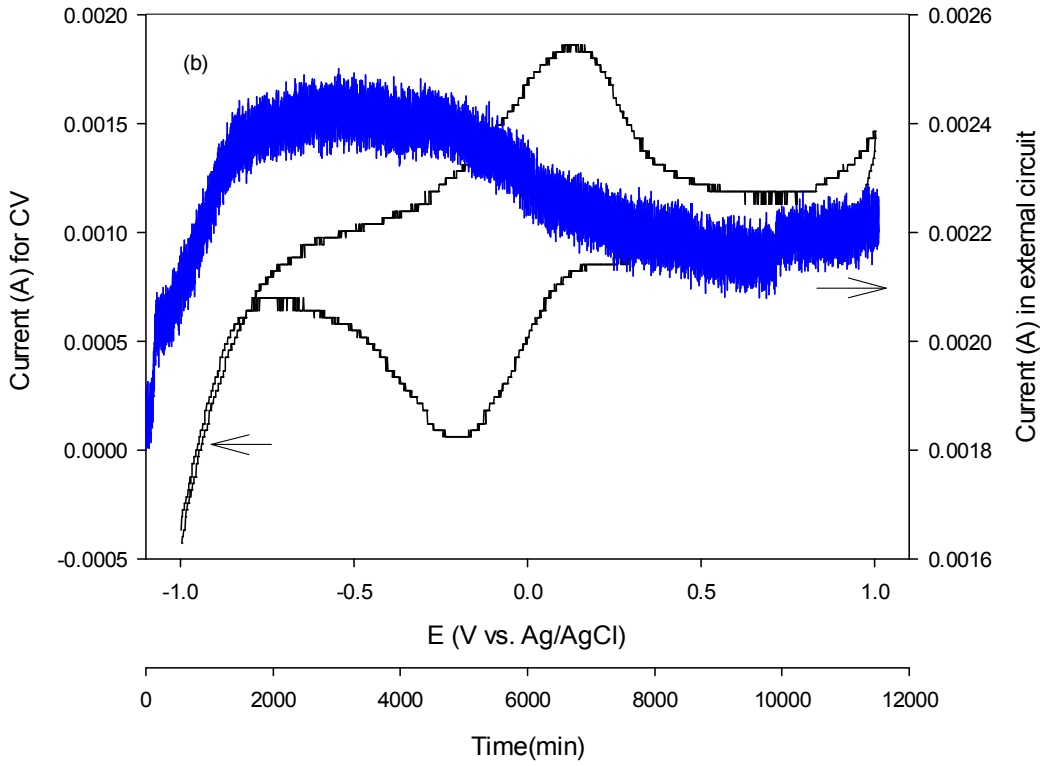


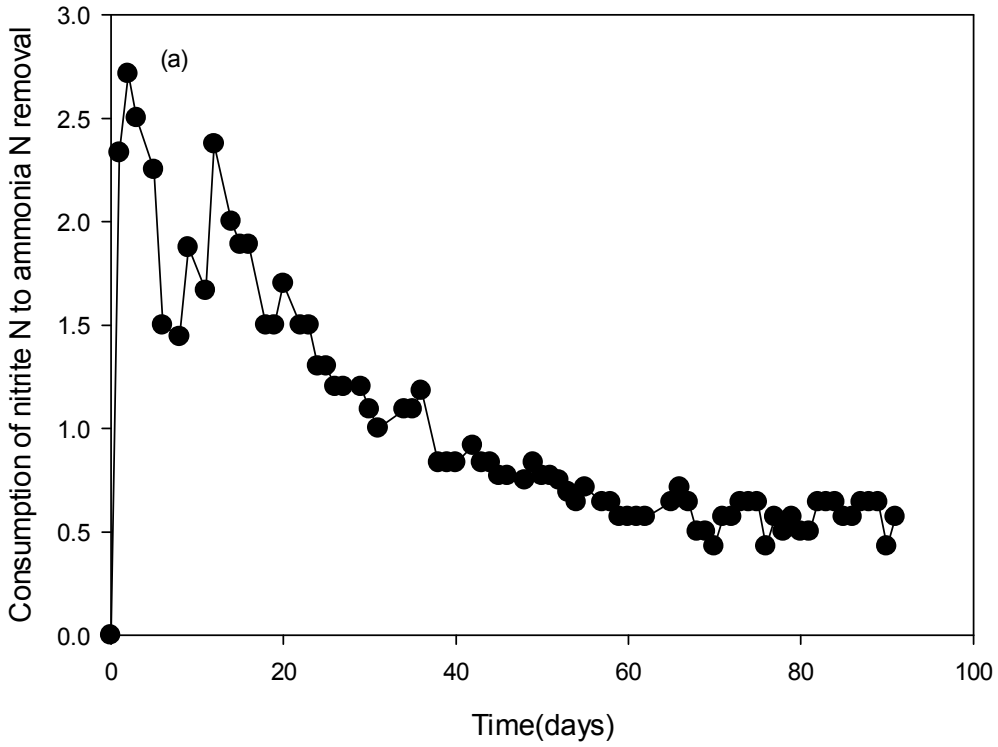
Fig.3.2 (a) Biogas gas production and the composition, (b) current and cyclic voltammogram for bulk solution during the 9th batch cycle of bioelectrochemical reactor.

The electric circuit was opened from the 92nd days to confirm the role of the polarized electrode on the electron transfer. The removals of ammonium and nitrite were stopped and the alkalinity and pH were no longer decreased, indicating that the driving force for the bDIET as well as the eDIET is the polarization of the electrode. It has been revealed that the electric field affects the electron transfer by changing the free energy changes (Lu et al., 2004; Yang et al., 2018). In the bioelectrochemical reactor, an electric field is formed between the polarized electrodes. The electric field is likely to promote the bDIET between the AOE and DNE. This is similar to the bDIET for methane production that observed in the bulk solution (Feng et al., 2018). In the bulk solution, after the repetition of the sequencing batch operation, the VSS was stabilized

to 2,000 mg/L, but the VSS decreased exponentially when the electric circuit was opened (Fig. 3.4a). This suggests that the microbial communities, including AOE and DNE, in the bulk solution are composed of absolute electroactive microorganisms that require an electric field to transfer the electrons for their energy metabolism.

3.3.2 Nitrite and alkalinity consumption for anaerobic ammonia oxidation

The consumption of nitrite required for ammonium removal was over 2.5 mg NO_2-N/mg NH_4-N for the first batch cycle but gradually decreased to 0.58 mg NO_2-N/mg NH_4-N as the batch cycle was repeated several times (Fig. 3.3a).



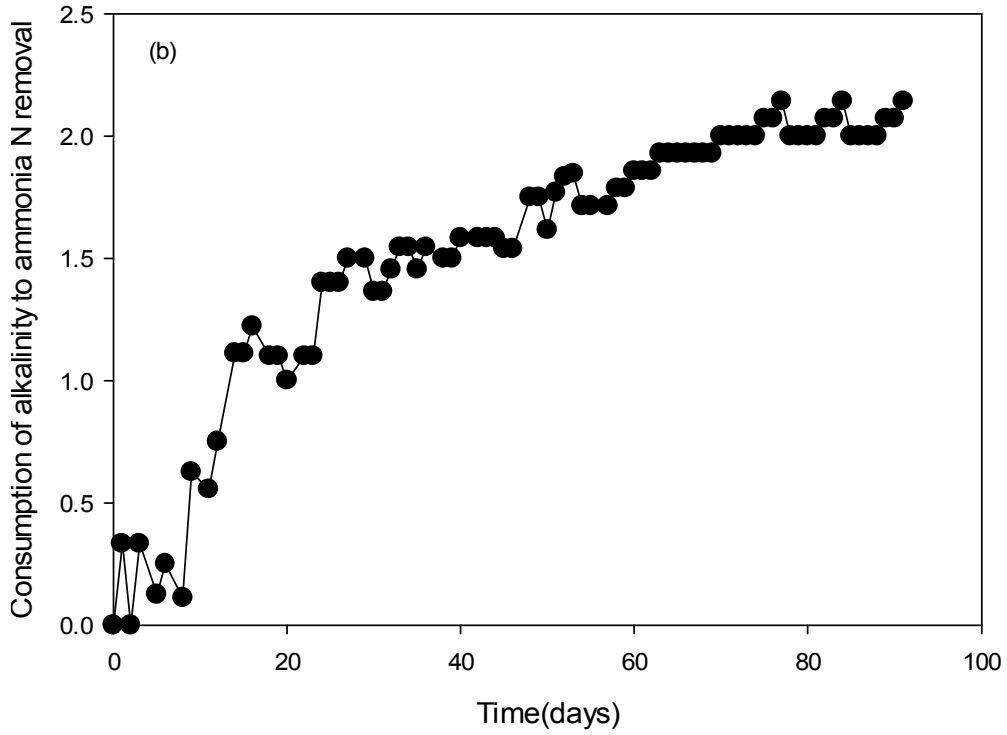


Fig.3.3 (a) Nitrite N and (b) alkalinity consumptions relative to the removal of ammonia N with operation time of the bioelectrochemical batch reactor.

In Anammox reaction, the amount of nitrite required for the ammonium removal is 1.32 mg NO_2-N/mg NH_4-N (Zhang et al., 2008; Jetten et al., 2009; Zhang et al., 2016). In the bioelectrochemical reactor, the amount of nitrite required for the ammonium removal was only 44% of the Anammox process, which is one of the great advantages of the bioelectrochemical nitrogen removal. The large requirement of nitrite for ammonia removal is a limiting factor for the wide application of Anammox process for nitrogen-rich wastewater (Jetten et al., 2009; Zhang et al., 2016; Szatkowska et al., 2014). The VSS concentration in the bioelectrochemical reactor, inoculated with the anaerobic sludge from sewage treatment plant, was initially 7,000 mg/L, but gradually decreased to 2,000 mg/L with the repetitions of the sequential batch operation (Fig. 3.4a). The bicarbonate alkalinity in the artificial wastewater was the carbon

source available for autotrophic bacteria in the bioelectrochemical reactor, but there were no carbon sources for heterotrophic bacteria. It is likely that the VSS was decreased by the lysis of the heterotrophic bacteria. This suggests that the large consumption of nitrite in the earlier sequential batch cycles was attributed to the oxidations of organic matter as well as ammonium. With the repetition of the sequential batch operation, the decrease in the nitrite consumption indirectly indicates that the dominant microbial group was gradually changed from heterotrophs to autotrophs in the microbial community. However, it seems that the amount of nitrite required for the ammonium removal was partly reduced in the bioelectrochemical reactor by the methane production, compared to the Anammox.

Contrary to the nitrite requirement for the ammonium removal, the alkalinity consumption increased from an initial 0.33 mg as $CaCO_3$/mg NH_4-N to 2.00 mg as $CaCO_3$/mg NH_4-N as the sequential batch operation was repeated (Fig. 3.3b). It seems that autotrophic microorganisms were getting more abundant with the repetition of the sequential batch operation. In the Anammox, the amount of alkalinity required for the ammonium removal is only 0.24 mg as $CaCO_3$/mg NH_4-N (Jetten et al 2009; Zhang et al 2016; Szatkowska et al., 2014). The large consumption of alkalinity in the bioelectrochemical reactor suggests that the growth rates of AOE and DNE are possibly higher than the Anammox bacteria.

The specific removal rate of ammonium in the bioelectrochemical reactor gradually increased and stabled at 34.8mg NH_4-N/g VSS/d (Fig. 3.4b). In Anammox process, the specific removal rate of the ammonium is 43.1-193 mg NH_4-N/g VSS/d (Zhang et al 2016; Szatkowska et al., 2014). Although the specific removal rate of ammonium in the bioelectrochemical reactor was lower than the Anammox, the rate could be further improved by optimizing the conditions such as temperature, pH, substrate, and applied voltage.

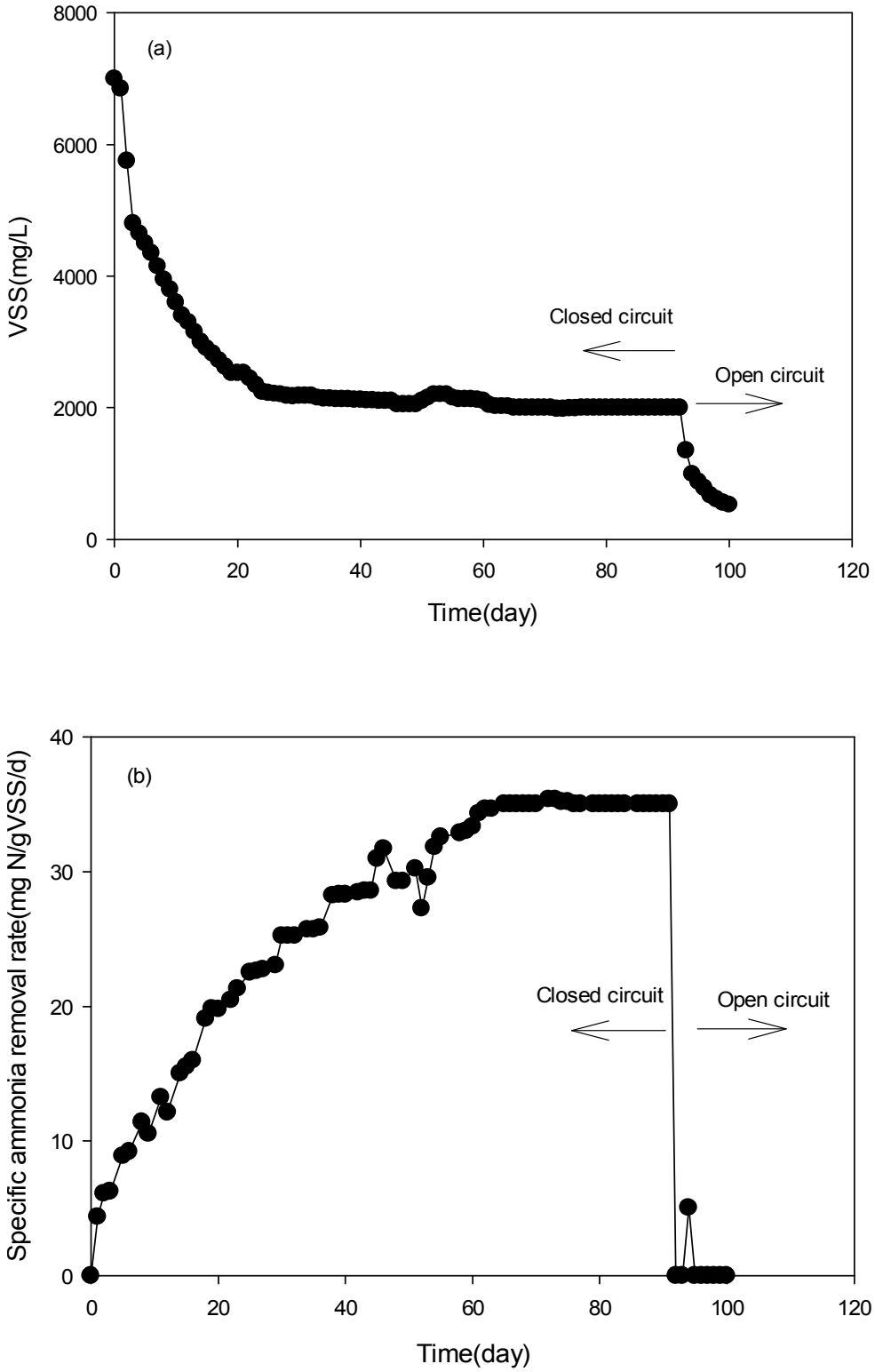


Fig.3.4 Changes in (a) VSS and (b) specific ammonia removal rate with operation time in bioelectrochemical reactor.

For the nitrogen balance in the bioelectrochemical reactor, the average removals of ammonium and nitrite estimated from the last three batch cycles were 35.0 mmoles for NH_4-N and 20.2 mmoles for NO_2-N, respectively, and the number of nitrogen moles recovered as biogas was only 23.3 mmoles (Table 3.1). This indicates that the percentage of the nitrogen in the forms of ammonium and nitrite converted to nitrogen gas is 84.4%. In the electron balance, the electrons released from the NH_4-N oxidation are 105.0 mmoles. The number of electron moles recovered as N_2 gas by reducing the NO_2-N was 69.9 mmoles, and the electrons of around 7.7 mmoles was converted to methane by reducing the carbon dioxide. This indicates that the percentage of electrons converted into biomass or lost was 26.1% (Table 3.1).

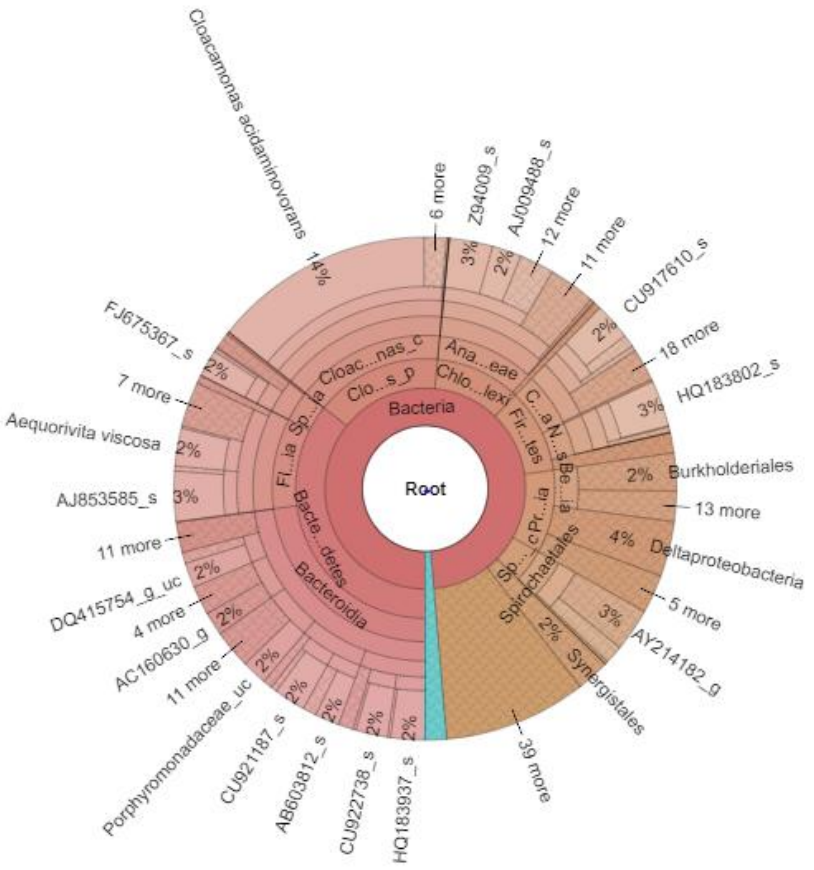
Table 3.1 Nitrogen and electron balance in the bioelectrochemical nitrogen removal

Contents	NH_4-N removal	NO_2-N removal	N_2 gas production	CH_4 gas production	Others (biomass, losses, etc)
Nitrogen (10^{-3} mole)	35.0	20.2	46.6	-	8.6
Electron (10^{-3} mole)	105.0	-	69.9	7.7	27.4

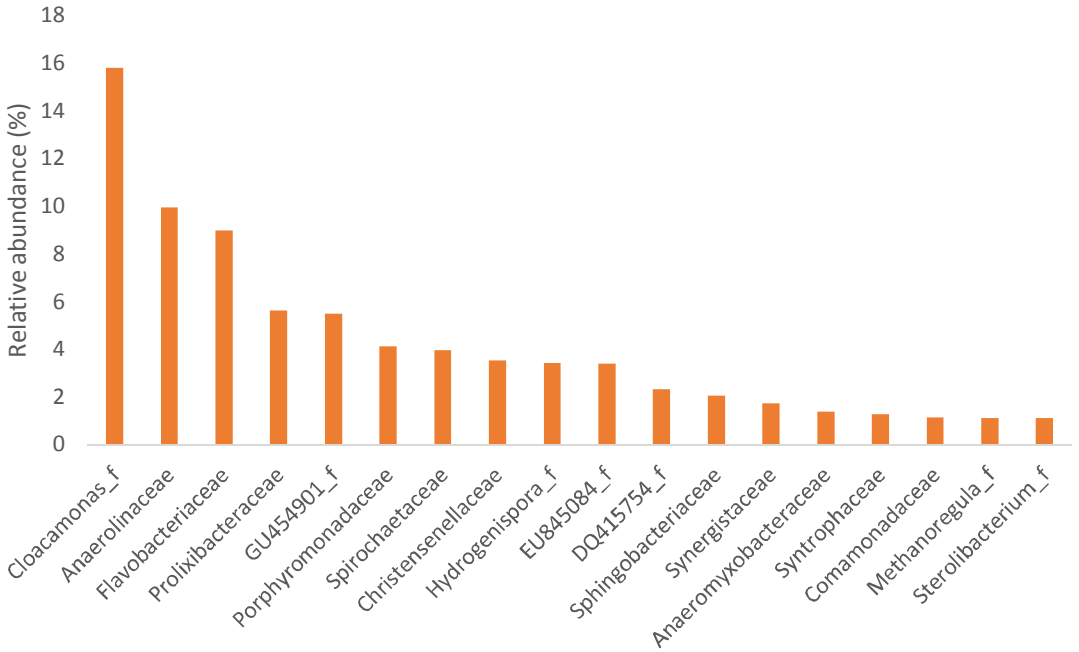
3.3.3 Microbial communities

In the bacterial communities, the known species of ammonia oxidation microorganisms were not observed, but there were lots of unidentified species. In the bacterial community, the most dominant phylum was Bacteroidetes (35.7%) and followed by Cloacamonas_p (15.8%), Chloroflexi (10.7%), Firmicute (10.5%), Proteobacteria (10.4%), and Spirochaetes (4.2%). In

the genus level, *Cloacamonas* (15.6%) were the most abundant and followed by *Vitellibacter* (3.5%), *Aequorivita* (2.6%), and *Draconibacterium* (2.4%) (Fig 5 a,b). However, the unidentified genera including *AJ009469_g* (6.9%), *AB239481_g* (3.2%), *EU878324_g* (3.0%) and *AY214182_g* (2.8%) were also abundant. Genus *Brocadia* known as Anammox bacteria was only 0.005%, and other Anammox bacteria such as *Kuenenia, Scalindua, Jettenia, Anammoxoglobus* were not observed (Zhang et al., 2008). At the species level, *Cloacamonas acidaminovorans* (CU466930, 100% 16S rRNA similarity) represented 14.2%, followed by *Aequorivita viscosa* (jgi.1076166, 97.6% 16S rRNA similarity) represented 2.4% of the total bacteria population.



(a)



(b)

Fig 3.5. Bacterial community (a) composition (b) family level in the bioelectrochemical reactor for nitrogen removal.

C. acidaminovorans are a syntrophic bacterium that takes the carbon and nitrogen for growth from the fermentation of amino acid. *C. acidaminovorans* are the species commonly observed from a bioelectrochemical reactor for methane production (Feng et al., 2016). It is a possibility that *C. acidaminovorans* are involved in the ammonia oxidation under bioelectrochemical anaerobic condition. *A. viscosa* is a member of Flavobacteriaceae family, and are the aerobic bacteria producing H_2S (Liu et al., 2013). *Vitellibacter aquimaris* (JRWG01000018, 97.4% 16S rRNA similarity) are known as the bacterial species reducing the nitrous oxide to nitrogen gas (Thevarajoo et al., 2017). It is likely that nitrous oxide is one of the intermediates in the bioelectrochemical nitrogen removal. Genus *Draconibacterium* is known as a chemoorganotrophic facultative anaerobic bacteria, but their role involved in nitrogen removal was not reported yet (Du et al., 2014). However, genus *Nitrosomonas* known as AOB was less than 0.01% (Hatzenpichler 2012). During the whole experimental period, the bicarbonate and

ammonia were the only carbon source and electron donor contained in the artificial wastewater. It seems that the unidentified bacterial species in the bioelectrochemical reactor are also autotrophic AOE and DNE or have a syntrophic relation with them.

Fig 3.6. Archaeal community in the bioelectrochemical reactor for nitrogen removal.

The diversity and evenness of the archaeal community were less than the bacteria. In the archaeal community, the most dominant phylum was Euryaechaeota (95.1%) and followed by Bathyarcheaota (4.8%), Thaumarchaeota (0.2%), and Bacteroidetes (0.01%). In the genus level, *Methanomassiliicoccus* (33.9%), *Methanosaeta* (11.0%), *Methanocorpusculum* (10.3%) and *Methanoculleus* (3.6%), *Methanobacterium* (2.9%) were dominant and the unidentified genus including *LNJC_g* (27.0%) and *AF424768_g* (4.6%) were also abundant. In the species level, the most dominant *LNJC_s* (LNJC01000028, 99.0% 16S rRNA similarity) (26.4%) were unidentified species and followed by *Methanomassiliicoccus luminyensis* (CAJE01000013, 98.1% 16S rRNA similarity) (23.7%), *Methanocorpusculum bavaricum* (AUMX01000033,

99.0% 16S rRNA similarity) (10.1%), *Methanosaeta concilii* (CP002565, 99.1% 16S rRNA similarity) (9.6%), *Methanomassiliicoccus intestinalis* (CP005934, 97.2% 16S rRNA similarity) (9.3%) and *Methanoculleus receptaculi* (DQ787476, 97.4% 16S rRNA similarity) (2.6%). *M. luminyensis* are the species producing methane from methanol with hydrogen as electron donor (Dridi et al., 2012). *M. bavaricum* are abundant hydrogenotrophic methanogens in bioelectrochemical reactor (Zellner et al., 1989). *M. concilii* is the acetoclastic methanogens that use acetate and carbon dioxide as the carbon source (Patel and Sproot 1990). It is known that *M. concilii* is an electrotrophic methanogens (Rotaru et al., 2014). *M. receptaculi* are the hydrogenotrophic methanogens (Cheng et al., 2008). However, *Nitrosopumilus maritmu*s as known AOA were not observed but genus *Nitrososphaera* was noted less than 0.1%. It revealed that the chemolithotrophic growth species using ammonia are belonged to *Thaumarchaeota* phylum. However, the abundance of *Thaumarchaeota* was only 0.02% (Hatzenpichler 2012). It seems that some of the unidentified species are AOA. In the bioelectrochemical reactor, a small portion of the biogas was the methane. It is possible that the methanogens including *M. luminyensis, M. bavaricum*, *M. concilii*, *M. intestinalis*, *M. receptaculi* produce methane by taking the electrons from the ammonia oxidizer such as AOB or AOA (Zhan et al., 2014; Feng et al., 2017). However, the nitrite inhibits methanogenesis at a low COD/NO_2^-, and the methane is produced after the nitrite reduction (Akizuki et al., 2015). It is likely that the dominant unidentified archaeal species, *LNJC_s*, are the DNE in the bioelectrochemical reactor, but further study is needed.

3.3.4 Implications

The nitrogen overabundance in natural water system causes serious water environmental problems such as algal bloom (Angenent et al., 2004; Ghafari et al., 2008). The removal of excess nitrogen in wastewater before the discharging into the rivers or sea is a major issue to

protect the natural water environment. The nitrogen in wastewater has been generally removed by biological nitrogen removal process, which is a consecutive step of nitrification and denitrification (Ahn 2006; Ge et al., 2015; Nancharaiah et al., 2016). However, the autotrophic nitrifying bacteria can be dominated after the organic matter is removed and the large amounts of oxygen and alkalinity are required for the nitrification (Ahn 2006; Nancharaiah et al., 2016). In addition, the carbon source as an electron donor is needed for the denitrification. In recent years, the economical nitrogen removal processes such as short-cut nitrogen removal or Anammox process have been introduced to reduce the requirements of oxygen, alkalinity, and carbon source (Ge et al., 2015; Ma et al., 2016; Pelletier et al.,2008). These nitrogen removal processes generally require the selective production of nitrite. For the nitrite production, it requires the growth of AOB dominantly by the suppression of NOB (Ma et al., 2016; Nancharaiah et al., 2016).

In this study, the simultaneous removal of ammonium and nitrite was found in an anaerobic bioelectrochemical reactor in which anaerobic sludge was inoculated and a small voltage of 0.6V was applied to the electrodes. The bioelectrochemical nitrogen removal does not produce nitrate as a by-product, and the consumption of nitrite as an electron acceptor is smaller, compared to Anammox. It seems that the nitrogen is removed by the DIET between AOE and DNE in the bioelectrochemical reactor. The ammonium was the only electron donor, and nitrite and carbon dioxide were the electron acceptors in the bioelectrochemical reactor. In the electron balance, the electrons transferred from the ammonium into nitrogen gas and methane were 66.6% and 7.3%, respectively, and the remaining was used for cell synthesis or was lost during the electron transfer process. In previous studies (Qu et al 2014; Zhan et al., 2013; Huang et al., 2013), the ammonium and nitrite were removed by the anodic oxidation and the cathodic reduction, respectively, in the bioelectrochemical reactor. However, the amount of nitrogen removed via the electrodes estimated from the current in the external circuit was only

23.3% of the total nitrogen removal. Intriguingly, the oxidation and reduction peaks for the suspended microorganisms were observed in the cyclic voltammogram, suggesting that there are electroactive microorganisms, including the AOE and DNE, in the bulk solution. This implies that the bDIET between AOE and DNE in the bulk solution is the main electron transfer pathway for the nitrogen removal in the bioelectrochemical reactor.

The amount of nitrite required for the ammonium removal was 0.58 mg NO_2-N/mg NH_4-N, which was about 44% in Anammox. This is a big advantage of bioelectrochemical nitrogen removal compared to the Anammox. However, the alkalinity consumption relative to the ammonium removal was around 2.0 mg as $CaCO_3$/mg NH_4 -N, more than the Anammox. During the experimental period, the NO_3-N was not observed in the bioelectrochemical reactor. This indicates that the nitrogen could be completely removed in the bioelectrochemical reactor. The specific activity of ammonia removal in the bioelectrochemical reactor was 34.8mg N/g VSS/d, which was lower than 43.1-193 mg N/g VSS/d of the Anammox (Zhang et al., 2016; Fernandez et al., 2008). The further studies on the optimal environmental conditions are needed to improve the removal rate of NH_4–N and to reduce the alkalinity requirement in the bioelectrochemical reactor. It is well known that the electron transfer depends on the voltage of polarized electrode in the bioelectrochemical reactor. This indicates that the nitrogen removal rate can be further improved by controlling the applied voltage to the electrode. The bioelectrochemical nitrogen removal is a novel economic and energy saving process that can complement the disadvantages of shortcut processes for nitrogen-rich wastewater.

3.4 Conclusions

A bioelectrochemical anaerobic nitrogen removal, which is different from the Anammox, is demonstrated in a bioelectrochemical anaerobic reactor equipped with a pair of bioelectrode polarized. The requirements of nitrite and alkalinity for the removal of ammonium are around

0.58 mg NO_2-N/mg NH_4-N and 2.0 mg as $CaCO_3$/mg NH_4-N, respectively, and the bioelectrochemical nitrogen removal does not produce nitrate as a by-product. The bacterial groups involved in the bioelectrochemical nitrogen removal are the autotrophic AOE and DNE, which are enriched in the bulk solution from anaerobic sludge by the polarized bioelectrode. The ammonium and nitrite are simultaneously removed in the bulk solution by the bDIET mainly between the AOE and DNE. A bioelectrochemical nitrogen removal is a novel approach recommended for treatment of nitrogen-rich wastewater.

Chapter 4 - Electroactive microorganisms enriched from activated sludge for bioelectrochemical nitrogen removal

4.1 Introduction

The nitrogen overabundance in natural water system causes serious water environmental problems such as eutrophication (Angenent et al., 2004; Ghafari et al., 2008). So biologically nitrogen removal technologies were widely used to remove the nitrogen from wastewater and protect natural water quality. In conventional wastewater treatment plant, ammonia nitrogen is oxidized to nitrite and nitrate nitrogen by autotrophic nitrification under aerobic condition. The nitrate nitrogen is reduced into nitrogen gas by heterotrophic denitrification, using electrons donated by organic matter (Ahn 2006). However, this process requires a significant amount of oxygen and alkalinity for nitrification and carbon source as an electron donor for the denitrification. Sharon process is one of the short cut nitrogen removal technology that was introduces to mitigate the excess burden of oxygen, alkalinity and carbon source in the conventional BNR (Biological Nitrogen Removal) process (Ahn 2006; Shalini and Joseph, 2012; Ge et al., 2015; Nancharaiah et al., 2016). The shortcut nitrogen removal is a process oxidizing ammonium into nitrite and then reducing the nitrite into nitrogen gas. The nitrite required for the shortcut nitrogen removal is obtained by the nitritation of ammonia. Ammonia is generally oxidized into nitrite by ammonia oxidation bacteria (AOB) such as *Nitrosomonas* and the nitrite oxidized into nitrate by the nitrite oxidizing bacteria (NOB) such as *Nitrobacter* (Ahn, 2006; Shalini and Joseph, 2012; Nancharaiah et al., 2016). Increasing temperature facilitate AOB to out compete NOB, and the optimum temperature for AOB and NOB are 35^{o}C and 38^{o}C, respectively (Shalini and Joseph, 2012; Ahn 2006; Ge et al., 2015). Recently, Anammox became the promising energy-neutral process for nitrogen removal from wastewater.

Anammox bacteria oxidize ammonium with nitrite as an electron acceptor. The anammox is more sustainable process than the conventional nitrification and denitrification process (Chen et al., 2011; Jin et al., 2013; Du et al., 2015). However, Anammox bacteria are slow in growth rate, and requires the nitritation of ammonium or partial denitrification from nitrate to obtain the nitrite, and is difficult to the removal of nitrogen completely due to the nitrate generation as a product of Anammox reaction.

In recent, a bioelectrochemical technology that activates direct electron transfer between bacterial species with the help of electrical energy has been attracted much attention (Feng et al., 2016; Blasco-Gómez et al., 2017). When the electron acceptors are outside the cells under anaerobic condition, some bacteria, called exoelectrogens, can transfer electrons to the external electron acceptor through c-type cytochrome that is over-expressed up to the outer membrane (Kiely et al., 2011; Lovely, 2011; Feng et al., 2017). Another type bacterium, called as electrotrophs, can accept the electrons directly from the exoelectrogens or electron donors outside the cell (Lovely 2011; Schroder and Harnisch 2017). Ammonium can be bioelectrochemically oxidized under anaerobic condition by exoelectrogens to generate the electrons (He et al., 2009; Qu et al., 2014; Zhan et al., 2014). In addition, some of electrotrophs use the oxidized forms of nitrogen as the electron acceptors, and reduce the electrons generated from the ammonium oxidation by exoelectrogens into nitrogen gas (Zhan et al., 2012; Huang et al., 2013; Kondaveeti et al., 2014). This implies that an anaerobic nitrogen removal reaction similar to anammox can be expected through the direct interspecies electron transfer between the exoelectrogens and the electrotrophs in bioelectrochemical reactor. The electroactive bacteria can be enriched selectively by the control of the polarized electrode potential. There is also great possibility to enrich the ammonium oxidizing exoelectrogens (AOE) and the denitritating electrotrophs (DNE) in bioelectrochemical reactor. In the bioelectrochemical

reactor, it is expected that the nitrogen could be efficiently removed by the DIET pathway between the AOE and DNE.

In this study, bioelectrochemical nitrogen removal was demonstrated by enriching the electrochemically active microorganisms from activated sludge inoculum in an anaerobic bioelectrochemical reactor with a pair of polarized bioelectrodes. The ratio of nitrite and alkalinity to ammonium for the bioelectrochemical nitrogen removal was estimated, and the microbial community involved in the bioelectrochemical nitrogen removal were also identified.

4.2 Experimental methods and analysis

4.2.1 BES configuration and operation

Anaerobic bioelectrochemical reactor (diameter 10 cm, height 16 cm, effective volume 1L) of anode (7cm × 10 cm) and cathode (7cm × 10cm) facing each other were installed at an interval of 5cm inside the reactor, which was prepared with acrylic resin. As described in earlier studies (Feng et al., 2018; Feng et al., 2016; Feng et al., 2017; Song et al., 2018) the anode and cathode were constructed by coating the surface of a GFF (Graphite Fabric Fiber, Samjung C&D Co., South Korea) with MWCNT (Multiwall Carbon Nanotube, Carbon Nano-material technology Co., Ltd., South Korea). The MWCNT were pre-treated in concentrated nitric acid solution for 24 hours to improve hydrophilicity and to remove impurities and then washed under running tap water until the pH was noted as neutral. 1g MWCNT, 0.25g nickel chloride, and 0.5g polyethyleneimine were dissolved into the 1L distilled water to prepare the electrolyte solution. For the electrophoretic deposition, the GFF sheet and a stainless-steel mesh (STS, 316L) were used as the working and counter electrodes by depositing the MWCNT and Ni on the GFF surface by applying 30 V for 30 min using a direct current (DC) power source (OPM series, ODA technologies Co. Ltd, Incheon, South Korea). Both anode and the cathode electrodes inside the

bioelectrochemical reactor were connected to a DC power supply (OPM series, ODA (Technologies Co. Ltd, Incheon, Korea) using conductive wire.

The anaerobic reactor was prepared by covering the top with the acrylic resin plate which have one biogas sampling port and one liquid sample collection port which were covered with butyl rubber stopper. The biogas sampling port was connected to a floating type gas collector that was filled with water - acidified with sulphuric acid and saturated with salt - to prevent the resolution of biogas (Feng et al., 2017) and the liquid sample port have a tube extended into the reactor until it was submerged inside the waste wastewater. The synthetic wastewater was prepared as follows 0.3 g/L KH_2PO_4, 1.0 g/L $Na_2HPO_412H_2O$, 0.5 g/L NaCl, 2.0 g/L $NaHCO_3$, 0.1 g/L $MgSO_47H_2O$, 0.01 g/L $CaCl_2$, and 1.91 g/L NH_4Cl, as in previous study (Zhan et al., 2012). The reactor was setup with the synthetic waste water (0.460 L) and inoculum (0.540 L). The activated sludge, used as the inoculum was collected screened to remove impurities by thickening with the gravitational force for a day.

The VSS (Volatile Suspended Solids) and pH of the activated sludge as inoculum was noted as 14,000mg/L and 7.89, respectively. The reactor was maintained at the applied voltage of 0.6V between the electrodes. The reactor was operated at room temperature with constant stirring using a magnetic bar at 500rpm. The initial concentration of NH_4-N and NO_2-N was 500 mg/L and 300 mg/L. NO_2-N was supplemented when the concentration decreased to a smaller level and when the concentration of NH_4-N was decreased, the synthetic wastewater was replaced with a fresh one after settling suspended sludge for 30 minutes. After the reactor got stabilized, the batch experiment was conducted in open circuit to investigate the role of polarized electrodes.

4.2.2 Analytical techniques and calculation

The pH was monitored everyday using a pH meter (Orion Model 370) and alkalinity was measured by titration with 0.02M HCl to a pH of 4.5, respectively in the bioelectrochemical

reactor. NH_4 -N, NO_2 -N, and VSS were measured every day according to the Standard methods for the examination of water and wastewater (APHA, 2005). The anode and cathode potentials were intermittently measured against an Ag/AgCl reference electrode (RE-1B, ALS Co., Ltd, Japan) using a portable digital multimeter (DM-1010, Dong Hwa Electronics, Co., Korea). The current in the external circuit was monitored using a digital multimeter (DMM, Ni cDAQ-9174, National Instruments), installed between the electrodes and the DC power source. The percentage of NH_4-N oxidized by the AOB on the anode surface was estimated in the bioelectrochemical reactor through the total amount of NH_4-N removed and the current in the external circuit. The equation as follows,

$$= \frac{\int idt}{nF(\Delta NH_4 - N)V} \times 100 \qquad (4.1)$$

Where, i is the electric current (A), n is the number of electrons for NH_4-N oxidation to NO_2-N, F is the Faraday constant (96,485C/e^- mole), ΔNH_4-N is the concentration of NH_4-N removed, and V is the liquid volume (1L) of the anaerobic batch reactor. Cyclic voltammogram (CV) for the bulk solution in the batch reactor was also obtained in the potential range of -1.0 V to 1.0 V (vs. Ag/AgCl) with a 10mV s^{-1} scan rate using the electrochemical instrument (ZIVE SP1, WonA Tech, South Korea). For the cyclic voltammetry test, stainless meshes (1cm × 1cm) were used as the working and counter electrodes. The peak currents and peak potentials of oxidation and reduction were obtained from cyclic voltammetry data using the analysis software, 'SMART Manager' (Zive Lap Wonatech, co., Korea)

The biogas production was monitored using the floating type gas collector, and the biogas composition was analyzed every day using a GC (Gaw-Mac Instrument Co., PA, USA) with Porapak-Q column (6 ft×8^{th} SS) and thermal conductivity detector. The biogas production including nitrogen, methane and carbon dioxide was estimated from the measurement of the

biogas volume and the biogas composition and converted to the volume at standard temperature and pressure (STP) using Eq. (4.1) (Feng et al., 2017).

$$V_{Biogas\ at\ STP}(L) = V_{Biogas\ at\ T} \times \frac{273}{273+T} \times \frac{760-W}{760} \quad (4.2)$$

Where, T is the experimental temperature (25^{o}C), and W is the water vapor pressure at that temperature (23.76 mm Hg). In the electron balance for the nitrogen removal, the total electrons released from the electron donor was obtained from the mole number of the NH_4-N removal. The electrons converted into nitrogen gas via the biological DIET in the bulk solution was estimated by subtracting the number of electrons transferred via the electrode (eDIET) from the total electrons consumed for nitrogen gas production. The difference in the electron moles contained in between the NH_4-N removed and nitrogen gas produced was considered to be the electrons converted into biomass or lost.

4.2.3 Bacterial community analysis

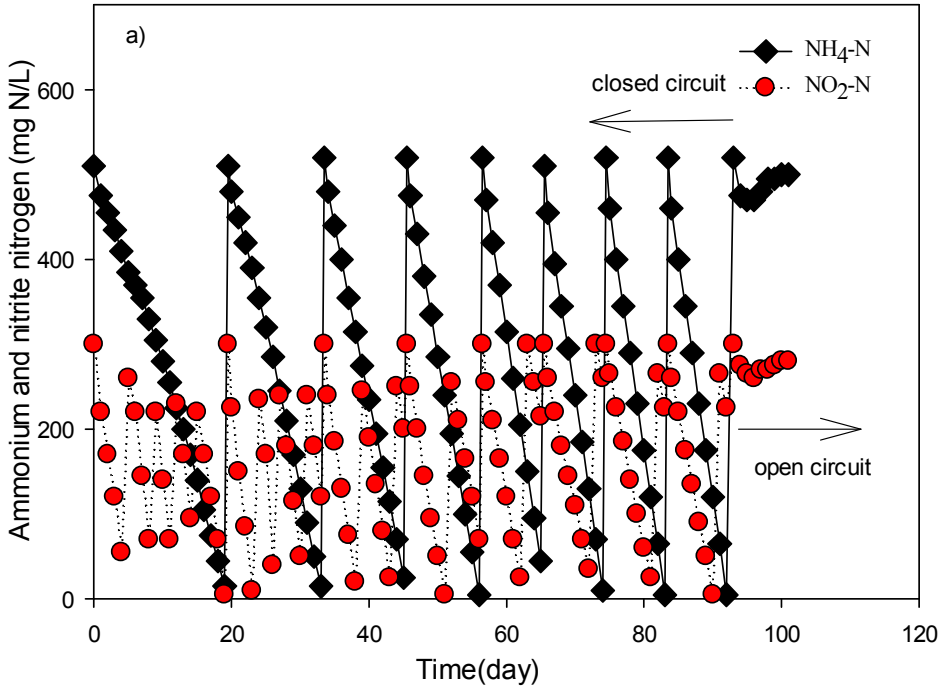
Microbiome Taxonomic Profiling was performed to investigate 16S rRNA bacterial communities in the bioelectrochemical reactor by collecting the samples from the reactor after it is allowed to settle at the end of the close circuit experiment. The fusion primer is used to amplify the variable region (V3V4) of the bacterial 16S rRNA gene in the genomic DNA (Hur et al., 2011). The 16S rRNA was amplified from the metagenomic DNA and pooled for sequencing on the MiSeq Personal Sequencer (Illumina, San Diego, CA, USA). The amplification, construction of the sequencing library, and sequencing and bioinformatic analysis were performed using previous studies (Chun et al., 2010). Chimera was checked and taxonomic assignments of these readings were done using the extended EzTaxon database (http://eztaxon-e.ezbiocloud.net/). Microbial community and the statistical taxonomical assignments were obtained through the operational taxonomic units. Comprehensive bioinformatic analysis like species-level classification of microbes, cluster analysis, microbial

origin tracking, fast unifrac analysis of inter-sample diversity, hierarchical clustering and various indicators of species diversity was conducted by CLcommunity software (Chunlab, Inc., Seoul, Republic of Korea).

4.3 Results and discussion

4.3.1 Bioelectrochemical nitrogen removal

The simultaneous removal of NH_4-N and NO_2-N were observed in the anaerobic bioelectrochemical reactor when a pair of electrodes are polarized at 0.6 V after the reactor was inoculated with activated sludge (Fig. 4.1a). This indicates that the NH_4-N and NO_2-N were removed by the redox reactions in the bioelectrochemical reactor, which were induced by the polarization of the electrodes under anaerobic condition.



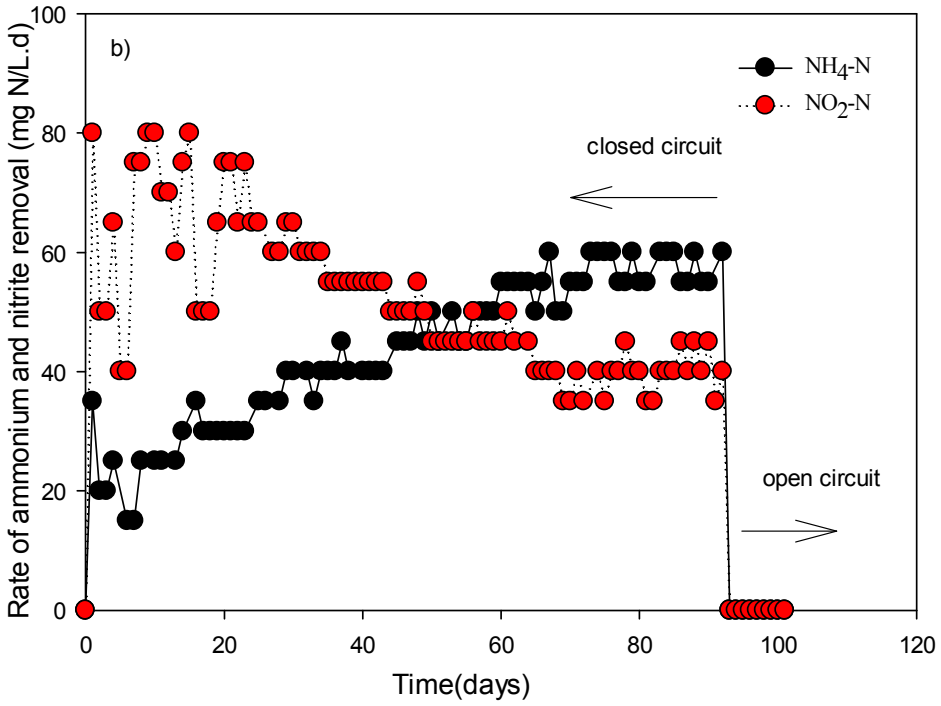
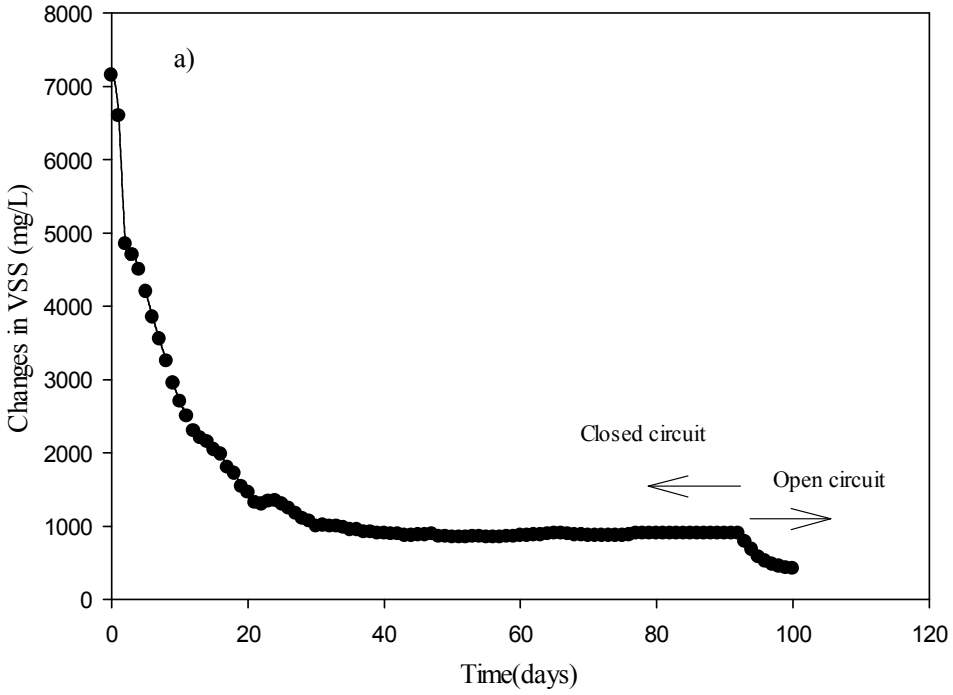


Fig.4.1 (a) Changes in ammonium and nitrite nitrogen and (b) their removal rates in bioelectrochemical batch reactor

There was no molecular oxygen as an electron acceptor in the anaerobic bioelectrochemical reactor except NO_2-N, although the artificial medium contained a small amount of SO_4^{2-}, added in the form of $MgSO_4.7H_2O$ as a macro nutrient. The NO_2-N was the main substance that can be used as an electron acceptor. In previous studies, the NH_4-N and NO_2-N were simultaneously removed under anaerobic condition only by Anammox microorganisms such as Genus *Brocadia, Kuenenia, and Scalindua* (Zhang et al., 2008; Sonthiphand et al., 2014). However, Anammox microorganisms grow slowly and can be enriched in the coastal sediment (Zhang et al., 2008; Engstorm et al., 2005). In general, Anammox microorganisms are not abundant in the activated sludges used as the inoculum. It was a very interesting finding that the NH_4-N and NO_2-N were immediately decreased after the start of the bioelectrochemical

reactor inoculated with the activated sludge. It suggests that some microbial species in the activated sludge plays a role in the oxidation of NH_4-N and the reduction of NO_2-N under anaerobic condition in the bioelectrochemical reactor with polarized electrodes.

The removal rate of NH_4-N was initially 20 mg NH_4-N/L. d and gradually increased to a stable value of about 57 mg NH_4-N/L.d. (Fig. 4.1b). The removal rate of NO_2-N was as high as 80 mg NO_2-N/L. d in the first cycle of the sequential batch operation but decreased to about 40 mg NO_2-N/L. d as the sequential batch operation was repeated. The bioelectrochemical reactor was started up with a high VSS concentration of 7,000 mg/L. However, the VSS was exponentially decreased during the initial three sequential batch cycles and then stabilized at 900 mg/L (Fig. 4.2a).



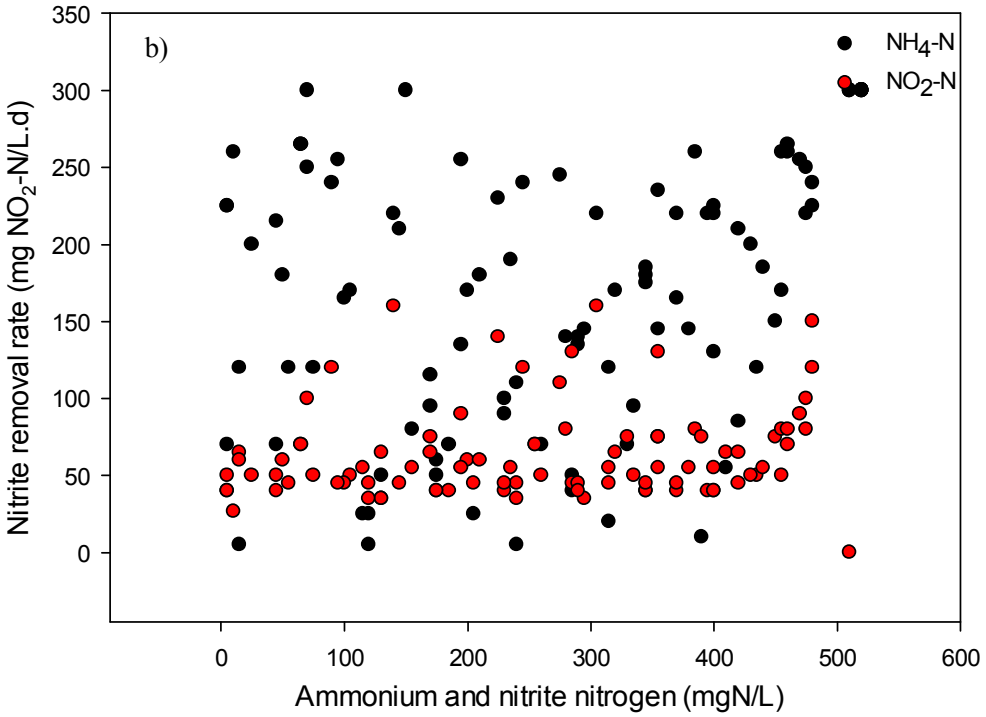


Fig.4.2 (a) VSS decreases in bioelectrochemical reactor and b) dependence of nitrite removal rate on the ammonium and nitrite nitrogen

The bioelectrochemical reactor was operated with the artificial medium without organic carbon source under anaerobic condition. It seems that most of the aerobic heterotrophs from the inoculated sludge in the bioelectrochemical reactor were decayed and so the organic matter from the heterotrophs were released into the bulk solution. The organic matter of the microbial cells consists mainly proteins, polysaccharides, lipids, DNA, RNA and other substances like sugar and amino acids (Tchobanoglous et al., 2014) which were used as the carbon source and electron donor. During the initial three batch operation cycles, the two main electron donors were the organic matter released from the decay of the aerobic heterotrophs and the NH_4-N supplied to the medium. However, NO_2-N was the main electron acceptor in the anaerobic bioelectrochemical reactor without molecular oxygen. The removal rate of NO_2-N was not

dependent on the NH_4-N or the NO_2-N during the whole experimental period (Fig.4.2b). This indicates that the bioelectrochemical nitrogen removal is a zero-order reaction to the NH_4-N and the NO_2-N.

During the early stages of the batch operational cycles, it shows that a large amount of NO_2-N was used as an electron acceptor for the oxidation of the organic matter released from the heterotrophs decay. During the operation of the bioelectrochemical reactor, the NO_2-N was supplemented to the initial value of 300 mg NO_2-N/L when the NO_2-N was decreased to a low value, indicating that the NO_2-N considerably was fluctuated in the bioelectrochemical reactor (Fig.4.1b). From the initial high fluctuations in the NO_2-N removal rate, it can be deduced that the oxidation of the organic matter was significantly influenced by the NO_2-N concentration.

In the bioelectrochemical reactor, the pH and the alkalinity were also decreased simultaneously with the removals of NH_4-N and NO_2-N. The pH and the alkalinity were decreased more as the repetition of the batch operation cycle and gradually stabilized from the third cycle (Fig.4.3a). The bicarbonate, a main component of the alkalinity at neutral pH, is the carbon source for autotrophs. This indicates that the ammonium oxidizing bacteria and the nitrite reducing bacteria in the bioelectrochemical reactor are the autotrophs.

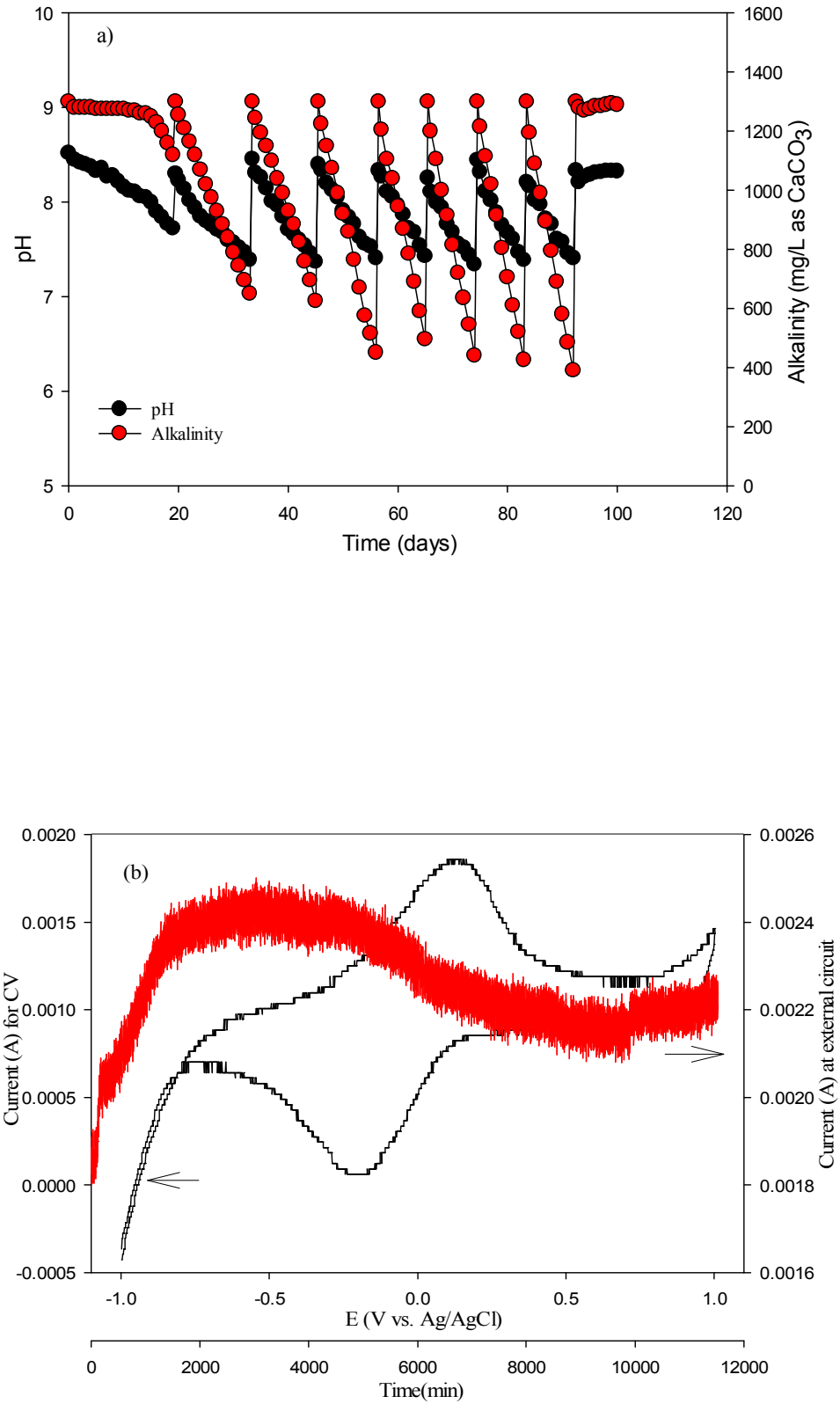


Fig.4.3 (a) Changes in pH and alkalinity and (b) cyclic voltammogram (black) and electric current (red) during the 6th batch cycle

At the end of the 6th cycle of the batch operation, cyclic voltammogram (CV) for the bulk solution was obtained to confirm the electrochemical activity of microorganisms in the bioelectrochemical reactor (Fig.4.3b). In the CV, the redox peaks were observed at 0.14 V vs Ag/AgCl for the oxidation and -0.20 V vs Ag/AgCl for the reduction, indicating the presence of electrochemically active substances in the bulk solution (Feng et al., 2016; Feng et al., 2017; Feng et al., 2018). In general, the electrochemically active substances include electroactive microorganisms such as exoelectrogens and electrotrophs, as well as the electron transfer mediators such as flavin compounds, quinone compounds, and humic substances (Newman and Kolter, 2000; Canstein et al., 2008). During the sequential batch operation of the bioelectrochemical reactor, the supernatant was replaced with the artificial medium prior to the start of every new batch cycle. However, the removals of NH_4-N and NO_2-N, as well as the decreases of pH and alkalinity did not significantly change after the 6th cycle, which is believed to be steady state (Fig.4.1, Fig.4.3a). This indicates that the redox peaks were originated from the electroactive microorganisms rather than the electron transfer mediators. This suggests that the electroactive species are the AOE and DNE.

During the 6th batch operation cycle, the electric current monitored increased from about 1.5 mA to 2.5 mA, and then slightly decreased again (Fig.4.3b). Generally, when the electroactive microorganisms are in the bulk solution, it is believed that the electroactive species are simultaneously enriched on the surface of polarized electrodes in the form of biofilm (Feng et al., 2018). The electric current in the external circuit monitored during the 6th batch cycle is evidence that AOE oxidizes the NH_4-N on the anode and DNE reduce the NO_2-N on the cathode. The electroactive microorganisms in the bulk solution can transfer the electrons directly by a direct contact for the electric connection between the cells, called as biological direct interspecies electron transfer (bDIET) (Feng et al., 2018; Song et al., 2019). The

electroactive microorganisms on the electrode surface mainly transfer the electrons directly through the electrode and the external circuit for their metabolism, called as the electrode mediated direct interspecies electron transfer (eDIET) (Feng et al., 2018; Song et al., 2019). In the bioelectrochemical reactor, it suggests that the NH_4-N and the NO_2-N are removed by the DIET between AOE and DNE in the bulk solution and on the electrode surface. It seems that the DIETs for the nitrogen removals in the bulk solution and on the electrode surface are driven by the electric field and the electric polarization of the electrodes. In order to confirm the role of the polarization of electrodes on nitrogen removal in the bioelectrochemical reactor, the external circuit was opened during the 9th batch cycle by disconnecting the electrodes with the voltage source. The decreases in NH_4-N and NO_2-N were abruptly disappeared, and the pH and the alkalinity were no longer decreased too (Fig.4.1a, Fig4.3a). This is an evidence that the electric polarization of the electrodes drives the electron transfer between AOE and DNE in the bioelectrochemical reactor.

At steady state, the specific removal rate of NH_4-N was 62.77 NH_4-N/g VSS.d. In the case of Anammox process, the specific removal rate of NH_4-N was in the range of 43.1-193 mg/L g VSS/d, depending on the operational parameter and environmental conditions (Zhang et al., 2016; Szatkonska et al., 2014). This suggests that the bioelectrochemical nitrogen removal process can compete with the Anammox process for nitrogen removal if optimized. However, in VSS, the biomass concentration of AOE and DNE, start to decrease at the open circuit condition. This suggests that the metabolisms of AOE and DNE are significantly dependent on the electric polarization of the electrodes. This can be a drawback in the bioelectrochemical nitrogen removal process, and it is necessary to study further to cope with power failure to put it into practical use in the field.

4.3.2 Nitrogen balance and electron transfer pathway

The nitrogen and the electron balances during the 6th batch cycle was evaluated for the NH_4-

N oxidation and the NO_2-N reduction in the bioelectrochemical reactor. The amount of NH_4-N removal was 500 mg NH_4-N, from initial 510 mg NH_4-N/L to the final 10 mg NH_4-N/L, in 1.0 L of the bioelectrochemical reactor (Fig.4.1a). However, the amounts of nitrogen and methane recovered as the biogas were 658.7 mL and 3.9 mL, respectively (Fig.4.4a). So, the number of moles removed for the NH_4-N and the NO_2-N were 35.7 mmoles and 21.8 mmoles, respectively. However, the nitrogen moles recovered as the biogas was 58.8 mmoles (29.4 mmoles as N_2 gas) (Fig.4.4b). The methane content was very small in the biogas. This means that most of the NH_4-N and the NO_2-N removed were converted to nitrogen gas. The imbalance between the removals of NH_4-N and NO_2-N and the product of nitrogen as biogas was 1.3 mmloes, which appears to be originated from the experimental errors. In the electron balance, the oxidation reaction releases 107.1 mmoles of the electrons from 35.7 mmoles of NH_4-N. The reduction reaction accepts 65.4 mmoles of the electrons for 21.8 mmoles of NO_2-N (Fig.4.4b). However, the number of the electrons used for the reduction of carbon dioxide to methane was only 1.4 mmoles. Thus, the unknown part of the electrons is 40.4 mmoles, which is 37.7% of the electrons released from the NH_4-N oxidation. It can be seen that the unknown part of the electrons was mainly used for the biomass synthesis or lost during the electron transfer process. However, there may be another electron transfer pathway between the electron donor and the acceptor for the nitrogen removal.

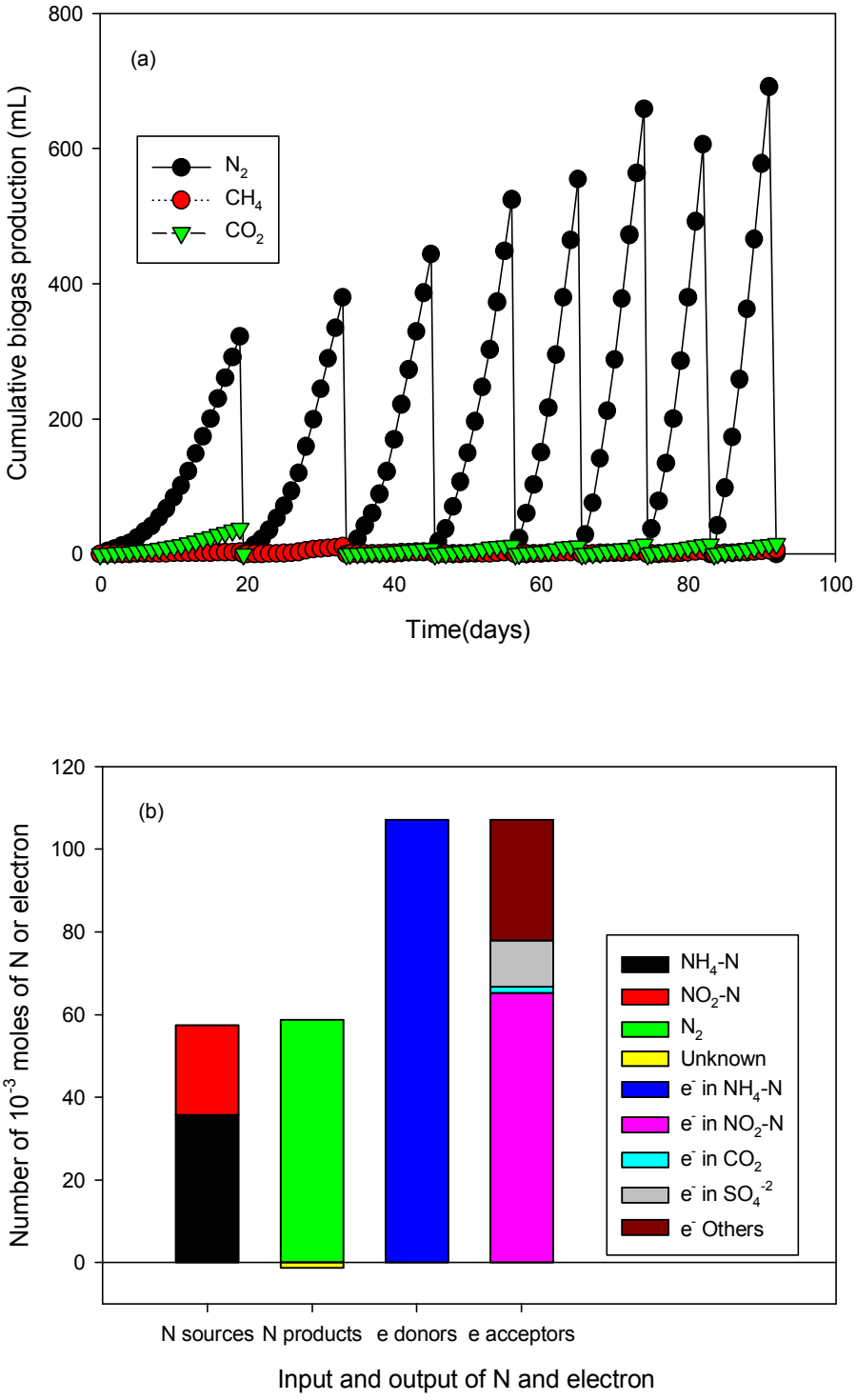


Fig.4.4 (a) Cumulative biogas production in the bioelectrochemical reactor and (b) nitrogen and electron balance during the 6th batch cycle

In the artificial medium, the sulfate was 39 mg/L, which was added in the form of $MgSO_4.7H_2O$ as a macro-nutrient. The sulfate can be used as an electron acceptor for the ammonia oxidation, as follow (Yang et al., 2009).

$$2NH_4^+ + SO_4^{-2} \rightarrow N_2 + S + 4H_2O \quad (4.3)$$

The number of electron moles acceptable from the 39 mg/L of SO_4-2 is 11.2 mmoles, which is 10.5% of the total electrons released from the NH_4-N oxidation. During the 6th batch cycle, the total coulomb based the current monitored in the external circuit was 1,569 C, corresponding to 16.3 mmole of the electrons. Thus, around 15.2% of the NH_4-N was removed by the eDIET via the bioelectrode, and the remaining of 84.8% was removed by the bDIET in the bulk solution of the bioelectrochemical reactor. This suggests that the bDIET between AOE and DNE in the bulk solution plays a more important role in nitrogen removal in the bioelectrochemical reactor.

4.3.3 Requirements of nitrite and alkalinity for ammonium removal

The amount of nitrite and alkalinity required for ammonium removal are the most important factors determining the economics of the anaerobic nitrogen removal process. The amount of NO_2-N used for the NH_4-N removal was initially 3.2 mg NO_2-N/mg NH_4-N, but it was gradually increased to a stable value of 0.72 mg NO_2-N/mg NH_4-N by the repetition of the batch cycle, which was less than 1.32 mg NO_2-N/mg NH_4-N of the Anammox reaction (Fig.4.5) (Zhang et al., 2008; Jetten et al., 2009; Zhang et al., 2016). The methane was observed in the biogas in the bioelectrochemical reactor for the nitrogen removal. This implies that the electrons released from the NH_4-N oxidation were used to reduce carbon dioxide to methane. In the previous study, sulfate was also an electron acceptor for the NH_4-N oxidation (Yang et al., 2009). These suggest that the reduced substances including CO_2 and SO_4^{-2} in the bioelectrochemical reactor reduce the amount of NO_2-N for the NH_4-N oxidation.

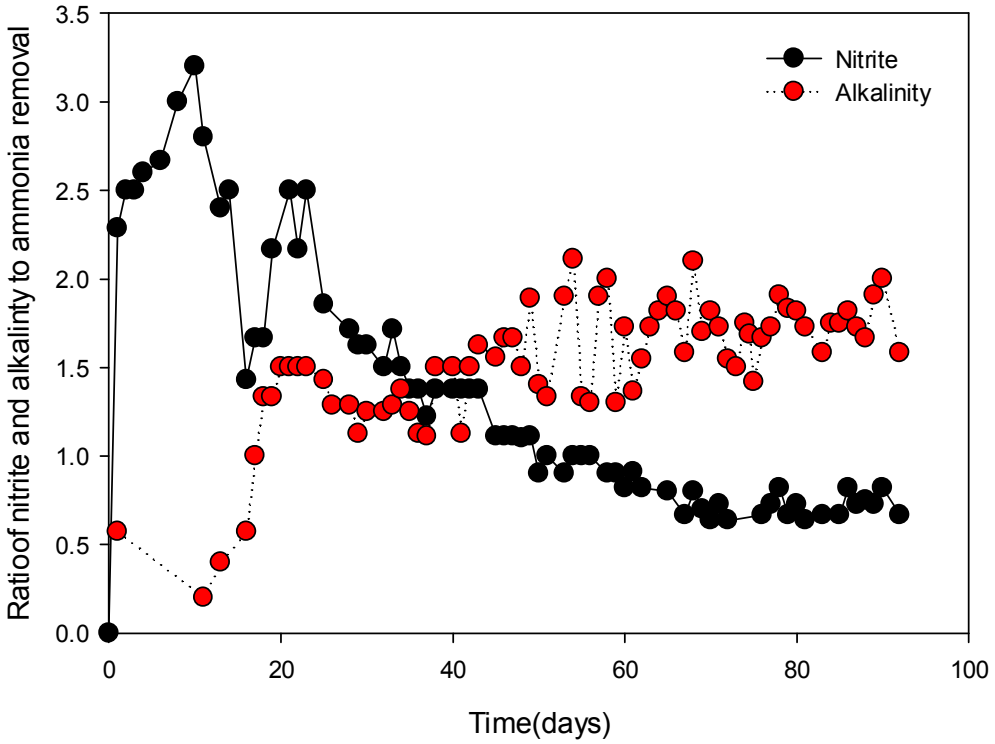


Fig.4.5 Changes of nitrite and alkalinity ratio for ammonium nitrogen removal

From the start-up, the alkalinity consumption for the NH_4-N oxidation was gradually increased and stabilized at around 1.73 mg as $CaCO_3$/mg NH_4-N (Fig.4.4). In the Anammox reaction, the amount of the alkalinity required for the NH_4-N oxidation is stoichiometrically 0.24 mg as $CaCO_3$/mg NH_4-N (Zhang, et al., 2008; Yang et al., 2009; Szatkowska and Paulseund, 2014; Zhang et al., 2016). It can be considered that the bioelectrochemical nitrogen removal process requiring more alkalinity was disadvantageous compared to the Anammox process. However, AOE and DNE are autotrophs that use the bicarbonate alkalinity as a carbon source. The high amount of the alkalinity required for the bioelectrochemical nitrogen removal indicates that the metabolic rates of AOE and DNE for the growth are high. In general, the Anammox microorganisms grow slowly and are difficult to enrich in the bioreactor (Zhang, et al., 2008; Yang et al., 2009; Zhang et al., 2016). However, the bioelectrochemical nitrogen removal can

be affected by the polarized potential of the electrode, microbial communities, and other environmental factors. It was revealed that the nitrogen can be removed in the anaerobic bioelectrochemical reactor with the polarized electrode, but further studies are needed to demonstrate the factors that affect the process performance for the field application.

4.3.4 Microbial communities

The microbial community in the bulk sludge was analyzed by pyrosequencing at the end of the 8th batch cycle. The total pyrosequencing reads for the bulk sludge sample were 72,555, and the valid reads were 44,760 (61.7%). The number of reads identified at the species level was 33,184 (74.1%). The number of OTUs found in the sample was 2,348, and the Good's coverage of library was 99.8%. The statistical estimates in the richness and evenness indicate that the microbial diversity in the bulk sludge was slightly lower (Table 4.1), compared to the activated sludge in previous studies (Kim et al., 2010). It seems that the microbial species capable of the metabolism through DIET and the species with the syntrophic relationship with them were only enriched in the bioelectrochemical reactor with polarized electrodes.

Table 4.1. Diversity induces based on the number and pattern of the operational taxonomic units (OTUs) observed in the bulk sludge.

Content	ACE	Chao1	Jackknife	Shannon	Simpson	NPShannon	Phylogenetic diversity
lci	2,369.3	2,351.2	2,454.0	5.325	0.022		
value	2,383.2	2,355.2	2,454.0	5.347	0.022	5.44	2,347.0
hci	2,397.6	2,363.9	2,454.0	5.269	0.022		

(lci and hci are rarefied 95% low and high confidence intervals, respectively)

Of the OTUs identified taxa, the first dominant phylum was Bacteroidetes (46.9%), followed by Proteobacteria (17.2 %), Firmicutes (11.6%), Spirochaetes (8.9%) and Chloroflexi (6.2%) (Fig.6). Kim et al (2010) reported that the first dominant phylum in the activated sludges collected from three domestic wastewater treatment plants was Proteobacteria. It seems that the AOE and the DNE mainly belong to the Bacteriodetes. At the class level, the dominant groups were Bacteroidia (35.9%), Gammaproteobacteria (10.5%), and Spirochaetes_c (8.8%). At the order level, Bacteroidales (35.9%) was the most abundant, but Spirochaetales, Pseudomonadales, Anaerolinaeles, and Flavobacteriales were also more than 5%. At the family level, the most dominant group was EU845084_f (17.7%), which was uncultured group belong to order Bacteroidales, and followed by Porphyromonadaceae (11.5%), Spirochaetaceae (8.6%), Pseudomonadaceae (7.1%), and Anaerolinaceae (5.5%). At the genus level, interestingly, the number of the uncultured groups increased to 8 genera among the 18 genera that are abundant more than 1%. In particular, the most abundant group was *DQ677001_g* (17.0%), and followed by *Sphaerochaeta* (8.1%), *Pseudomonas* (4.3%), *Petrimonas (*4.1%), *AM982608_g* (4.0%), *AJ009469_g* (3.0%), and *Thiopseudomonas* (2.55%). Genus *DQ677001_g* are the uncultured member of Order Bacteroidales, which are likely to be mainly the AOE or DNE species. Genus *Sphaerochaeta* is the group that produces acetate, formate, and ethanol, and enriched when exposed to high NH_4-N (Esquivel-Elizondo et al., 2016). Genus *Petrimonas* are the fermenters of carbohydrates and are predominant on the electrode in microbial electrolysis cell (Grabowski et al., 2005; Liu et al, 2016). It seems that Genus *Sphaerochaeta* and *Petrimonas* are the groups that have the syntrophic relationship with the AOE and DNE. The uncultured groups *AM982608_g and AJ009469_g* are the members of order Flavobacteriales and family Anaerolinaceae, respectively. Some members of Anaerolinaceae are the fermenters assimilating proteins and amino acids, and known to grow with hydrogenotrophic methanogens (Zehnder et al., 1979). Genus *Thiopseudomonas* is a

sulfide oxidizer anaerobically with nitrate as the electron acceptor (Tan et al., 2015). However, genus *Nitrosomonas* known as AOB has not appeared and genus *Nitrobacter* known as NOB was only 0.002%. The Anammox bacteria including genus *Brocadia, Kuenenia, Scalindua, Jettenia,* and *Anammoxoglobus* were also not appeared. This suggests that the nitrogen removal mechanism in the bioelectrochemical reactor is clearly different from the conventional nitrification-denitrification process or Anammox process.

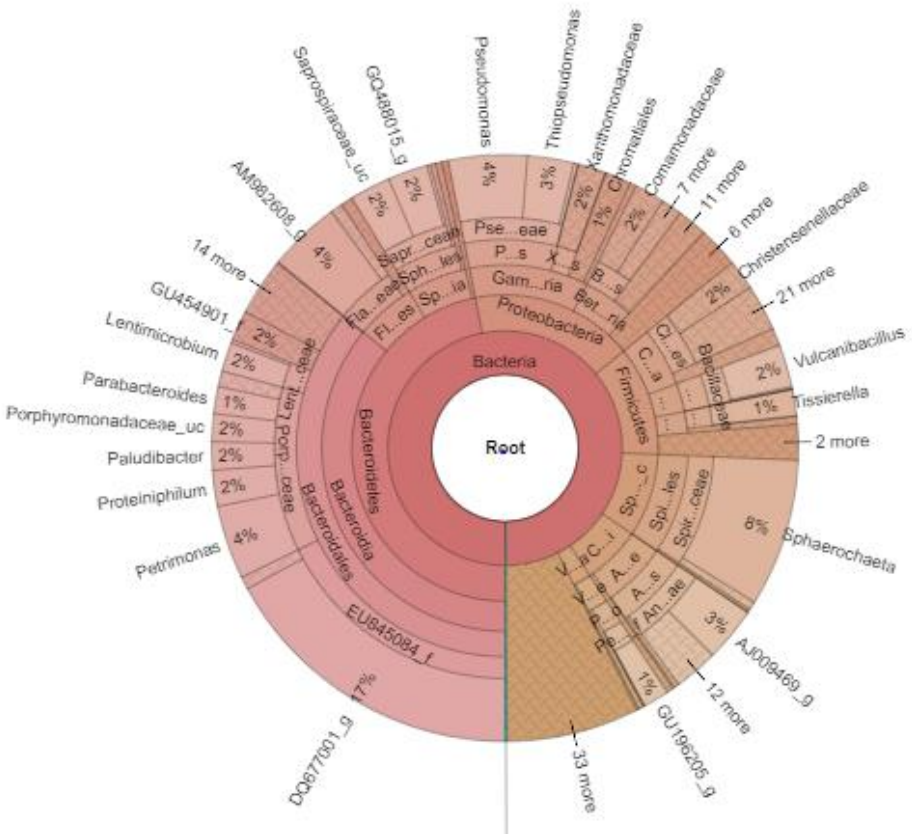


Fig.4.6**.** Microbial community in the bulk solution of bioelectrochemical reactor.

The features of the microbial community in the bulk sludge were more evident at the species level. Most of the abundant microorganisms were interestingly uncultured species except only a few species. The microbial species that are abundant more than 4% were *FJ535014_s* (9.9%), *Aj488100_s* (4.1%), and *FJ825540_s* (4.1%). *FJ535014* is the species observed on the

biocathode surface, likely to be the electrotrophs (Yun et al., 2018). *Aj488100_s* and *FJ825540_s* are the uncultured species, and their functions have not yet been reported. The microbial species between 2 % and 4 % in the abundance are *Pseudomonas caeni* (EU620679, similarity 98.6%) (3.9%), *Petrimonas sulfuriphila* (AY570690, Similarity 99.7%) (3.7%), *AM982608_s* (2.8%), *GQ488015_s* (2.1%), and *AB243814_s* (2.1%). *Pseudomonas caeni* is the denitrifier that reduces nitrite and nitrate, which was isolated in the anaerobic ammonium-oxidizing bioreactor (Xiao et al., 2009). *Petrimonas sulfuriphila* is an anaerobic fermenter that uses element sulfur and nitrate as electron acceptors (Grabowski et al., 2005). According to GenBank, *AM982608_s* is a species belong to Family Flavobacteriaceae, which was isolated from pig sawdust, and *GQ488015_s* is a species of Family Saprospiraceae, which was isolated from soil polluted by heavy metals. *AB243814_s* is a species of order Bacteroidales, which was isolated from Nigata oil well. In the microbial species being abundant ranging from 1% to 2%, most of the species including *EF586003_s* (1.6%), *EU358741_s* (1.6%), *HQ183755_s* (1.5%), *GU946455_s* (1.3%), *HQ602914_s* (1.3%), and *EU542487_s* (1.0%) were uncultured species, except for *Macellibacteroides fermentans group* (HQ020488, Similarity 98.8%) (1.4%) and *Sphaerochaeta globosa group* (CP002541, Similarity 98.8%) (1.0%). *Macellibacteroides fermentans* is an obligately anaerobic fermenter that produces lactate, acetate, butyrate, isobutyrate, which was isolated from upflow anaerobic filter treating abattoir wastewater (Jabari et al., 2012). *Sphaerochaeta globosa group* was an exoelectrogenic fermenter that reduces Fe (III) to Fe (II), which was isolated from freshwater sediment (Ritalahti et al., 2012). *Thiopseudomonas denitrificans* (KJ567598, Similarity 99.8%) was a minority species (0.9%), which is a species that oxidizes sulfide anaerobically with nitrate as an electron acceptor (Tan et al., 2015).

4.4 Conclusion

The bioelectrochemical anaerobic nitrogen removal was demonstrated in an anaerobic sequential batch reactor after inoculating activated sludge and polarizing the electrodes to 0.6V. The requirements of nitrite and alkalinity for the removal of ammonia nitrogen are around 0.72 mg NO_2-N/mg NH_4-N and 1.73 mg as $CaCO_3$ /mg NH_4-N, respectively, and nitrate as a by-product was not produced from the bioelectrochemical ammonia oxidation, indicating that the bioelectrochemical nitrogen removal differ from the Anammox process. The bacterial groups involved in the bioelectrochemical nitrogen removal is electroactive autotrophs and can be enriched from activated sludge by polarized electrode. The bioelectrochemical nitrogen removal is a novel approach recommended for treatment of nitrogen-rich wastewater.

Chapter 5 - Nitrite and nitrate as electron acceptors in bioelectrochemical ammonium oxidation through the electrostatic field

5.1 Introduction

Nitrogen that causes eutrophication in the water system has been a major concern in biological wastewater treatment for decades. Nitrogen contained in wastewater is generally removed by autotrophic oxidation of ammonium under aerobic condition and by heterotrophic reductions of nitrite and nitrate under anoxic condition (Ahn 2006; Ma et al., 2016). However, there are still challenges in the treatment of nitrogen-rich wastewater such as recycle water and industrial wastewater. Anammox is one of typical processes currently recommended for the treatment of nitrogen-rich wastewater. In the Anammox, nitrite is the only electron acceptor to oxidize ammonium to nitrogen gas under anaerobic condition (Chen et al., 2011; Jin et al., 2013; Du et al., 2015; Nancharaiah et al., 2016). Hence, the Anammox process can significantly save energy required for oxidation of ammonium and reduction of nitrite and nitrate compared to autotrophic and heterotrophic combined process. However, the Anammox process requires a special strategy to enrich Anammox microorganisms and strict nitritation to supply nitrite only as the electron acceptor. In addition, nitrogen compounds cannot be completely removed from wastewater by the Anammox reaction because nitrate is produced as a by-product (Shalini and Joseph 2012; Ge et al., 2015; Nancharaiah et al., 2016).

Recently, it has been revealed that these limitations of the Anammox process can be mitigated by bioelectrochemical processes. The bioelectrochemical process for nitrogen removal uses an anaerobic reactor with polarized electrodes. In the bioelectrochemical reactor, ammonium oxidizing exoelectrogens (AOE) grow on the anode surface to oxidize ammonium. Electrons released from the oxidation are then directly transferred to the anode (He et al., 2009; Zhan et

al., 2012; Qu et al., 2014; Joicy et al., 2019). Denitrifying electrotrophs (DNE) on the cathode surface can take these electrons directly from the cathode to reduce nitrite or nitrate into nitrogen gas (Mook et al., 2013; Joicy et al., 2019). This means that ammonium, nitrite, and nitrate nitrogen can be removed anaerobically in the bioelectrochemical reactor by AOE and DNE. However, bioelectrochemical nitrogen removal on polarized electrode surfaces has been difficult to put into practical use in the field because it is directly controlled by surface areas of the anode and the cathode (Call et al., 2009; Ren et al., 2012). It is well known that electroactive microorganisms are found in an anaerobic or nutrient limited natural environment where insoluble electron acceptors are outside cells (Doyle and Marsili 2015; Chaert et al., 2015). However, in a bioelectrochemical reactor, the bulk solution in which suspended microorganisms grow is exposed to the electrostatic field that is created by polarized electrodes. The electrostatic field enriches exoelectrogens and electrotrophs by expressing electroactive genes of microorganisms suspended in the bulk solution (Feng et al., 2018; Song et al., 2019). These suspended exoelectrogens and electrotrophs in the bulk solution can be electrically connected by physical contact between adjacent species for direct interspecies electron transfer (DIET) without a mediator (Kiely et al., 2011; Lovely, 2011; Feng et al., 2017; Schroder and Harnisch, 2017).

Interestingly, when ammonium as an electron donor and nitrite as an electron acceptor were added into a bioelectrochemical reactor, AOE and DNE were enriched in the bulk solution by electrostatic field from activated sludge or anaerobic sludge as inoculum (Joicy et al., 2019; Song et al., 2019). Ammonium and nitrite in the bulk solution were removed by DIET between AOE and DNE through an Anammox-like mechanism. However, species of AOE and DNE in the bioelectrochemical reactor were different from Anammox species (Zhang et al., 2008; Van Niftrik and Jetten 2012; Sonthiphand et al., 2014). In addition, nitrate, a by-product in the Anammox reaction, was not observed in the bulk solution. Meanwhile, change in free energy

for ammonium oxidation into nitrogen gas is a negative value when nitrite or nitrate is used as electron acceptor (Rittmann & McCarty, 2001). This means that not only nitrite, but also nitrate can be used as electron acceptor in the bioelectrochemical reactor. In the Anammox reaction, the number of moles of nitrite, the electron acceptor required to ammonium oxidation, is similar to that of ammonium. However, strict partial nitritation which oxidizes only about half of ammonium contained in wastewater to only nitrite is a tricky and difficult process (Ge et al., 2015; Nancharaiah et al., 2016). This suggests that the use of nitrate as an electron acceptor for ammonium oxidation can have great advantage for bioelectrochemical nitrogen removal. However, nitrate as an electron acceptor for bioelectrochemical ammonium oxidation has not studied in detail yet.

In the present study, electroactive nitrogen removal microorganisms including AOE and DNE were enriched in a bulk solution that was exposed to an electrostatic field in a bioelectrochemical nitrogen removal reactor (BENR). It was demonstrated that bioelectrochemical ammonium oxidation depended on nitrate fraction of the electron acceptor composed of nitrite and nitrate. In addition, nitrate as well as nitrite could be used as electron acceptor for the bioelectrochemical ammonium oxidation. These findings provide a great advantage of bioelectrochemical nitrogen removal process compared to Anammox process which requires strict nitritation to selectively produce only nitrite from ammonium.

5.2. Materials and Methods

5.2.1 Artificial medium, seed sludge, and electrode preparation

Artificial medium containing 0.3 g/L KH_2PO_4, 1.0 g/L $Na_2HPO_4 \cdot 12H_2O$, 0.5 g/L NaCl, 2.0 g/L $NaHCO_3$, 0.1 g/L $MgSO_4 7H_2O$, and 0.01 g/L $CaCl_2$ was prepared as described in previous studies (Zhan et al., 2012; Joicy et al., 2019). Concentrations of NH_4-N, NO_2-N, and NO_3-N were controlled with NH_4Cl, $NaNO_2$, and $NaNO_3$, respectively, depending on the experimental

purpose. Activated sludge was collected from Y-WWTP (B-Metrocity, Korea) and screened to remove impurities. The activated sludge was settled for a day and used as an inoculum. VSS (Volatile Suspended Solids) and pH of the inoculum were 4,500 mg/L and 7.89, respectively. Two copper foils (0.3T, copper 99.9%, KDI Co.) of small (5.5cm × 7cm) and large in size (26cm × 9cm) were prepared. The surface of foils was coated with a dielectric polymer (Alkyd enamel, VOC 470g/L, Noroo paint Co., South Korea) to prepare small and large insulated electrodes.

5.2.2 Bioelectrochemical nitrogen removal reactor (BENR)

A cylindrical anaerobic bioelectrochemical reactor (effective volume 0.5L) was prepared with an acrylic tube (Fig. 5.1). The bioelectrochemical reactor was covered with an acrylic plate and flanged to maintain anaerobic condition. Small and large electrodes rolled into the annulus were placed in the center of the reactor and on the inner surface of the reactor, respectively. The spatial distance from the inner electrode to the outer electrode was about 3 cm. These electrodes were connected to the terminals of an external DC voltage source (ODA Technologies, Co., Incheon, South Korea) with titanium wires. A blade driven by a DC motor was installed inside the bioelectrochemical reactor to mix the liquid medium. Portholes for liquid and biogas samplings were installed on the cover plate. The top of the gas sampling porthole was covered with an n-butyl rubber stopper and the bottom of the liquid sampling porthole was attached with a sealing tube extended into the liquid. A biogas venting valve was also installed on the cover plate. The biogas venting valve was connected to a floating-type gas collector. An acidic solution saturated with NaCl was filled to the gas collector to prevent dissolution of biogas as described in previous study (Song et al., 2019).

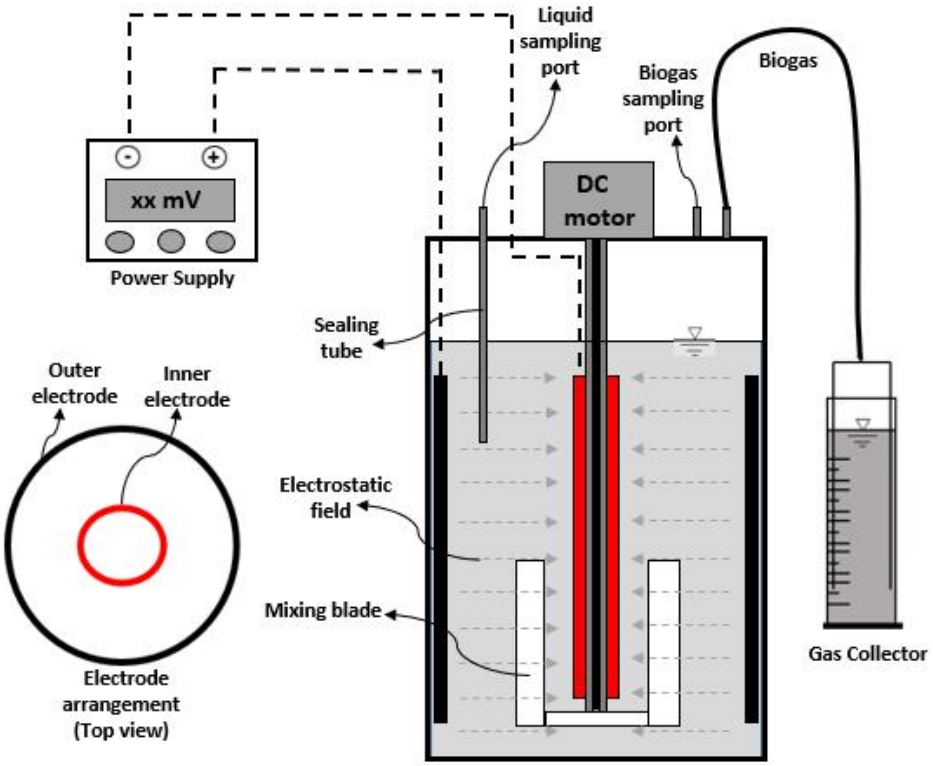


Fig. 5.1. Schematic diagram of bioelectrochemical nitrogen removal reactor

5.2.3 Start-up and operation of bioelectrochemical reactor

For the experiment, the artificial medium (250 mL) and inoculum (250 mL) were added into three bioelectrochemical nitrogen removal reactors (BENRs), respectively. In all BENRs, initial concentration of NH_4-N was 500 mg/L and total concentration of the electron acceptor composed of NO_2-N and NO_3-N was 300 mg/L. However, the fraction of NO_3-N in the electron acceptor was initially adjusted to 1/3, 2/3, and 3/3 for BENR1, BENR2, and BENR3, respectively. These electrodes were polarized by applying a voltage of 0.6 V to create an electrostatic field of 0.20 V/cm between the electrodes. Operation of BENRs was started at room temperature (25 ± 2°C). Concentrations of NO_2-N and NO_3-N were intermittently monitored and replenished when their concentrations were reduced to a low value during batch operation. Batch operation of BENRs was sequentially repeated by replacing the medium

inside the reactor with a fresh one after settling the suspended sludge for 30 minutes when NH_4-N was depleted in the BENR. Initial concentrations of NH_4-N and the electron acceptor in every sequential batch operation were controlled to their values.

5.2.4 Analytical techniques and calculation

During sequential batch operation, physicochemical properties including alkalinity, NH_4-N, NO_2-N, NO_3-N, and VSS in the bulk solution of BENRs were analyzed daily according to Standard methods (Eaton et al., 2005) and pH was monitored by using a pH meter (YSI pH1200). At the end of the batch experiment, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) for the bulk solution were performed using an electrochemical instrument (ZIVE SP1, WonA Tech, South Korea) as described previously (Marsili et al., 2008; Joicy et al., 2019). For CV, small pieces of stainless mesh (1cm×1cm) were used as working and counter electrodes. The potential window was in the range of -1.0 V to 1.0 V (vs. Ag/AgCl) and the scan rate was 10 mV/s. From the cyclic voltammogram, redox peak height and potential were obtained using 'SMART Manager' software (ZIVE BP2 Series, WonATech Co., Korea) to investigate electroactive bacteria. In the EIS test, the Ag/AgCl electrode was used as a reference electrode. The potential wave signal was 25 mV and the frequency range were from 100 kHz to 10 MHz. Impedance responses were fitted into a Randles equivalent circuit model. The Randles equivalent circuit model included a solution ohmic resistance in series with a double-layer capacitor in parallel with Faradaic reaction impedance consisting of a charge-transfer resistance and Warburg element in series (Feng et al., 2017).

5.2.5 Bacterial community analysis

At the end of the experiment, Microbiome Taxonomic Profiling was performed to investigate microbial communities using 16S rRNA in the bulk solution. DNA was extracted from suspended sludge in the bulk solution using a Power soil DNA isolation kit (MO BIO Laboratories, Inc., CA, USA) according to the kit protocol. Fusion primer was used to amplify

the variable region of V3V4 for bacteria of the 16S rRNA gene in genomic DNA. 16S rRNA was amplified from the metagenomic DNA, pooled, and sequenced on a MiSeq Personal Sequencer (Illumina, San Diego, CA, USA). Amplification, construction of the sequencing library, and bioinformatic analysis were performed as described previously (Chun et al., 2010). Chimera was checked and taxonomic assignments of these readings were done using EzBioCloud database (http://ezbiocloud.net/). Microbial community and statistical taxonomical assignments were obtained through operational taxonomic units. Comprehensive bioinformatic analysis such as species-level classification of microbes, cluster analysis, microbial origin tracking, hierarchical clustering, and various indicators of species diversity were conducted with EZ Biocloud (Chunlab, Inc., Seoul, Korea). Bacterial species similarity of more than 1% of inter microbial communities was obtained from correlation analysis by principal component analysis using factoextra package in R.

5.3. Results and discussion

5.3.1 Bioelectrochemical nitrogen removal depending on nitrate and nitrite

In BENRs, ammonium was removed from the bulk solution exposed to an electrostatic field of 0.2 V/cm as nitrite and nitrate decreased (Fig.5.2). This indicates that ammonium was bioelectrochemically oxidized under anaerobic condition by an Anammox-like mechanism. However, it was noteworthy that nitrate besides nitrite was used as an electron acceptor to oxidize ammonium in these BENRs. This indicates that microbial metabolism for bioelectrochemical ammonium oxidation is different from the Anammox reaction. In previous studies, when only ammonium and nitrite were electron donor and acceptor in the bioelectrochemical reactor, respectively, electroactive species including AOE and DNE were enriched in the bulk solution (Qu et al., 2014; Zhan et al., 2014; Joicy et al., 2019). Ammonium and nitrite were simultaneously removed by biological direct interspecies electron transfer

(DIET) via an electrical connection with physical contact between adjacent AOE and DNE (Jetten et al., 2009; Zhan et al., 2012; Lovley 2011; Feng et al., 2017; Feng et al., 2018). However, as far as we know, this is the first report showing that ammonium can be bioelectrochemically oxidized under anaerobic condition by nitrate besides nitrite as electron acceptor. The Anammox process is now known as a viable process for removing nitrogen-rich wastewater (Chen et al., 2011; Jin et al., 2013; Du et al., 2015; Ma et al., 2016). However, the Anammox process uses nitrite only as the electron acceptor to oxidize ammonium under anaerobic condition and produce nitrate as a by-product. Partial nitritation to selectively produce nitrite only from ammonia-rich wastewater is one of the major obstacles that hinder wide application of the Anammox process.

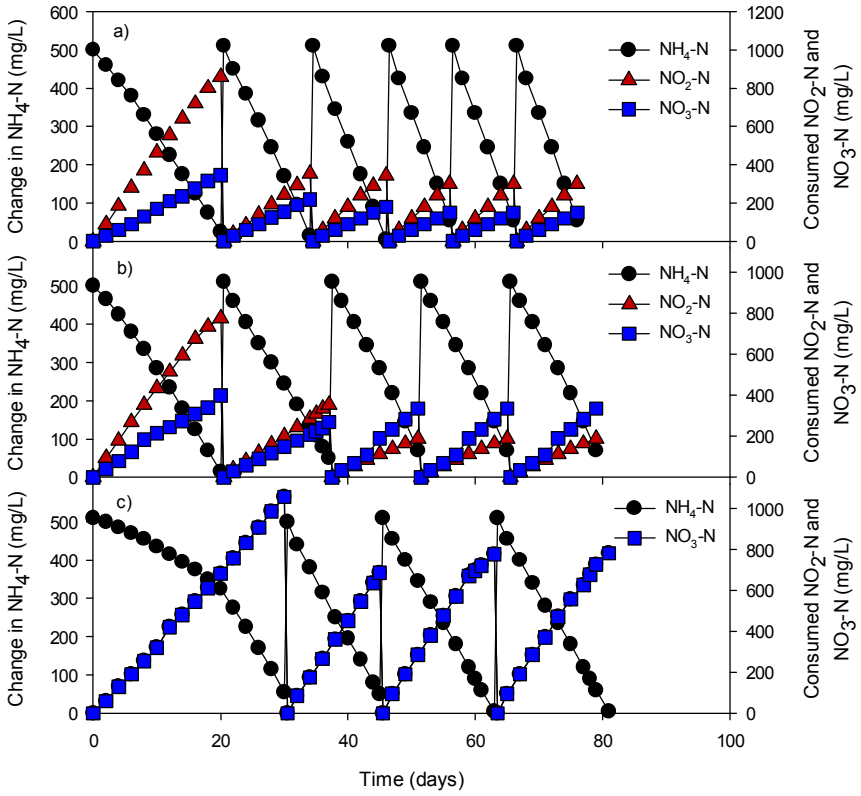


Fig. 5.2. Concentration changes in ammonium and consumed nitrite and nitrate. a) BENR1, b) BENR2, and c) BENR3

In BENRs, ammonium removal started from the first batch cycle after start-up (Fig. 5.2). This indicates that some species in the activated sludge inoculum can anaerobically oxidize ammonium in the electrostatic field. Interestingly, the decrease of ammonium and decreases of nitrite and nitrate were almost linear. It seems that bioelectrochemical oxidation of ammonium is a pseudo-zero order reaction that is less affected by their concentrations. The removal rate of ammonium in BENR1 was gradually improved to 48.4 mg NH_4-N/L.d as the batch cycle repeated and the consumption rate of nitrite was higher than that of nitrate. The consumption rate of nitrate exceeded that of nitrite in BENR2. However, the removal rate of ammonium in BENR2 was 32.5 mg NH_4/L.d, which was lower than that in BENR1. In the electron acceptor composed of nitrite and nitrate, the fraction of nitrate was 1/3 in BENR1 and 2/3 in BENR2. In a previous study, the rate of ammonium removal rate was 57 mg NH_4-N/L.d when nitrite was the only electron acceptor (Joicy et al., 2019). This indicates that ammonium oxidation rate is dependent on nitrate fraction in the electron acceptor. In BENR3 with only nitrate as the electron acceptor, ammonium removal was observed. However, its rate was the smallest at 28.7 mg NH_4-N/L.d. At standard condition (pH = 7.0, 25℃), free energy changes ($\Delta G^{0'}$) for half reaction of nitrite and nitrate reductions to nitrogen gas are –92.56 kJ/e^- and –72.20 kJ/e^-, respectively, at neutral pH while free energy change ($\Delta G^{0'}$) for half reaction of ammonium oxidation to nitrogen gas is -26.70 kJ/e^- (Rittman & McCarty, 2001). This indicates that although nitrate is thermodynamically less effective than nitrite, it can be used for the electron acceptor for bioelectrochemical ammonium oxidation.

5.3.2 Changes in pH, alkalinity, and VSS

Decreases in pH and alkalinity associated with nitrogen removal were observed in BENRs. As batch cycle repeated, values of pH and alkalinity were gradually stabilized. However, their

decreases were dependent on nitrate fraction in the electron acceptor (Fig. 5.3). During the last batch cycle, the decrease in alkalinity was 850 mg/L as $CaCO_3$ in BENR1, which was a little less than 890 mg/L as $CaCO_3$ in BENR2 and 955 mg/L as $CaCO_3$ in BENR3 (Table 5.1). BENRs were started with high initial VSS concentration of 4,500 mg/L. However, VSS decreased exponentially with repetition of batch cycles and then stabilized at 960 mg/L in BENR1, 1,000 mg/L in BENR2, and 1,110 mg/L in BENR3 (Table 5.1). This indicated that alkalinity consumptions were correlated with VSS concentration in BENRs. It seems that electroactive species in BENRs are autotrophs that use bicarbonate, a main component of alkalinity at neutral pH, as a carbon source. Bicarbonate can be also converted to carbon dioxide. However, carbon dioxide content in biogas is only around 5-6% in previous studies (Joicy et al., 2019; Song et al., 2019). The initial decline in VSS observed in BENRs might be due to a process that rapidly decayed heterotrophic bacteria and selectively enriched autotrophic electroactive nitrogen removal species including AOE and DNE.

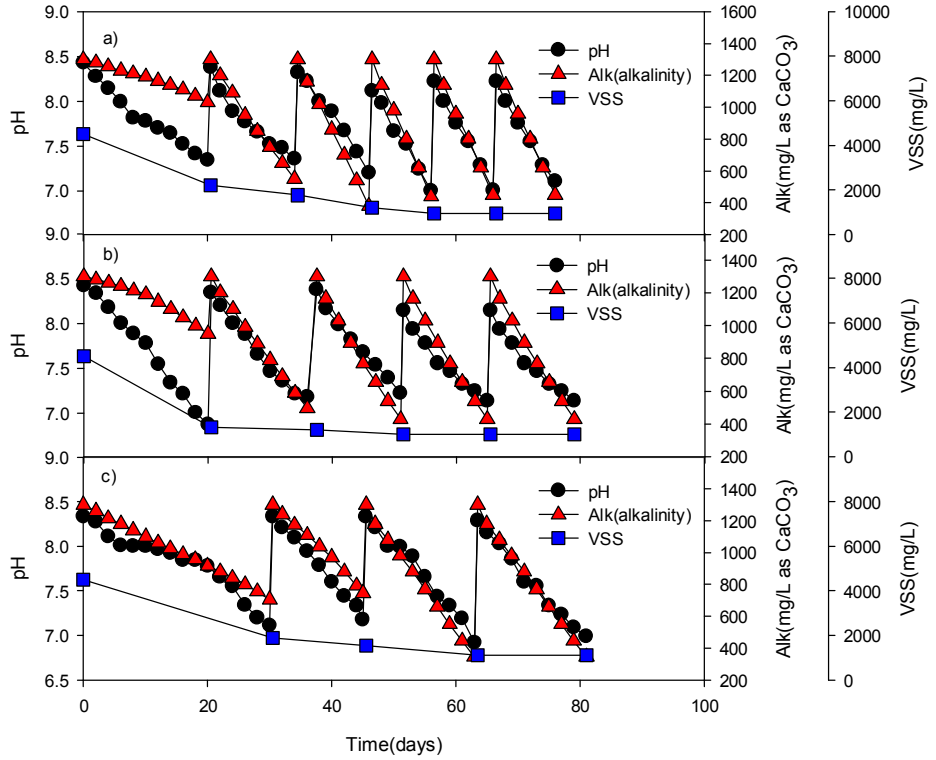


Fig. 5.3. Changes in pH, alkalinity, and VSS concentrations. a) BENR1, b) BENR2, and c) BENR3

The alkalinity used for ammonium oxidation was 1.72 mg $CaCO_3$/mg NH_4-N in BENR1. It was slightly increased when nitrate fraction was increased in the electron acceptor. In the Anammox process, the amount of alkalinity required for ammonium removal is only 0.24 mg as $CaCO_3$/mg NH_4-N (Joicy et al., 2019; Song et al., 2019). More alkalinity requirement for ammonium oxidation than that for the Anammox process can be a weakness of BENR process in terms of economic aspect. However, AOE and DNE are autotrophs that can use bicarbonate as a carbon source. This indicates that metabolic rates of AOE and DNE for their growth are possibly higher than those of Anammox microorganisms. The high growth rate of AOE and DNE can be an advantage of BENR in retaining more biomass required for process stability.

In general, Anammox microorganisms grow slowly. It is difficult to enrich them in the bioreactor (Zhang et al., 2008; Yang et al., 2009; Zhang et al., 2016).

The specific removal rate of ammonium based on biomass is an indicator that shows abilities of AOE and DNE to exchange electrons for nitrogen removal in the BENR. The specific removal rate of ammonium was 48 mg NH_4-N/g VSS.d in BENR1, which was higher than that in BENR2 of 39 mg NH_4-N/g VSS.d or in BENR3 of 24 mg NH_4-N/g VSS.d (Table 5.1). The specific removal rate of ammonium in Anammox process was in the range of 43.1 to 193 mg NH_4-N/g VSS.d, higher than those in BENRs (Szatkowska and Paulsrud, 2014; Zhang et al., 2016). However, there are still several issues to be solved in Anammox process, including nitrite requirement as electron acceptor, enrichment of Anammox bacteria, and nitrate production as a by-product. In BENRs, nitrate can be used as an electron acceptor for bioelectrochemical ammonium oxidation. However, it is not a by-product. This is a great advantage of BENRs compared to Anammox process. The specific removal rate of NH_4-N was 62.8 NH_4-N/g VSS.d in the BENR using nitrite as the only electron acceptor (Song et al., 2019; Joicy et al., 2019). It is believed that optimization still has a significant potential to improve the specific removal rate of ammonium in BENR.

5.3.3 Electron balance and preferential electron acceptor

The amount of nitrite and nitrate required for ammonium oxidation which depends on nitrate fraction in the electron acceptor is an essential parameter in the operation of bioelectrochemical nitrogen removal process (Joicy et al., 2019; Song et al., 2019). After repetition of the batch cycle, consumptions of nitrite and nitrate required for ammonium removal varied depending on nitrate fraction in the electron acceptor. In BENR1, consumptions of nitrite and nitrate required for ammonium removal were 0.73 mg NO_2-N/mg NH_4-N and 0.35 mg NO_3-N/mg NH_4-N, respectively (Table 5.1). This indicated that nitrite was preferentially used as the electron acceptor to oxidize ammonium at a low nitrate fraction of 1/3. However, in BENR2,

nitrate consumption was as high as 0.77 mg NO_3-N/mg NH_4-N while nitrite consumption was just 0.45 mg NO_2-N/mg NH_4-N (Table 5.1). This suggests that nitrite is not always used preferentially as the electron acceptor in the BENR, although nitrite is a thermodynamically better electron acceptor than nitrate. In BENR3, nitrate consumption was as high as 1.63 mg NO_3-N/mg NH_4-N while nitrite was not observed (Table 5.1). The only electron acceptor in BENR3 was nitrate. It seems that the preferential use of nitrite or nitrate as the electron acceptor in BENR is thermodynamically determined by relative fractions of nitrite and nitrate.

Bioelectrochemical nitrogen removal can be better understood by electron balance for nitrite and nitrate used for ammonium oxidation. In BENR1, electrons released from the ammonium oxidation was 32.5 mmoles, which was close to the sum of electron moles accepted by nitrite (21.4 mmoles) and nitrate (10.7 mmoles). In previous studies, nitrite required for ammonium oxidation in a BENR was in the range of 0.58 to 0.72 mg NO_2-N/mg NH_4-N when nitrite was used as the only electron acceptor (Joicy et al., 2019; Song et al., 2019). It seems that the amount of electron acceptor required for bioelectrochemical ammonium oxidation increases when nitrate fraction in the electron acceptor increases. In electron balance for BENR2, electron acceptor that could accept electrons of 6.1 mmoles (16.3%) was excessively used for ammonium oxidation. However, the amount of electron acceptor required for ammonium oxidation was 1.19 mmoles. In the Anammox process, nitrite requirement for ammonium oxidation is theoretically 1.32 mg NO_2-N/mg NH_4-N (Ma et al., 2016; Song et al., 2019). This indicates that nitrate fraction acceptable in the electron acceptor is about two thirds in the BENR compared to that in the Anammox process. In the electron balance for the BENR3, electron acceptors that can accept electron of 23.5 mmoles (42.4%) were excessively used for ammonium oxidation. Interestingly, the increase in electron acceptor requirement depending on nitrate fraction was in agreement with increases in alkalinity consumption and VSS concentration (Table 5.1). This suggests that an increase in nitrate fraction in the electron

acceptor can increase biomass growth, thus increasing the number of electron acceptors used in biomass degradation.

Table 5.1. Parameters for bioelectrochemical nitrogen removal depending on nitrate fraction in the electron acceptor (nitrite and nitrate) during the last batch cycle

Contents	BENR1	BENR2	BENR3
Nitrate/(Nitrite + Nitrate) (mg N/mg N)	1/3	2/3	3/3
Specific NH_4-N removal rate (mg NH_4-N/g VSS.d)	48.0	39.0	24.0
NO_2-N/NH_4-N (mg N/mg N)	0.73±0.05	0.45±0.06	-
NO_3-N/NH_4-N (mg N/mg N)	0.35±0.07	0.77±0.05	1.63±0.08
Alkalinity/NH_4-N (mg/L as $CaCO_3$/mg N)	1.72±0.03	1.78±0.07	1.80±0.06
Decreased alkalinity (mg/L)	850±9.36	890±5.59	955±10.61
VSS (mg/L)	960±8.58	1000±10.77	1110±10.45

5.3.4 Electrochemical analysis of bioelectrochemical reactor

The bulk solution in BENRs showed one oxidation and two reduction peaks in the cyclic voltammogram (CV). These peaks in the CV provide perception of electroactive microorganisms involved in bioelectrochemical nitrogen removal (Joicy et al., 2019; Song et al., 2019). In previous studies, when only nitrite was the electron acceptor in BENR, peaks in the CV were observed at 0.13 ~ 0.14 V vs. Ag/AgCl for oxidation and -0.20 ~ -0.21 V vs. Ag/AgCl for reduction (Joicy et al., 2019; Song et al., 2019). These redox peaks were mainly contributed by electroactive microorganisms including AOE and DNE. However, redox peak potentials in BENRs were somewhat complicated when nitrate was used as the electron acceptor. In BENR1, the oxidation peak was observed at -0.20 V vs. Ag/AgCl, which was more negative than previous studies. It seemed that AOE species enriched in the BENR varied depending on types of electron acceptor. In BENR1, reduction peaks were observed at -0.02 V vs. Ag/AgCl and -0.48 V vs. Ag/AgCl (Fig. 5.4a) likely involved in reductions of nitrite and nitrate, respectively, based on changes in free energy. However, further studies are needed to explain the peak height of 0.73 mA at -0.48 V vs. Ag/AgCl, which was higher than 0.30 mA at -0.02 V vs. Ag/AgCl. In BENR2, peak potentials for oxidation were similar to those in BENR1. However, reduction peaks were slightly shifted toward increase of overpotentials. In addition, peak heights in BENR2 were slightly smaller compared to those in BENR1. However, in BENR3, peak potentials of oxidation and reduction were shifted to more positive potentials. This indicated that the overpotential for electron transfer to oxidize ammonium was increased more. Peak heights in BERN3 were significantly smaller compared to those in BENR1 or BENR2. It seemed that activities of AOE and DNE were relatively low when nitrate was used as the only electron acceptor.

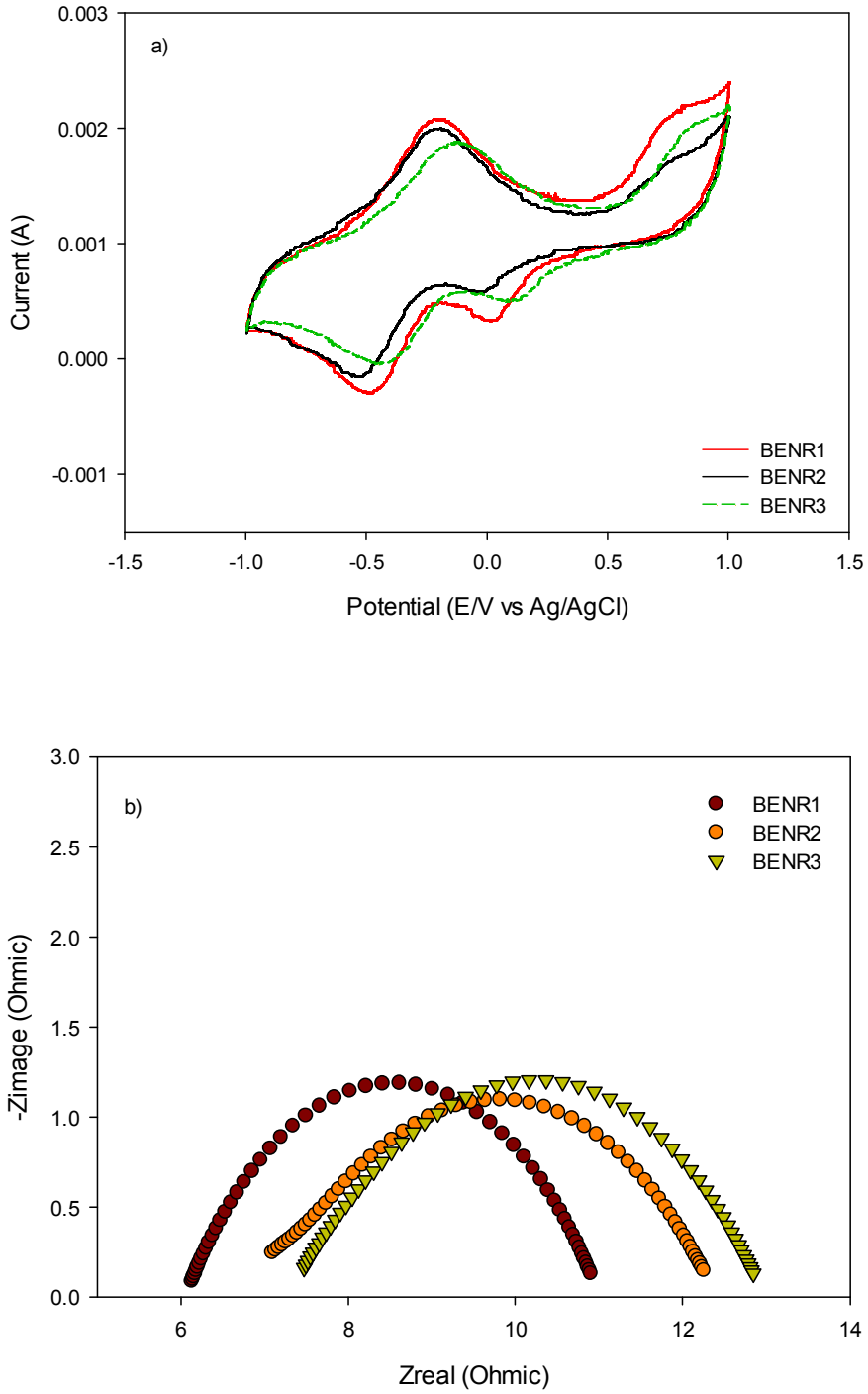


Fig. 5.4. Electrochemical analysis for the bulk solution of BENRs. a) Cyclic voltammogram, b) Nyquist plot for the EIS data

Electroactive AOE and DNE species enriched in the bulk solution and their activity can also be identified by electrochemical impedance spectrum (EIS). All EIS data obtained from the bulk solution for BENRs appeared with semicircle shapes on the Nyquist plot (Fig. 5.4b). On the horizontal axis of the Nyquist plot, the left intersection of the semicircle is the solution resistance (R_s) and the diameter of the semicircle is the charge transfer resistance (R_{ct}) (Sekar and Ramasamy 2013; Doyle and Marsili 2015). The conductivity of microbial consortium increases when electroactive bacteria with a high conductive redox protein are abundant (Shrestha et al., 2014; Li et al., 2016). In BENRs, it is believed that VSS in the bulk solution represents concentration of biomass composed of electroactive species. However, solution resistance in BENR1 was 6.1 Ω, which was lower than 7.0 Ω in BENR2 and 7.5 Ω in BENR3 (Fig. 5.4b). This indicates that solution conductivities in BENRs coincide with peak heights of CVs in the order of BENR1, BENR2, and BENR3. It seems that solution resistance is more dependent on the type of electroactive species more than on total concentration of bacterial species. The activity of electroactive species can be described by charge transfer resistance (R_{ct}) obtained from the EIS in a bioelectrochemical reactor (Sekar and Ramasamy 2013; Doyle and Marsili 2015; Feng et al., 2017). In BENR1, charge transfer resistance (R_{ct}) was 4.8 Ω, which was also lower than 5.2 Ω in BENR2 and 5.4 Ω in BENR3 (Fig. 5.4b). This indicates that the charge transfer resistance increases slightly with increasing nitrate fraction in the electron acceptor. Solution resistance and charge transfer resistance in BENRs coincided with decreased ammonium removal rate as nitrate fraction in electron acceptor increased.

5.3.5 Bacterial community analysis

Bacterial communities in the bulk solution were analysed by microbiome taxonomic profiling at the end of batch experiment. Concentrations of VSS in BENRs were well reflected in the number of OTUs. Statistical estimates showed that species diversity in richness and evenness in BENR3 was higher than that in BENR2. It was the lowest in BENR1. Regarding taxonomic

composition, dominant bacterial phyla were Bacteroidetes, Proteobacteria, Firmicutes, and Spirochaetes in all BENRs (Table 5.2).

At genus level, abundant bacterial groups in BENR1 were *Proteiniphilum* (7.96%), *Thiopseudomonas* (7.49%), *DQ677001_g* (7.01%), *Pseudomonas* (5.26%), and *Petrimonas* (4.51%). However, in BENR2, *Arcobacter* (24.62%), *Lentimicrobium* (6.82%), and *Petrimonas* (5.41%) were abundant. These were significantly different from those in BENR1. It is known that genus *Arcobacter* can contribute to denitrification in activated sludge system (Heylen et al., 2006). However, abundances of *Proteiniphilum* (0.91%), *Thiopseudomonas* (0.01%), and *DQ677001_g* (1.82%) were significantly less in BENR2. It seems that these bacterial genera are involved in ammonium oxidation with only nitrite as the electron acceptor through the electrostatic field. Interestingly, *Arcobacter* (6.1%) and *DQ677001_g* (5.6%) were abundant in BENR3, although there are no predominant genera. However, abundances of *Lentimicrobium* (2.40%) and *Petrimonas* (1.91%) were less in BENR3 compared to those in BENR2. Genus *Petrimonas* was more abundant in BENR2 followed by that in BENR1 and BENR3. Genus *Petrimonas* is known as fermenters of carbohydrates. It is predominant on the electrode in a microbial electrolysis cell (Grabowski et al., 2005; Liu et al., 2016). It seems that *Petrimonas* is the genus that uses nitrite as an electron acceptor. Genus *Pseudomonas* was more abundant in BENR1 than that in BENR2 or BENR3. It has been revealed that genus *Pseudomonas* is capable of bidirectional electron transfer (Su et al., 2012). It can also reduce nitro-aromatics (Kong et al., 2017) and enhance denitrification in BES (Srinivasan and Butler, 2017). However, Anammox bacteria such as *Brocadia, Kuenenia, Scalindua, Jettenia,* or *Anammoxoglobus* were not observed in any BENR.

Table 5.2. Taxonomic compositions of bacterial communities in bulk solutions of BENR reactors

Classifications	Taxonomic composition	BENR1 (%)	BENR2 (%)	BENR3 (%)
Phylum	Bacteroidetes	43.43	37.19	37.38
	Proteobacteria	37.60	45.27	34.78
	Firmicutes	8.65	6.37	6.09
	Spirochaetes	1.43	2.15	3.36
	Others	8.89	9.02	18.39
Genus	*Proteiniphilum*	7.96	0.91	0.47
	Thiopseudomonas	7.49	0.01	0.15
	DQ677001_g	7.01	1.82	0.60
	Pseudomonas	5.26	3.21	2.76
	Petrimonas	4.51	5.41	1.90
	Arcobacter	2.21	24.62	6.10
	Lentimicrobium	1.44	6.82	2.40
	Others	64.12	57.20	85.62

	Thiopseudomonas HQ183821_s	7.10	0.05	0.11
	Proteiniphilum_uc	6.51	0.31	0.00
	Porphyromonadaceae_uc	4.87	0.55	0.00
	Pseudomonas caeni	4.80	3.16	2.66
	DQ677001_g FJ535014_s	3.88	1.44	4.14
	Petrimonas sulfuriphila	3.64	4.53	1.68
	Parabacteroides chartae group	1.43	2.02	0.33
	Lentimicrobium AY570585_s	0.84	5.20	1.43
Species	*Sulpfurospirillum arsenophilum group*	0.83	2.16	2.49
	Nitrospira defluvii group	0.80	0.05	1.41
	Sphaerochaeta AJ488100_s	0.74	1.74	0.27
	Arcobacter AM084124_s	0.73	24.16	0.00
	Arcobacter AY570594_s	0.70	0.02	3.20
	Dechloromonas denitrificans group	0.40	1.41	1.37
	Cloacamonas AB513440_s	0.30	0.00	2.15
	Others	62.43	53.20	78.76

Bacterial communities at species level in BENRs varied depending on nitrate fraction in the electron acceptor based on PCA analysis. Variances of bacterial communities in BENRs could be explained by two principal components (PC1 40.1%, PC2 33.7%) (Fig. 5.5). The biplot showed that bacterial species in BENR1 was positively correlated with PC1 and PC2. In BENR1, the feature of bacterial species was mainly affected by *Thiopseudomonas HQ183821_s* (Accession HQ183821, 7.1%), uncultured species *Proteiniphilum_uc* (6.5%),

Porphyromonadaceae_uc (4.9%), and *Pseudomonas caeni* (4.8%). *T. HQ183821_s* is the ammonium utilizing species isolated from leachate sediment (Liu et al., 2011). *Proteiniphilum* is a facultatively anaerobic fermenter and proteolytic bacterium (Chen and Dong, 2005). *Porphyromonadaceae_uc* are uncultured bacteria belonging to family *Porphyromonadaceae*. *P. caeni* is known as a denitrifying species that reduces nitrite and nitrate. It is isolated in an anaerobic ammonium-oxidizing bioreactor (Xiao et al., 2009). *P. caeni* is also identified in a bioelectrochemical reactor with polarized electrode for nitrogen removal as well as in microbial fuel cells (Zhan et al., 2014; Joicy et al., 2019). It seemed that these species were electroactive species enriched in the electrostatic field by lower nitrate fraction than by nitrite in the electron acceptor. However, in BENR2, bacterial species was positively correlated with PC1, but negatively with PC2. This was significantly different from that in BENR1. The feature of bacterial species in BENR2 was mainly affected by *Arcobacter AM084124_s* (Accession AM084124, 24.2%) and *Lentimicrobium AY570585_s* (Accession AY570585, 5.2%). *A. AM084124_s* is a nitrate reducing species belonging to Epsilon proteobacteria. It has been isolated from activated sludge (Heylan et al., 2006). *L. AY570585_s* is also a nitrate reducing bacterium isolated from low temperature biodegraded Canadian oil reservoir (Grabowski et al., 2005). These bacteria might be electroactive species more enriched in the higher nitrate fraction than those in the nitrite of the electron acceptor. The bacterial community in BENR3 was negatively correlated with PC2 only. The feature of bacterial species was mainly affected by *DQ677001_g FJ535014_s* (Accession FJ535014, 4.14%), *Arcobacter AY570594_s* (Accession AY570594, 3.2%), and *Cloacamonas AB513440_s* (Accession AB513440, 2.2%). *D. FJ535014_s* is an uncultured bacterium isolated from anaerobic fermentation reactor with a mixture of waste activated sludge and carbohydrate at pH 8.0 (Feng et al., 2009). *A. AY570594_s* is also an uncultured nitrate reducing bacterium isolated from low temperature biodegraded Canadian oil reservoir (Grabowski et al., 2005). *C. AB513440_s* is an uncultured

bacterium isolated from enrichment culture from a polychlorinated dioxin-treated composter (Narihiro et al., 2010). Abilities of *D. FJ535014_s* and *C. AB513440_s* to reduce nitrite or nitrate are unclear yet. However, these bacterial species were the electroactive bacteria enriched in the electrostatic field by nitrate only as electron acceptor. Bacterial species with the abundance over than 1% in all BENRs were *P. caeni and D. FJ535014_s.* The abundance of *P. caeni* was decreased with increasing nitrate fraction in the electron acceptor. However, *D. FJ535014_s* was more abundant in BENR3 than that in BENR1 or BENR2. It seems that species *P. caeni* prefers nitrite over nitrate for ammonium oxidation while species *D. FJ35014_s.* prefers nitrate as electron acceptor. However, further studies on activities and functions of these abundant bacterial species in BENRs are needed to elucidate the mechanism of bioelectrochemical nitrogen removal.

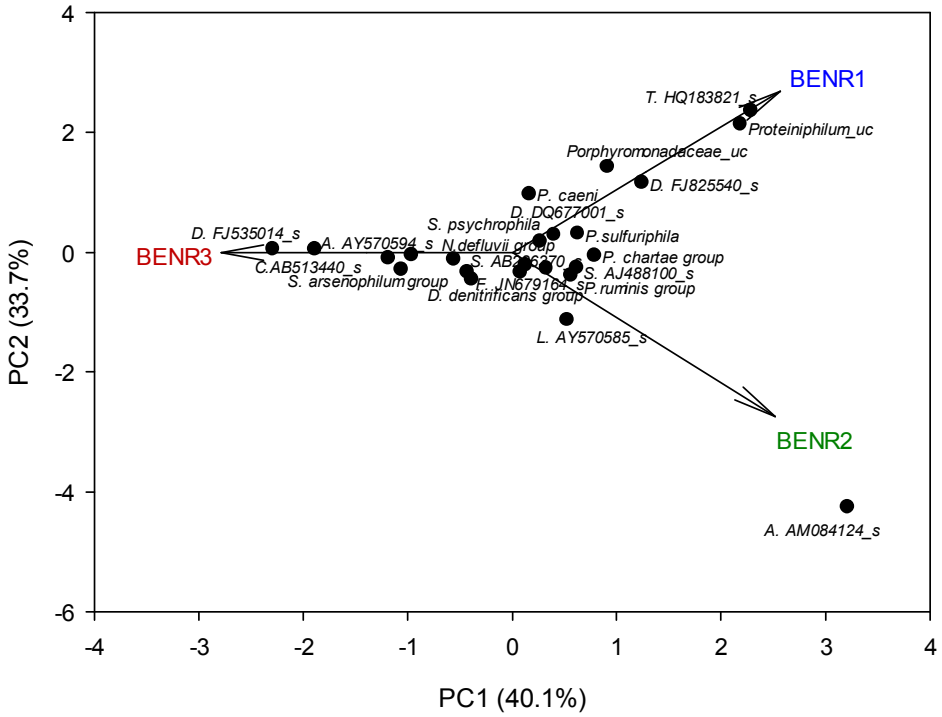


Fig. 5.5. Biplot for the bacterial species communities depending on nitrate fraction in the electron acceptor

5.4. Conclusion

Electroactive nitrogen removal species could be enriched by electrostatic field of 0.2 V/cm in bulk solution. Electrostatic field could drive bioelectrochemical ammonium oxidation under anaerobic condition using nitrite and nitrate as electron acceptors by promoting DIET between electroactive species. However, ammonium oxidation rate varied depending on fractions of nitrite and nitrate in the electron acceptor. Nitrate was a less effective electron acceptor than nitrite, although it could be used as an electron acceptor for bioelectrochemical ammonia oxidation under electrostatic field. This finding provides an advantage of avoiding stringent nitritation that selectively produces only nitrite from ammonium when treating ammonium-rich wastewater.

Chapter 6 - Electrostatic field facilitates direct interspecies electron transfer for bioelectrochemical anaerobic nitrogen removal

6.1 Introduction

The nitrogen compounds in the water environment over-stimulate the growth of aquatic life, significantly reducing the value of water use (Ahn 2006; Du et al., 2015). The nitrogen in the water environment is primarily associated with the discharge of the nitrogen-rich wastewater that is incompletely treated (Alshameri et al., 2014). The treatment of nitrogen-rich wastewater has been a concern in the management of the water environment. The nitrogen in wastewater has been usually removed by the conventional biological nitrogen removal (BNR) process consisting of aerobic autotrophic nitrification and anoxic heterotrophic denitrification (Ahn 2006; Ma et al., 2016). However, the aerobic nitrification requires enrichment of autotrophic nitrifier selectively by removing the organic matter first and supplying a large amount of oxygen and alkalinity (Ahn 2006; Shalini and Joseph 2012; Ge et al 2015). The heterotrophic denitrification requires an external carbon source as the electron donor (Komorowska-Kaufman et al., 2006; Nancharaiah et al., 2016). Thus, conventional BNR was an expensive process for the treatment of nitrogen-rich wastewater. The partial nitritation and denitritation technology have been developed to improve the economics in the conventional BNR process (Ahn 2006; Ge et al 2015; Nancharaiah et al., 2016). However, the several stringent operational conditions, including a high temperature, short retention time and low dissolved oxygen, required for the partial nitritation and denitritation have limited the field application (Ahn 2006; Shalini and Joseph 2012; Ge et al 2015). Recently, Anammox process that uses ammonium as an electron donor and nitrite as an electron acceptor in anaerobic condition have received significant attention (Du et al., 2015; Chen et al., 2011; Jin et al 2013). However, the growth

rate of Anammox microorganisms is too slow, and the partial nitritation of ammonium are required to provide the nitrite as the electron acceptor. The nitrate production as a by-product is also another disadvantage of the Anammox process (Ma et 2016; Ge et al., 2015; Nancharaiah et al., 2016; Komorowska-Kaufman et al., 2006).

Interestingly, it has been found that the polarized potentials of the electrode in anaerobic bioelectrochemical reactor enriches the ammonium oxidizing exoelectrogens (AOE) and the denitritating electrotrophs (DNE) on the surfaces of anode and cathode, respectively (He et al., 2009; Zhan et al., 2012; Huang et al 2103; Kondaveeti et al 2014; Qu et al., 2014). The AOE and DNE simultaneously remove ammonium and nitrite nitrogen as in the Anammox process by oxidizing the ammonium on the anode and reducing nitrite on the cathode, respectively (He et al., 2009; Zhan et al., 2012; Huang et al 2103; Qu et al., 2014; Mook et al., 2013; Joicy et al., 2019; Song et al., 2019). The polarized electrode mediates the direct interspecies electron transfer (DIET) between AOE and DNE for the nitrogen removal. It is noted, however, that the nitrogen removal through the electrode-mediated DIET is limited by the surface area of electrode, but the use of electrode with sufficient surface area is not cost effective in the field scale facilities (Logan 2010; Mousavi et al., 2012). In addition, the electrode-mediated DIET in bioelectrochemical reactor can be restricted by the ohmic resistance of the electrode and the activation overpotential for the electron transfer (Song et al., 2015; Feng et al., 2018a). Fortunately, in the bioelectrochemical anaerobic reactor with polarized electrode, it has been found that the electroactive bacterial species including AOE and DNE are enriched in the bulk solution as well as on the surface of the electrode (Joicy et al., 2019; Song et al., 2019; Feng et al., 2018a). It is noteworthy that the polarized electrode establishes the electrostatic field, potentially enriching the AOE and DNE in the bulk solution (Song et al., 2019). The exoelectrogens in the bulk solution can transfer the electrons directly to the electrotrophs that are electrically connected each other in close proximity, called as biological DIET (Song et al.,

2019; Feng et al., 2018a). The biological DIET has advantages in thermodynamics and kinetics in the electron transfer compared to the electrode mediated DIET (Feng et al., 2018a). Then, the electrode-mediated DIET can be excluded in bioelectrochemical reactor by insulating the electrode surface, which potentially promotes the biological DIET in the bulk solution. However, it is not yet known whether the electric field enriches the AOE and DNE and promotes the DIET for nitrogen removal.

In this study, we demonstrated that the electrostatic field enriches the electroactive bacteria (AOE and DNE) in the anaerobic reactor and facilitates the nitrogen removal via the biological DIET between the electroactive bacteria. The exoelectrogenic and electrotrophic activities of the electroactive bacteria were identified from the cyclic voltammogram, and the overpotentials for the DIET depending on the strength of electrostatic field were also investigated from the electrochemical impedance spectroscopy. The electroactive species enriched in the bulk solution were estimated from the abundant species in the microbial community analysis.

6.2. Materials and Methods

6.2.1 Bioelectrochemical reactor set up and operation

A cylindrical anaerobic batch reactor (effective volume 0.5L, diameter 8.5cm and height 10cm) was prepared using an acrylic resin as a bioelectrochemical nitrogen removal reactor (BENR) (Fig.6.1).

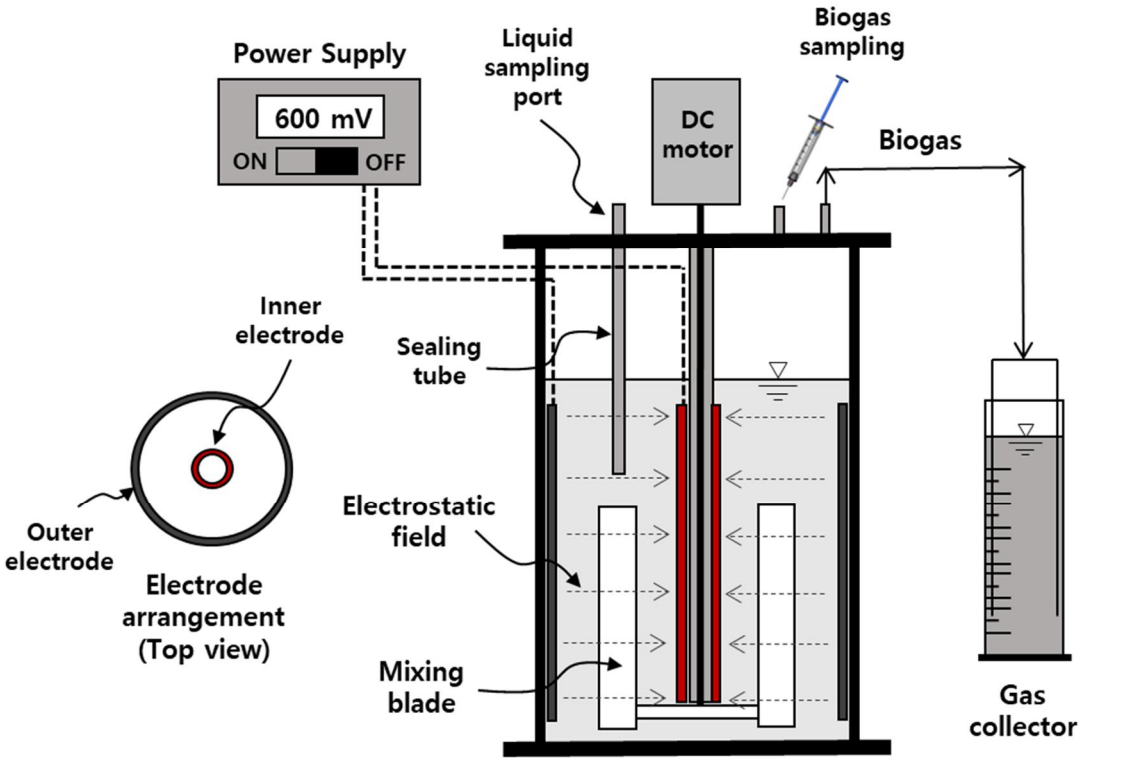


Fig.6.1 Schematic diagram of bioelectrochemical nitrogen removal reactor exposed to the electrostatic field.

The anaerobic batch reactor (fig. 6.1) was covered with the acrylic plate and flanged for sealing the reactor. A blade was installed for mixing the medium inside the reactor. The blade was connected to the DC motor over the acrylic cover plate using a steel shaft. The ports for biogas and liquid samples were installed on the cover plate and the ports were covered with n-butyl rubber stoppers to prevent oxygen ingress. The acrylic sealing tubes submerged into the medium solution were attached to the bottoms of the holes for liquid sampling port and steel shaft hole in the acrylic cover plate. A biogas venting valve was also installed in the cover plate, and the valve was connected to a floating type gas collector by a rubber tube. The gas collector was filled with water - acidified with sulfuric acid and saturated with salt to prevent the dissolution of biogas. Two copper foils (0.3T, copper 99.9%, KDI Co.) of a large (26cm × 9cm)

and small size (5.5cm × 7cm) were prepared and their surface were coated with a dielectric polymer (alkydenamel, VOC 470g/L, Noroo paint Co., South Korea) to obtain the surface insulated electrodes. The electrodes were installed at the inner wall of the reactor and the outer wall of the sealing tube for the steel shaft, respectively. The spatial distance from the inner electrode to the outer electrode was about 3cm. The electrodes were connected to the terminals of an external DC voltage source (ODA Technologies, Co., Incheon, South Korea) with titanium wires. The nitrogen rich medium containing 0.3 g/L KH_2PO_4, 1.0 g/L $Na_2HPO_4{\cdot}12H_2O$, 0.5 g/L NaCl, 2.0 g/L $NaHCO_3$, 0.1 g/L $MgSO_47H_2O$, 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 (Zhan et al., 2012, Joicy et al., 2019). The activated sludge was collected, screened to remove impurities, and then precipitated for a day to obtain the inoculum. The VSS and pH of the inoculum were 14,000mg/L and 7.89, respectively. For the experiment, the nitrogen (ammonium and nitrite) rich medium (250 mL) and inoculum (250 mL) were added into three different reactors, and the insulated electrodes were polarized by applying voltage ranged from 0.6 V to 2.0 V to establish the electrostatic fields (0.20 V/cm, 0.33 V/cm, and 0.67 V/cm). The completed BENRs were referred to as BENR20, BENR33 and BENR67, depending on the electrostatic field, respectively. The bioelectrochemical reactors were operated at room temperature. The initial concentrations of NH_4-N and NO_2-N were 500 mg/L and 300 mg/L, and the NO_2-N was intermittently supplemented during the batch operation when the concentration decreased to a small level. The operation of the BENRs were sequentially repeated by replacing the medium inside the reactor with a fresh one after settling suspended sludge for 30 minutes when the NH_4-N was depleted in the wastewater.

6.2.2 Analytical techniques and calculation

During the operation of the BENRs, the pH was daily monitored by using a pH meter (YSI pH1200 laboratory pH meter 115-230V (T1)). The changes in the physicochemical properties

including alkalinity, NH_4-N, NO_2-N, and VSS were also measured everyday according to the Standard methods for the examination of water and wastewater (Eaton 2005). The electrode potentials were intermittently checked against Ag/AgCl reference electrode (RE-1B, ALS Co., Ltd., Japan) using a portable digital multimeter (DM-1010, Dong Hwa Electronics, Co., Korea). At the end of the batch experiment, the cyclic voltammogram (CV) for the bulk solution in the anaerobic batch reactor was also obtained in the potential range of -1.0 V to 1.0 V (vs. Ag/AgCl) with a 10mV s^{-1} scan rate using the electrochemical instrument (ZIVE SP1, WonA Tech, South Korea). For the cyclic voltammetry test, the small pieces of stainless mesh (1cm×1cm) were used as the working and counter electrodes. The peak currents and peak potentials of oxidation and reduction were obtained from cyclic voltammogram using the software, 'SMART Manager' (ZIVE BP2 Series, WonATech Co., Korea). At steady state, the electrochemical properties of the bulk solution in the BENR were estimated by EIS (electrochemical impedance spectroscopy) test using electrochemical instrument (Zive SP1 WonATech, co., Korea). as described in previous studies (Marsili et al 2008). The impedance spectra were fitted to the Randle equivalent circuit model with mixed kinetic and diffusion control. The Randle equivalent circuit was consisted of a solution ohmic resistance in series with a double-layer capacitor, which is in parallel with the Faradaic reaction impedance, consisting of a charge-transfer resistance and Warburg element in series.

6.2.3 Bacterial community analysis

Microbiome Taxonomic Profiling was performed to investigate 16S rRNA bacterial communities in the BENR at the end of the experiment by extracting the DNA from the suspended sludge in the bulk solution using MO BIO Power soil DNA kit. The extraction of the DNA was followed by the kit protocol. The fusion primer was used to amplify the variable region (V3V4) of the bacterial 16S rRNA gene in the genomic DNA. The 16S rRNA was amplified from the metagenomic DNA and pooled for sequencing on the MiSeq Personal

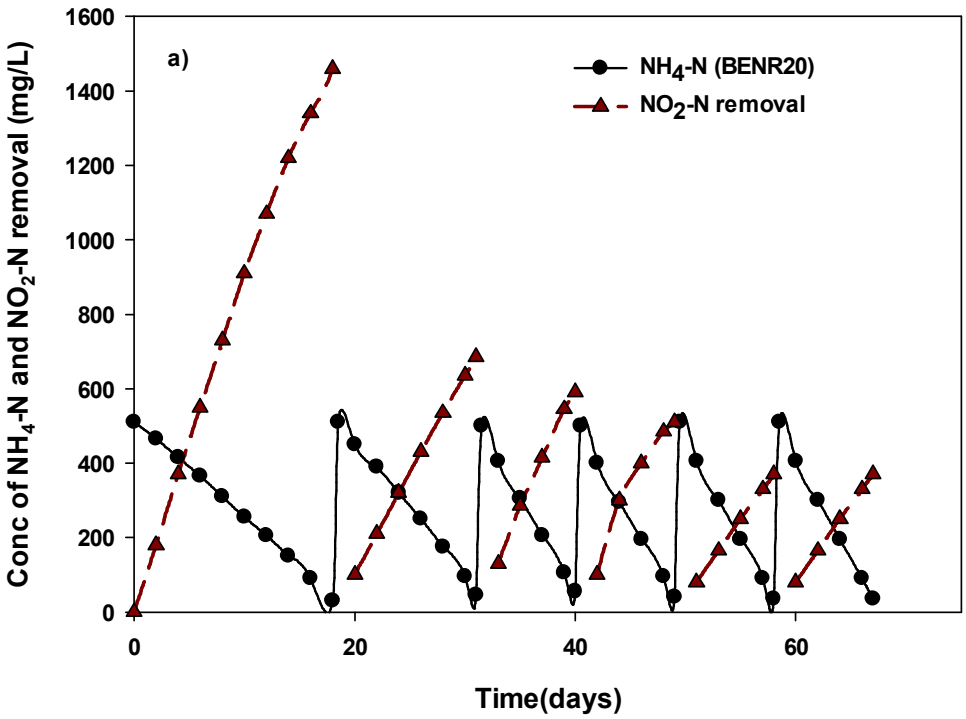
Sequencer (Illumina, San Diego, CA, USA). The amplification, construction of the sequencing library and bioinformatic analysis were performed as described in the previous study (Chun et al.,2010). Chimera was checked, and taxonomic assignments of these readings were done using the extended EzTaxon database (http://eztaxon-e.ezbiocloud.net/). Microbial community and the statistical taxonomical assignments were obtained through the operational taxonomic units. Comprehensive bioinformatic analysis like the species-level classification of microbes, cluster analysis, microbial origin tracking, fast unifrac analysis of inter-sample diversity, hierarchical clustering and various indicators of species diversity was conducted by the CLcommunity software (Chunlab, Inc., Seoul, Republic of Korea).

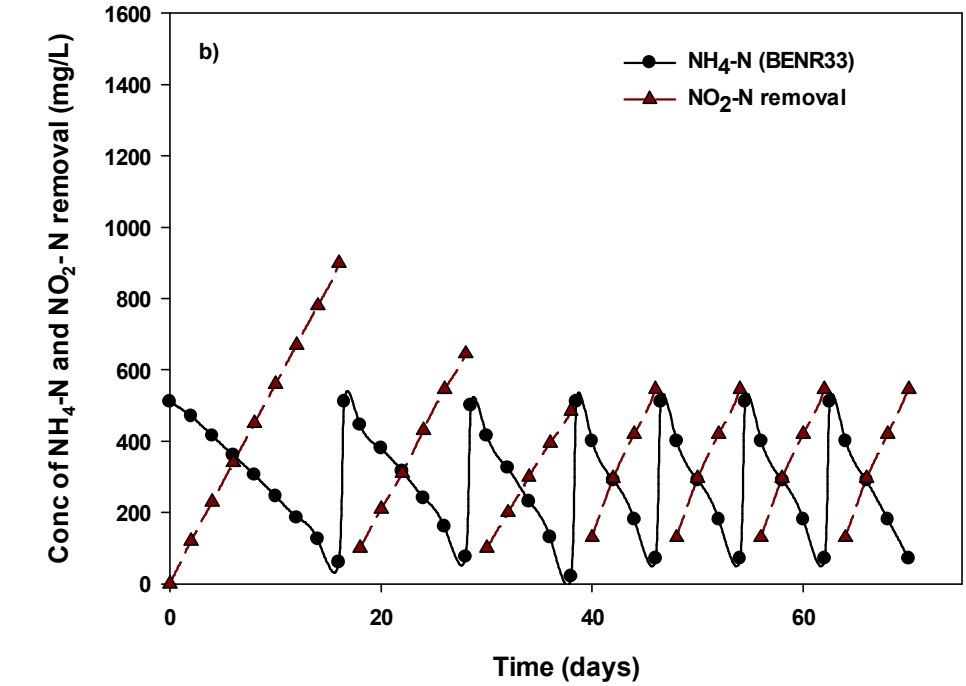
6.3. Results and Discussion

6.3.1 Bioelectrochemical nitrogen removal

The ammonium removal accompanied by the consumptions of nitrite and alkalinity was observed in the BENR where the bulk solution was exposed to the electrostatic field ranged from 0.2 V/cm to 0.67 V/cm (Fig. 6.2, Fig. 6.3). The removal rates of ammonium and nitrite nitrogen were gradually increased with the operation time of the BENRs. In the BENR67, the removal rate of ammonium was 72.5 mg NH4-N/L.d, the same as the nitrite nitrogen, after the repetition of the sequential batch operation. In the medium of BENR67, the ammonium and nitrite were the sole electron donor and acceptor for the microbial species, respectively, and there is no organic carbon source. This indicates that the mechanism of nitrogen removal in the bulk solution exposed to a high electrostatic field of 0.67 V/cm is similar to that of Anammox. However, Anammox bacteria grow slowly and are generally not abundant in activated sludge used as the inoculum (Joicy et al., 2019; Song et al., 2019; Zhang et al., 2008). This suggests that the nitrogen removal mechanism in the BENR67 is unlikely to be the Anammox. In previous studies, AOE and DNE including the genera *Nitrosomonas* and *Empedobacter* were

enriched on the polarized electrode surfaces in the anaerobic bioelectrochemical reactor (Qu et al., 2014; Zhan et al., 2014). The ammonium was oxidized at the anode surface by the AOE to release the electrons, and DNE reduced the nitrite to nitrogen gas at the cathode surface using the electrons (Zhan et al., 2012; Qu et al., 2014; Joicy et al., 2019). However, the electrode surface in the BENR67 was insulated with a dielectric polymer, and there was no electrode surface that AOE and DOE could attach for the electron transfer.





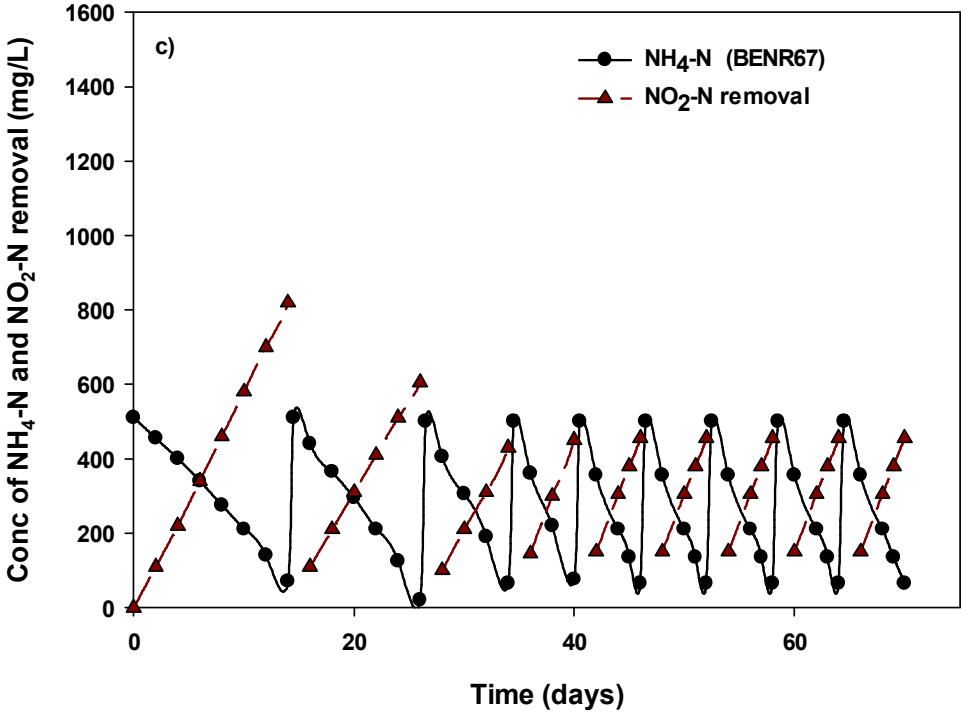
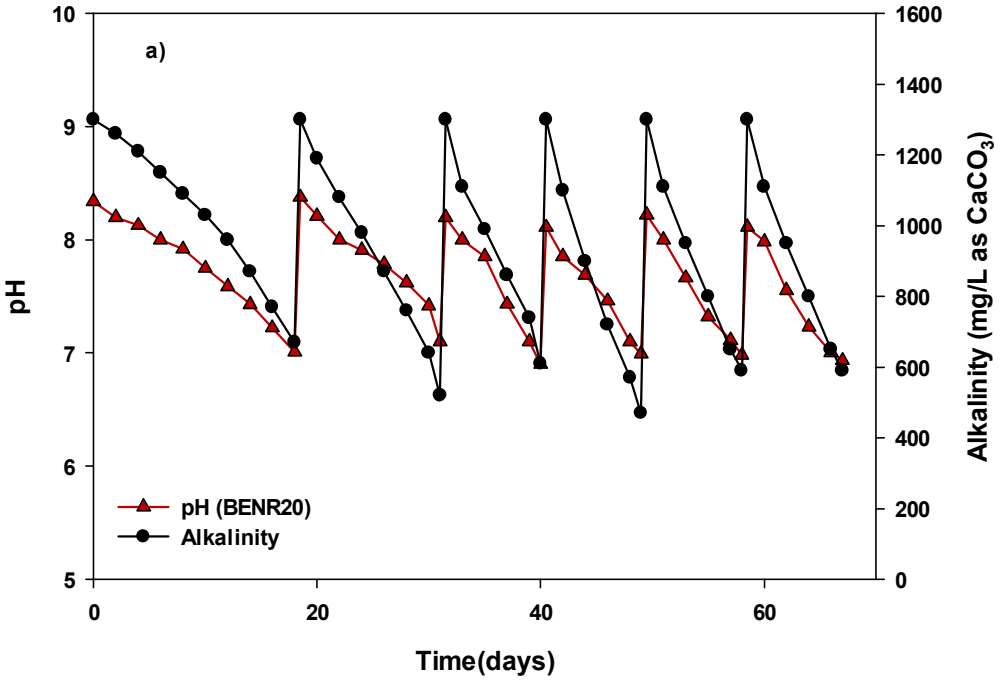
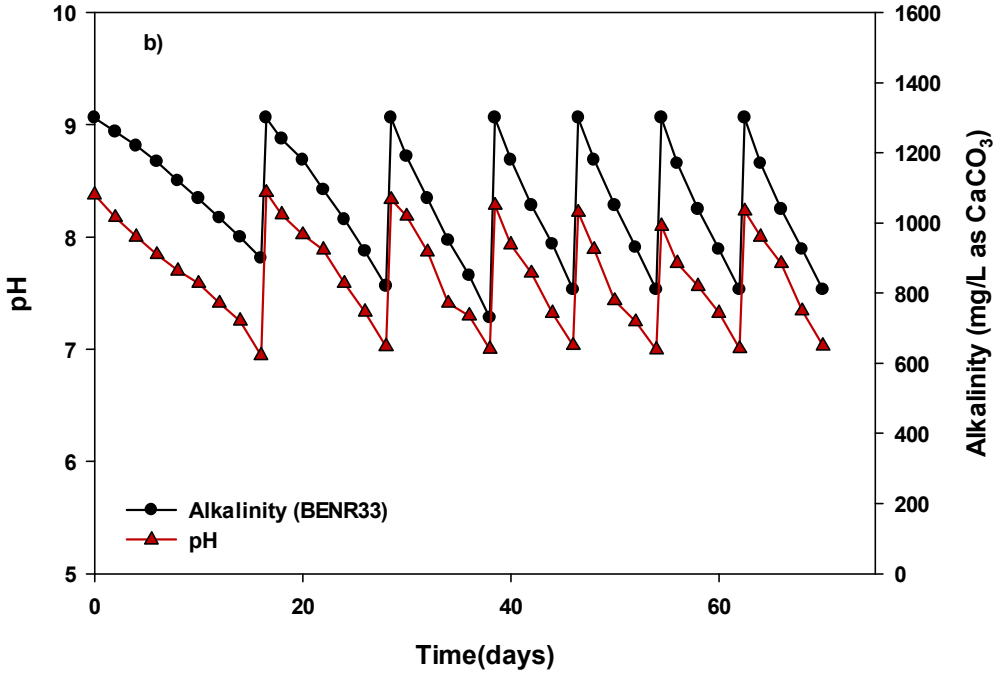


Fig.6.2 Changes in ammonium and nitrite nitrogen in bioelectrochemical nitrogen removal reactor: a) BENR20, b) BENR33 and c) BENR67


a)
pH
Alkalinity (mg/L as CaCO3)
Time(days)
pH (BENR20)
Alkalinity
10
9
8
7
6
5
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60


b)
pH
Alkalinity (mg/L as CaCO3)
Time(days)
Alkalinity (BENR33)
pH
10
9
8
7
6
5
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60

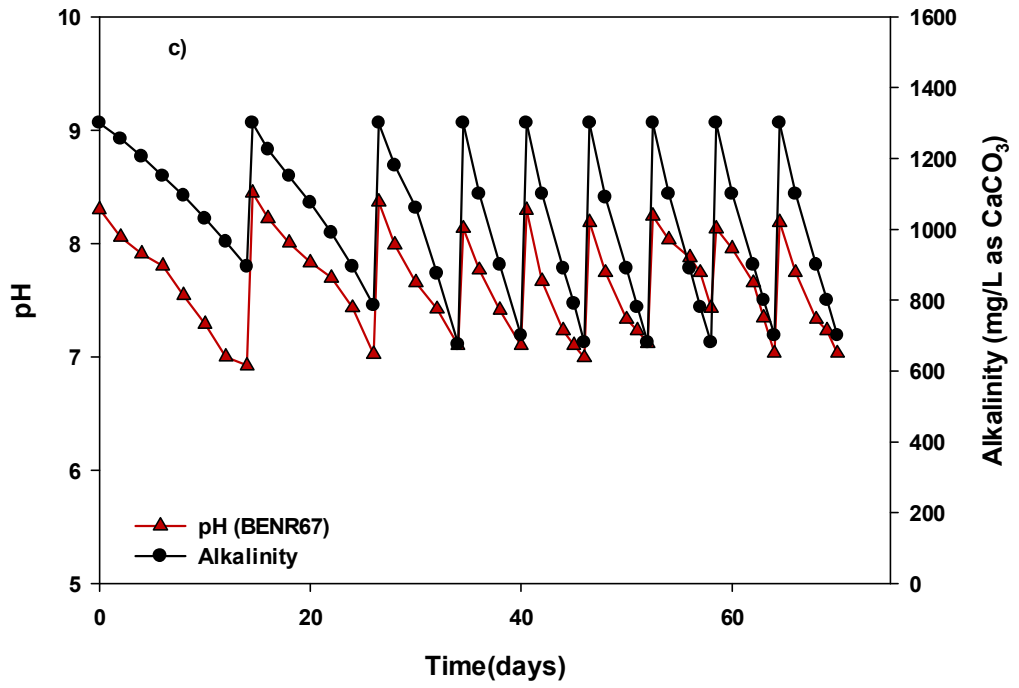


Fig. 6.3 Changes in pH and alkalinity in bioelectrochemical nitrogen removal reactor a) BENR20, b) BENR33, c) BENR67

In the BENR67, the pH and alkalinity decreased simultaneously with the removals of ammonium and nitrite nitrogen (Fig. 6.3). It seems that the bicarbonate, a main component of the alkalinity, at neutral pH was used as the carbon source for the growths of AOE and DNE. In previous studies, the AOE and DNE species in the bioelectrochemical reactor for nitrogen removal were the autotrophs (Joicy et al., 2019; Song et al., 2019). After the inoculation of the activated sludge, the VSS was initially 7,000 mg/L in the BENR67 but exponentially decreased to around 860 mg/L with the time (Fig. 6.4a). It seems that the aerobic heterotrophic microorganisms that inoculated initially were mostly decayed by the substrate and oxygen depletion. It is likely that the VSS retained in the bulk solution is mostly the autotrophic microorganisms, including AOE and DNE, which were enriched by the electrostatic field. This

implies that the AOE oxidize the ammonium in the bulk solution to release the electrons, and the electrons are transferred directly to the DNE by the electrical contact with the AOE in close proximity. This is a kind of biological DIET similar to the direct electron transfer between the fermentation exoelectrogens and electrotrophic methanogens in bioelectrochemical anaerobic reactor (Feng et al., 2018a; Lovley 2011; Shen et al., 2016; Feng et al., 2017; Feng et al., 2018b). Then, the removal rates of ammonium and nitrite decreased to 55.0 mg NH4-N/L.d and 63.8 mg NO2-N/L.d in the BENR33, respectively, compared to the BENR67, and further decreased to 52.5 NH4-N/L.d and 41 NO2-N/L.d in the BENR20. The strength of the electrostatic field was 0.33 V/cm in the BENR33, and 0.20 V/cm in the BENR33, indicating that the biological DIET for nitrogen removal decreases with the decrease in the strength of the electrostatic field. However, the VSS in the BENR33 and BENR20 retained between 860 mg/L and 890 mg/L, similar to the BENR67 (Fig. 6.4a). The ability of AOE and DNE to transfer the electrons can be described by the specific ammonium removal rate per biomass. The specific ammonium removal rate in the BENR67 was 78.7 NH4-N/g VSS.d. However, the specific ammonium removal rate in the BENR33 was decreased to 64.0 NH4-N/g VSS.d and further 61.8 NH4-N/g VSS.d in the BENR20 (Fig. 6.4b).

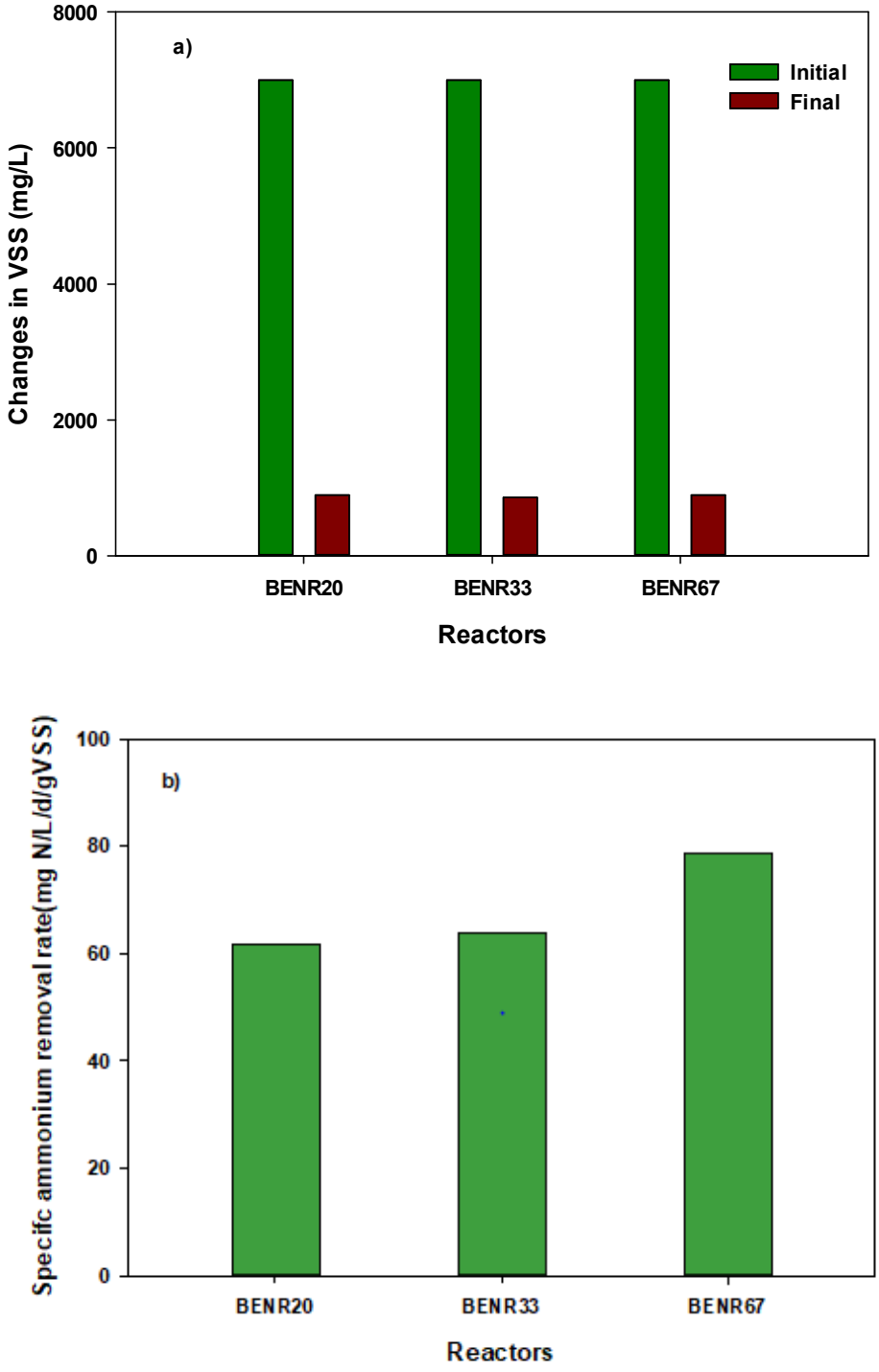
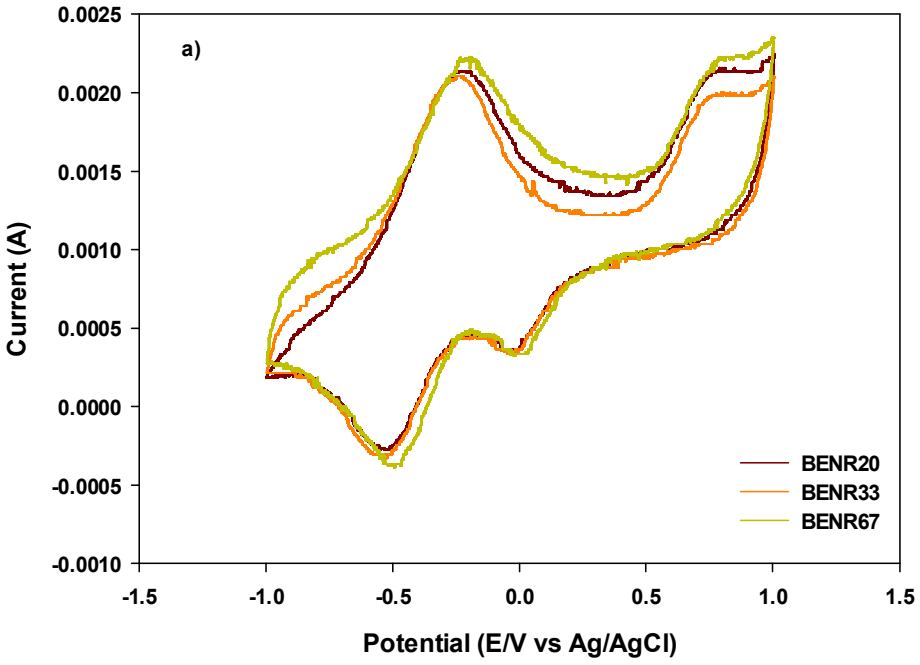


Fig.6.4 a) VSS levels retained and b) specific ammonium removal rate in BENR at steady state, depending on the electrostatic field

This suggests that the biological DIET for nitrogen removal between AOE and DNE for the nitrogen removal in the bulk solution is dependent on the strength of the electrostatic field. In the Anammox process, the specific ammonium removal rate was ranged from 43.1 mg NH4-N /L g VSS/d to 193 mg NH4-N /L g VSS/d, depending on the environmental conditions (Joicy et al., 2019; Szatkowska and Paulsrud 2014; Zhang et al., 2016). This suggests that the bioelectrochemical nitrogen removal driven by electrostatic field can compete with the Anammox process for nitrogen removal if optimized.

6.3.2 Electrochemical properties in the bulk solution

In the cyclic voltammogram (CV), one oxidation and two reduction peaks at non-turnover condition were observed from the bulk solution in the BENRs (Fig. 6.5a), and these peaks give the insight on the AOE and DNE suspended in the bulk solution (Joicy et al., 2019; Song et al., 2019).



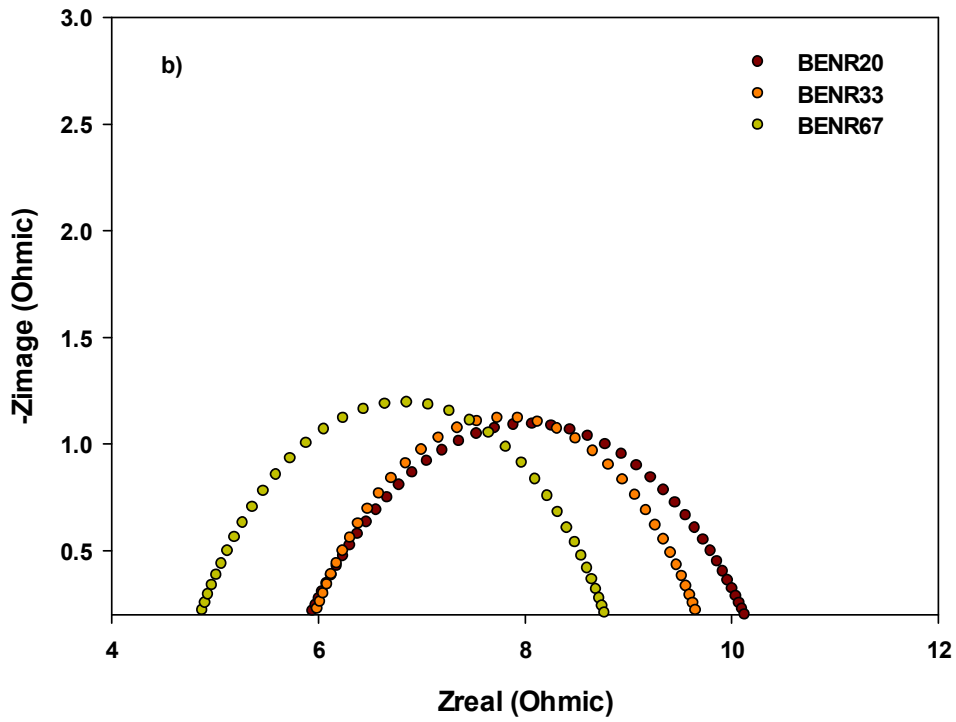


Fig. 6.5. Electrochemical analysis for the bulk solution of BENRs: a) CV b) Nyquist plot for the EIS data

The strength of the electric field exposed to the bulk solution affected the redox peaks in the voltammogram. In the BENR67 with the electrostatic field of 0.67 V/cm, the oxidation peak was 1.24 mA at -0.25V vs. Ag/AgCl, and the reduction peaks were 0.38 mA at -0.00 V vs. Ag/AgCl and 0.92 mA at -0.50 V vs. Ag/AgCl. In the bioelectrochemical reactor, the redox peaks in the CV are occurred by the redox compounds as well as electroactive bacteria species such as AOE and DNE (Newman and Kolter 2000; Canstein et al., 2008). There are several types of redox compounds, including flavin, quinone, and sulfur-based compounds, phenazines and humic substances, which are originated from the endogenous decay of microorganisms or supplied from the outside of bioreactor (Joicy et al., 2019; Canstein et al., 2008; Richter et al., 2012; Wu et al.,2013). However, the fresh nitrogen-rich medium did not have any redox

compounds. Besides, the nitrogen removal rate in the BENRs was not significantly affected by the fresh medium replaced prior to the new batch cycle during the operation. These indicate that the redox peaks in the CVs were mainly expressed by the electroactive bacteria in the BENRs rather than the redox compounds. These indicate that the electroactive bacteria suspended in the bulk solution contributed mainly to the nitrogen removal. However, when the strength of the electrostatic field was reduced to 0.33 V/cm in the BENR33 and 0.20 V/cm in the BENR20, the peak potentials were slightly shifted in the positive direction for oxidation peak and in the negative direction for the reduction peaks (Table 6.1). However, the redox peak heights were decreased in the BENR33, and further in the BENR20, increasing the overpotentials required to reach the redox peaks. These indicate that the AOE and DNE were enriched more at the higher strength of the electrostatic field, which were in good agreement with the removal rate of ammonium and nitrite (Fig. 6.2).

At the end of the batch experiment, the EIS data for the BENRs showed the half cycles in the Nyquist plots (Fig. 6.5b). When the EIS data were fitted to the Randle equivalent circuit, the solution resistance in the BENR67 was 4.7 Ω, which was increased to 5.9 Ω in the BENR20 (Table 6.1).

Table 6.1. Summary of electrochemical analysis in the bioelectrochemical reactor with the strength of electrostatic field ranged from 0.20 V/cm to 0.67 V/cm

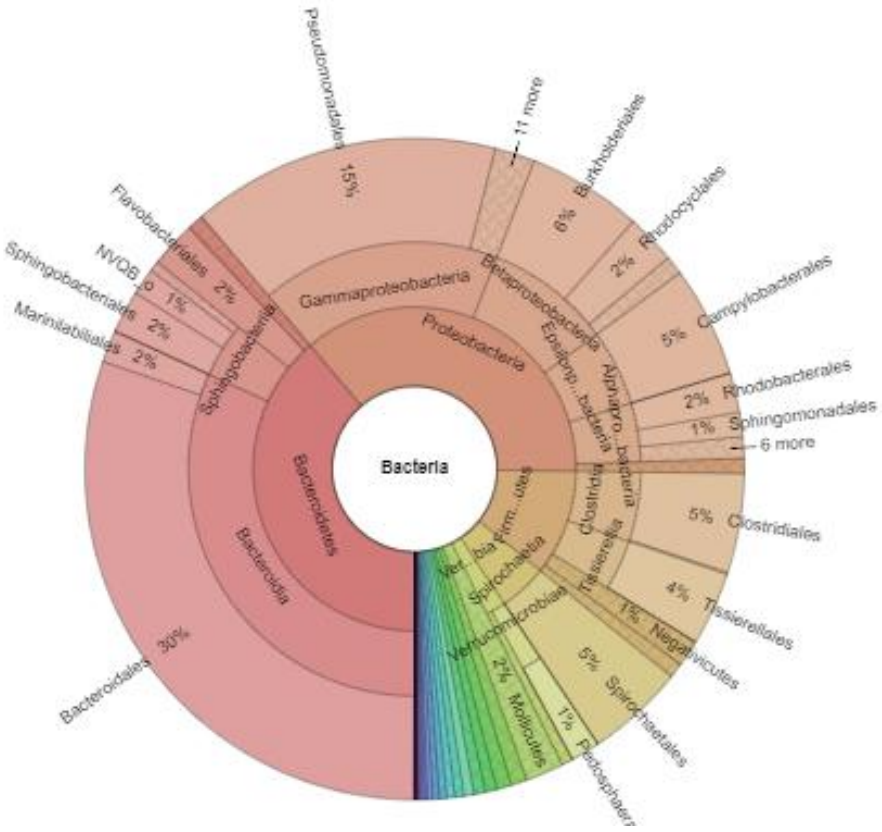
BENR	Electrostatic field (V/cm)	Redox	E_p (V)	I_p (mA)	R_s/R_{ct} (Ω)
BENR20	0.20	Oxidation	-0.20	1.06	5.87/4.38
		Reduction	-0.53	0.85	
			-0.03	0.31	
BENR33	0.33	Oxidation	-0.23	1.14	5.76/3.91
		Reduction	-0.52	0.87	
			-0.02	0.32	
BENR67	0.67	Oxidation	-0.25	1.24	4.71/3.30
		Reduction	-0.50	0.92	
			0.00	0.38	

The electroactive bacteria enriched in bulk solution affect the solution resistance in the bioelectrochemical reactor (Dubé et al., 2015; Doyle and Marsili 2015). The total biomass estimated from the VSS level was similar in the BENR67 and BENR20 (Fig. 6.4a). This means that the high electrostatic field of 0.67 V/cm enriched more the electroactive species including AOE and DNE in the bulk solution, compared to the low electrostatic field of 0.20 V/cm. Then, the charge transfer resistance was 3.3 Ω in the BENR67, which was also lower than 4.4 Ω in the BENR20, indicating that the charge transfer resistance is decreased as the increase in the strength of the electrostatic field. The charge transfer resistance in the BENR67 less than the BENR20 may be partly attributable to the electroactive bacteria enriched more by the high

strength of the electrostatic field. It is known, however, that the electrostatic field increases the enthalpy, and changes the entropy by the orientation of the permanent dipole (Wisseroth et al., 1977). The activation energy required for the reactant to be the transient state is reduced by the electrostatic field, and the electron transfer reaction becomes thermodynamically favourable (Wisseroth et al., 1977; Dignam et al., 1969; Pivrikas et al., 2011). This suggests that as the strength of electrostatic field increases in the range of 0.2 V/cm to 0.67 V/cm, the activation energy required for the electron transfer decreases, facilitating the DIET between the AOE and DNE for the nitrogen removal.

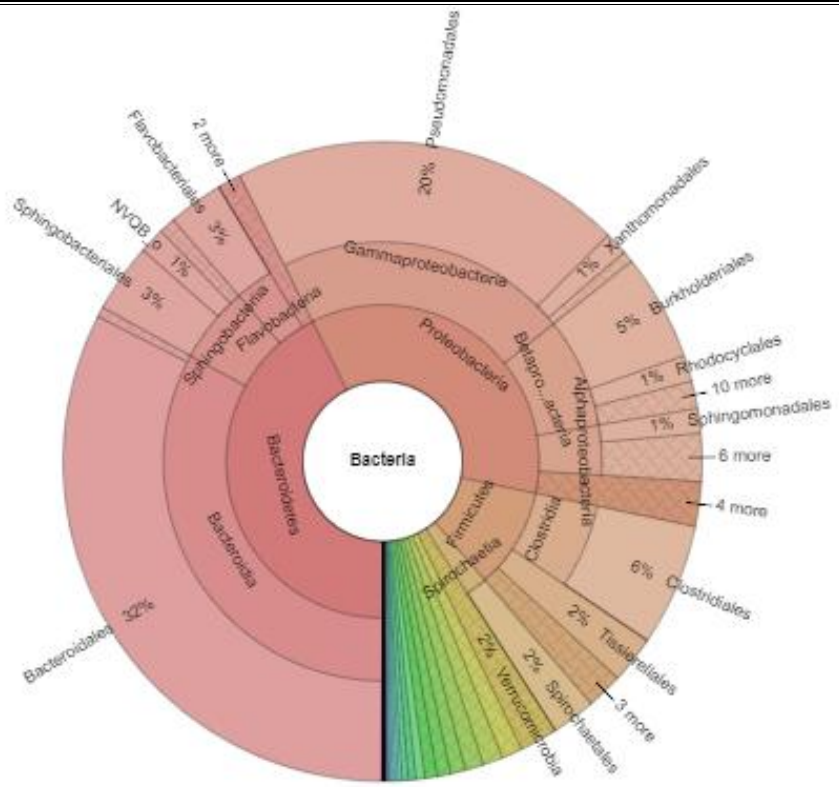
6.3.3 Microbial community analysis

In the bacterial communities, the dominant groups in the bulk solution were similar in all of the BENRs, but their abundances were varied, depending on the strength of the electrostatic field (Fig. 6.6).



a)

b)



c)

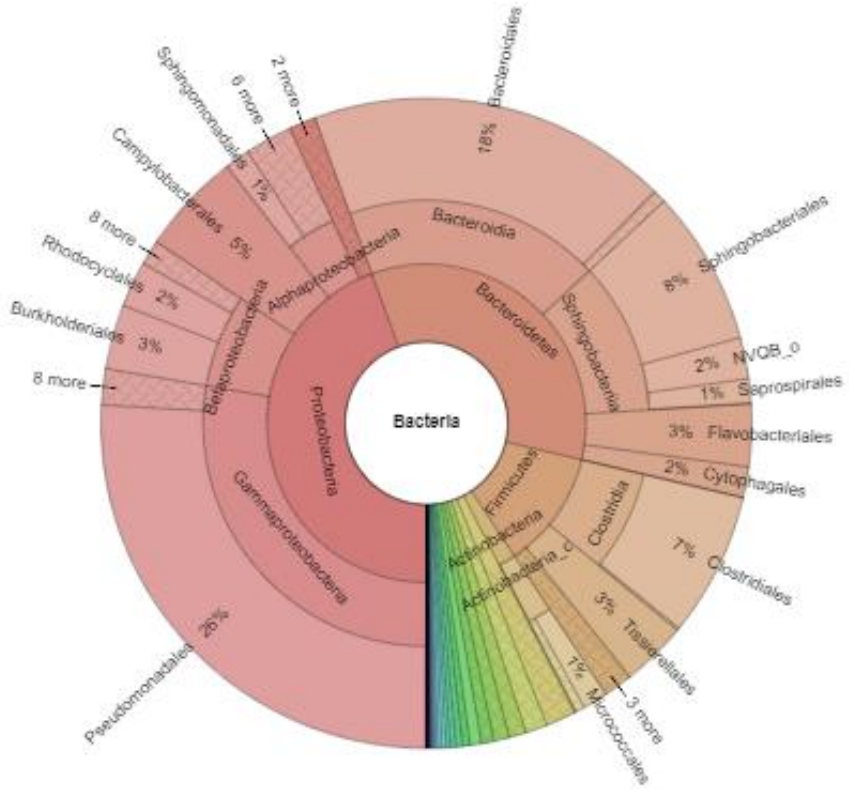


Fig.6.6. Pie charts showing the percentage of abundance of the phylum with species level of the microbial communities: a) BENR20, b) BENR33, c) BENR67.

The total pyrosequencing reads for the bulk solution samples obtained from the BENR20 and BENR 30 were varied from 73,860 to 102,148, which were higher than the BENR67 (54,878). However, the valid reads in the BENR67 (84.9%) was higher than the BENR20 (64.1%) or the BENR33 (65.8%). The number of reads identified of the valid reads at the species level was 38,159 (80.6%) in the BENR20 and 50,814 (75.6%) in the BENR33, but it was 40,048 (86.0%) in the BENR67. The OTUs of BENR67 was 1650, which was less than the BENR20 (2190) and the BENR33 (3019). However, the slope of the OTUs to the reads in the rarefaction curve was steeper in the BENR20 and followed by the BENR 33 and BENR67. The steeper slope of the rarefaction curve indicates the higher species diversity. The OTUs was approached to a plateau value in the rarefaction curve, indicating the microbial communities are analysed in depth (Fig. 6.7a). In the statistical estimates in the diversity indices, the species richness (ACE, Chao1, and Jack knife) and evenness (Shannon, Simpson, and NP Shannon) in the BENR67 were smaller than the BENR33 or BENR20 (Table 6.2). This indicates that the electroactive bacterial species were more selectively enriched as the increase in the strength of the electrostatic field.

Table 6.2. Pyrosequencing reads, number of the OTUs and diversity indices based on the number and pattern of the OTUs for the bulk solution exposed to electrostatic field

Contents	BENR20	BENR33	BENR67
Total reads (valid %)	73,860(64.1)	102,148(65.8)	54,878 (84.9)
Read length (bp)	114-454	86-446	114-446
Reads identified at the species level	38,159(80.6)	50,814(75.6)	40,048(86.0)
OTUs	2190	3019	1650
Good's coverage of library	99.6	99.7	99.5
ACE	2,271.2	3,117.1	1,766.9
Chao1	2,216.5	3,044.1	1,734.1
Jack knife	2383	3245	1864
Shannon	5.313	5.339	4.964
Simpson	0.019	0.044	0.052
NP Shannon	5.384	5.411	5.012
Phylogenetic diversity	2232	2561	2274

Of the OTUs identified taxa, three dominant groups at the phylum level were Proteobacteria, Bacteroidetes, and Firmicutes in the BENRs. In the BENR67, the first dominant phylum was Proteobacteria (44.4%), but Bacteroidetes were the most dominant phylum in the BENR33

(42.6%) and BENR20 (38.8%). The phylum Firmicutes was ranged from 10.4% to 12.0%, similar to all of the BENRs. In the previous study, the first dominant phylum in the conventional bioelectrochemical reactor for nitrogen removal was Bacteroidetes (Joicy et al., 2019). It seems that the dominance of the phyla Proteobacteria and Bacteroidetes depends on the strength of the electrostatic field exposed to the bulk solution. At the class level, it is noted that Gammaproteobacteria were higher in BENR67 (27.7%), compared with the BENR33 (21.6%) and BENR20 (17%), and followed by Bacteroidia (18.6%) and Sphingobacteriia (11.0%) belong to the phylum Bacteroidetes. However, Bacteroidia were the most dominant class in the BENR33 (33.0%) and BENR20 (31.9%). At the genus level, the bacterial abundance was distinctly different depending on the electrostatic field. In the BENR67, the most dominant genus was *Pseudomonas* (21.7%), followed by *Petrimonas* (4.6%), *Arcobacter* (3.9%), *Thiopseudomonas* (3.4%) and uncultured *EU234264-g (3.1%)*, but *Nitrospira* was only 0.5% (Fig.7b). In the BENR33, the abundance of the genera uncultured *DQ677001_g* (5.0%), *Paludibacter* (3.8%) and *Parabacteroides* (3.2%) were higher, compared to the BENR67, while the abundance of *Pseudomonas* (19.7%), *Petrimonas* (3.8%) and *Thiopseudomonas* (0.7%) were lower. In the BENR20, the abundance of *Pseudomonas* (7.2%) was significantly lower than the other two reactors, but the genera *Petrimonas* (7.4%), *Thiopseudomonas* (6.9%) and *DQ677001_g* (6.8%) were higher. The genera *Pseudomonas*, *DQ677001_g*, Petrimonas, *Thiopseudomonas*, were abundant in the bulk solution in the conventional bioelectrochemical reactor for nitrogen removal, in previous study (Joicy et al., 2019). However, the genera *Nitrosomonas* and *Nitrobacter*, which oxidize ammonium and nitrite, as well as the genera known as Anammox bacteria such as *Brocadia*, *Kuenenia*, *Scalindua*, *Jettenia*, and *Anammoxoglobus* were not observed in all of the BENR microbial community. This suggests that the nitrogen removal mechanism driven by the electrostatic field in the bioelectrochemical reactor is distinctly different from the Anammox process.

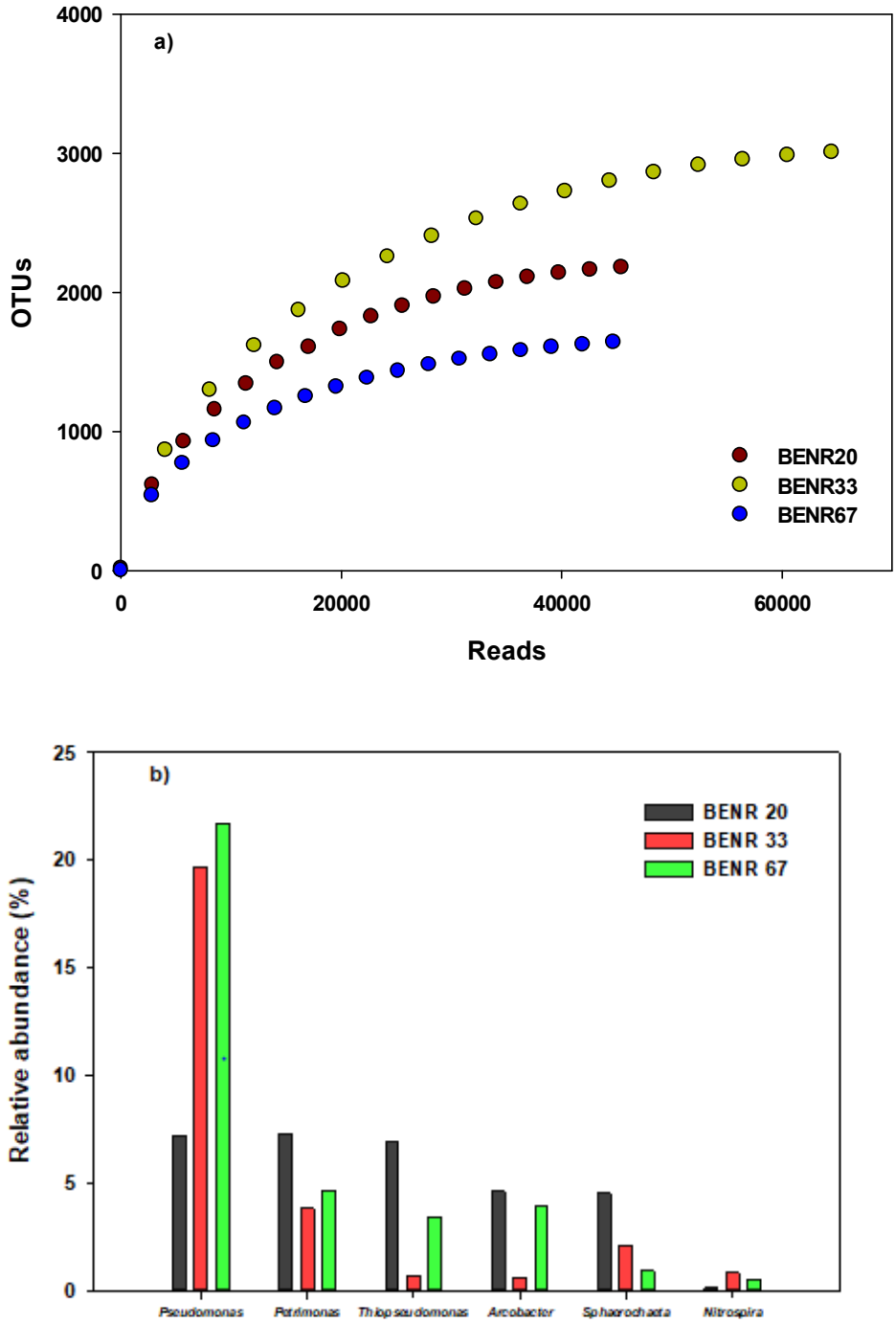


Fig.6.7 Rarefaction curves with number of OTUs of reads: a) refractive curves b)

Predominant bacteria at the genus level at BENRs

The bacterial abundance at the species level gives insight into the nitrogen removal in the bioelectrochemical reactor. It is noted that *Pseudomonas caeni* were the most abundant species in the BENR67 (21%) and BENR33 (19.6%), but the abundance was significantly decreased in the BENR20 (6.6%). This indicates that the growth of *P. caeni* is significantly dependent on the electrostatic field. *P. caeni* are known as a denitrifying species that reduces nitrite and nitrate, which were isolated in the Anammox reactor (Xiao et al., 2009). In recent, *P. caeni* were also identified in the bioelectrochemical reactor with the polarized electrode for nitrogen removal, as well as microbial fuel cells (Joicy et al., 2019; Tkach et al., 2017). This suggests that *P. caeni* are the most dominant electroactive species in which the electrostatic field drives the electron transfer in the BENR. The next abundant species in BENR67 were *Arcobacter AM084124_s* (3.4%) and *Petrimonas sulfuriphilia* (3.3%). *A. AM084124_s* are the denitrifying bacteria belonging to Epsilon proteobacteria and was isolated from activated sludge. It has been shown that genus *Arcobacter* can contribute to the denitrification in the activated sludge system (Heylen et al., 2006). *P. sulfuriphila* are the anaerobic fermenter that uses element sulphur and nitrate as electron acceptors (Grabowski et al., 2005), which were abundant in the bioelectrochemical reactor with the polarized electrode for nitrogen removal (Joicy et al., 2019). However, the genus *Petrimonas* are the fermenter of carbohydrates, and they were observed in the microbial fuel cells (Grabowski et al., 2005; Liu et al., 2016). It seems that the genus *Petrimonas* might have the syntrophic relationship with the AOE and DNE. Other abundant species in BENR67 was *Thiopseudomonas denitrificans* (2.8%) that is sulphide oxidizer anaerobically with nitrate as an electron acceptor (Tan et al., 2015). However, the abundances of *Thiopseudomonas sp.* and *Petrimonas sp.* were higher in the BENR20 than the BENR33 and BENR67. This indicates that *Thiopseudomonas sp.* and *Petrimonas sp.* are the species that directly transfer the electrons for nitrogen removal, but their metabolism is less affected by the strength of the electrostatic field. However, the functions of the abundant bacterial species

identified in the BENRs has not yet been elucidated. The metabolism of abundant bacterial groups for the ammonium oxidation and nitrite reduction need to be further clarified to describe the mechanisms of bioelectrochemical nitrogen removal in BENR.

6.4. Conclusion

The electroactive microorganisms including ammonium oxidizing exoelectrogens (AOE) and denitritating electrotrophs (DNE) are enriched from activated sludge as inoculum in the ammonium and nitrite-rich medium that exposed to the electrostatic field. Electroactive microorganisms remove ammonium in an anaerobic condition, companying with the consumption of nitrite and alkalinity, through the direct interspecies electron transfer (DIET) between AOE and DNE. The electrostatic field facilitates the DIET for nitrogen removal by reducing the charge transfer resistance that depends on the strength of the electrostatic field. The electrostatic field-driven DIET in anaerobic condition is a competitive novel approach for the treatment of nitrogen-rich wastewater.

Chapter 7 – Bioelectrochemical partial nitritation of ammonium rich wastewater in upflow reactor: electrostatic field and HRT effects

7.1 Introduction

To overcome the abundance of nitrogenous contamination in the wastewater, Anammox process has become the attractive technology in recent decades. Normally this anammox process converts ammonium to nitrogen gas by using nitrite as electron acceptor under anaerobic condition. Due to their importance in removing fixed nitrogen from both engineered and natural systems, it has become a replaceable for conventional biological nitrogen removal systems in wastewater treatment plants. As Anammox bacteria use nitrite as an electron acceptor to oxidize ammonia, the major challenge was the slow growth rate of Anammox bacteria and also requires special strategies to enrich Anammox microorganisms. Moreover, other challenges are, to resolve the limitations involved in the nitrite requirement and nitrate production from the Anammox reaction. The nitrite for the Anammox reaction is generally obtained by oxidizing about half of the ammonium contained in the wastewater, but sometimes, the nitrate is partially denitrified into nitrite. The partial nitration of ammonium is an essential step to economically remove nitrogen from ammonium-rich wastewater in biological nitrogen removal processes including Anammox and bioelectrochemical processes.

Recently, it has been revealed that a bioelectrochemical nitrogen removal process can mitigate these limitations of the Anammox process. Bioelectrochemical nitrogen removal process is based on the use of electrochemically active microorganisms. Electrochemically active microorganisms are capable extracellular electron transfer. So, in a bioelectrochemical reactor, ammonium oxidizing exoelectrogens (AOE) grow on the anode surface to oxidize ammonium, and the electrons released from the oxidation are directly transferred to the cathode.

Denitrifying electrotrophs (DNE) on the cathode surface take the electrons directly from the cathode to reduce nitrite or nitrate into nitrogen gas. This means that the ammonium, nitrite, and nitrate nitrogen can be removed bioelectrochemically by the AOE and DNE. Hence, electro active microorganism function as a catalyst for the electrochemical oxidation of the organic material. However, bioelectrochemical nitrogen removal through polarized electrode surfaces has been difficult to put into practical use in the field because it is directly controlled by the surface area of the anode and cathode. When the suspended microorganisms in the bulk solution are exposed to electrostatic field by polarized electrodes, it has been noted that the electrostatic field enriches the exoelectrogens and electrotrophs by expressing the electroactive genes in the bioelectrochemical reactor. These suspended exoelectrogens and electrotrophs in the bulk solution can be electrically connected by physical contact between adjacent species to transfer the electrons directly each other (DIET) without a mediator. Interestingly, electroactive microorganisms can be enriched on the surface of conductive materials such as activated carbon, carbon nanotube and magnetite. Conductive materials serve as the conduit for DIET between AOE and DNE, which is known as conductive material mediated DIET (cDIET). However, the influencing factors on the cDIET have not been elucidated in detail yet. As electrostatic field enriches the electroactive microorganisms and promotes DIET for nitrogen removal through previous studies, in this study we demonstrate the bioelectrochemical partial nitritation of ammonium rich wastewater in upflow reactor through series of electrostatic field and HRT effects as parameters. This study was conducted to suggest that the bioelectrochemical nitritation and nitrification are dependent on the intensity of electrostatic field as well as HRT.

7.2 Materials and Methods

7.2.1 Seed sludge, artificial medium and electrode preparation

The artificial medium containing 0.3 g/L KH_2PO_4, 1.0 g/L $Na_2HPO_4.12H_2O$, 0.5 g/L NaCl, 2.0 g/L $NaHCO_3$, 0.1 g/L $MgSO_47H_2O$, 0.01 g/L $CaCl_2$, 1.91 g/L NH_4Cl, was prepared for continuous bioelectrochemical reactor in accordance with previous studies (Zhan et al., 2012; Joicy et al., 2019). Powdered activated carbon was obtained from a local provider, and used as a conductive material (Power Carbon Technology Co., Ltd., Korea). For the inoculum, activated sludge was collected (Y-WWTP, B-Metrocity, Korea) and screened to remove impurities, then concentrated by gravity for a day and used in the bioelectrochemical reactors. The initial characteristics of the activated inoculum was 7.95 of pH and 4,500 mg/L of VSS (volatile suspended solids), respectively. Commercially available titanium foil obtained from a supplier (0.1 T, Grade 2; Baoji Hong Ya Da Nonferrous Metal Materials Co., Ltd; Baoji, China) was used as the polarized electrode. The foil was cut into a small size (5.5cm×23 cm) and a large size (24cm×25.5cm) to obtain a pair of ordinary electrodes (OE). The foil surfaces were coated with a dielectric material (alkyd enamel, 470 g/L volatile organic compounds, NOROO Paint & Coatings Co., Ltd. Anyang, Korea) to prepare the surface insulated electrode (IE).

7.2.2 Bioelectrochemical reactor set-up and operation

The upflow bioelectrochemical reactor used in this experiment was prepared using a cylindrical acrylic resin (effective volume 1.0 L, inner diameter 7 cm). A flanged cover plate was used for each reactor to ensure that the upper end of the reactor was airtight in each case. An inlet valve that flows into the wastewater is installed on the bottom wall of the reactor, and an outlet valve is installed on the wall below the headspace of the reactor. The upper portion of the reactor was sealed with an acrylic cover and one port was placed on the cover for aerator tube. The aerator

tube holding port was sealed by connecting an aerator tube immersed in the bulk solution to the bottom side of the cap plate. An upflow reactor was used as a control reactor without applying voltage as BENR-C in the experiment, and the other two upflow reactors were used as BENR-1 and BENR-2 reactors. For the experiment, the nitrogen (ammonium) rich medium (450mL) and inoculum (550 mL) were added into three different reactors, and the insulated electrodes were polarized by applying voltage ranged from 0.6 V, 1.0 V, 2.0 V and 5.0 V using the DC voltage source, and the electrostatic field between the polarized electrodes were 0.24 V/cm, 0.40 V/cm, 0.80 V/cm, 1.96 V/cm. The small and large electrodes were arranged in annular shapes and then installed on the outer wall of the steel shaft tube and on the inner wall of the reactor, respectively. Powdered activated carbon (3g) was then added to the bioelectrochemical reactors. The potential between the insulated electrodes in the BENR1 and BENR2 reactors was controlled by using a direct current (DC) power source (OPM series, ODA Technologies Co., Incheon, Korea). The prepared bioelectrochemical reactors were operated at room temperature (25 ± 2 ° C).

7.2.3 HRT and electrostatic field for bioelectrochemical nitrogen removal

The HRT for the three upflow bioelectrochemical reactor (Control, BENR1, BENR2) was varied with 1 day, 2 days and 4 days. At each HRT conditions the bioelectrochemical reactors were operated and the steady state was confirmed from the stability of some state variables, such as pH, Alkalinity, NO_2-N, NO_3-N and NH_4-N removal. The two bioelectrochemical reactors were started by setting the electrostatic field of BENR1 - 0.24 V/cm and BENR2 - 0.40 V/cm between the polarized electrodes at the room temperature. The electrostatic field maintained between the polarized electrodes were adjusted from 0.24 V/cm to 0.80 V/cm in BENR1 and 0.40 V/cm to 1.96 V/cm in BENR2 as the state variables were stabilized.

7.2.4 Analysis and calculation

During the operation of the upflow bioelectrochemical reactors, the properties of the influent and effluent including alkalinity, NH_4-N, NO_2-N and NO_3-N were monitored according to the Standard Methods for the examination of water and wastewater (APHA-AWWAWEF, 2005). The NH_4-N was analyzed by Phenate method, and the NO_2-N and NO_3-N were obtained by Ultraviolet Spectrophotometric Screening Method and Colorimetric Method, respectively. The pH was daily monitored by using pH meter (YSI pH1200 laboratory pH meter 115-230V (T1)) and the DO was monitered daily by using DO meter. For the CV, the small pieces of stainless mesh (1cm×1cm) were used as the working and counter electrodes. The potential window was in the range of -1.0 V to 1.0 V (vs. Ag/AgCl), and the scan rate was 10mV s-1. The redox peak height and potential were obtained from cyclic voltammogram using the software, 'SMART Manager' (ZIVE BP2 Series, WonATech Co., Korea).

7.2.5 Bacterial community analysis

At the end of the experiment, Microbiome Taxonomic Profiling was performed to investigate microbial communities using 16S rRNA in the bulk solution of the upflow bioelectrochemical reactors. The DNA was extracted from suspended sludge in the bulk solution using Power soil DNA isolation kit according to the kit protocol (MO BIO Laboratories, Inc., CA, USA). The fusion primer was used to amplify the variable region of V3V4 for bacteria of the 16S rRNA gene in the genomic DNA. The 16S rRNA was amplified from the metagenomic DNA, and pooled, sequenced on the MiSeq Personal Sequencer (Illumina, San Diego, CA, USA). The amplification, construction of the sequencing library and bioinformatic analysis were performed as described in the previous study (Chun et al., 2010). Chimera was checked, and taxonomic assignments of these readings were done using the EzBioCloud database (http://ezbiocloud.net/). Microbial community and the statistical taxonomical assignments were obtained through the operational taxonomic units. Comprehensive bioinformatic analysis like the species-level classification of microbes, cluster analysis, microbial origin tracking,

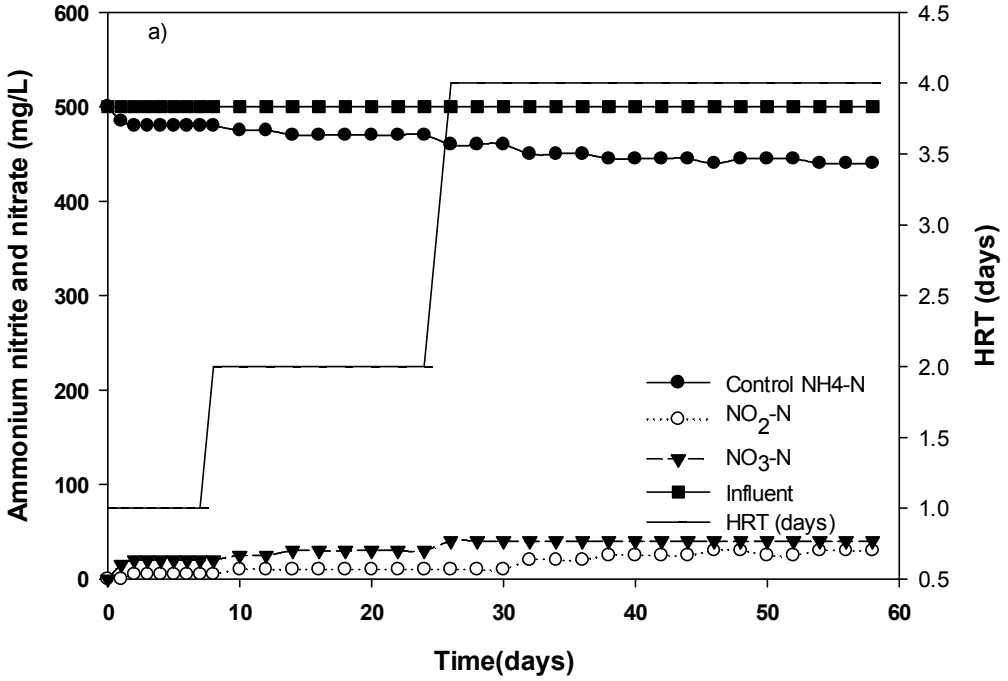
hierarchical clustering and various indicators of species diversity were conducted by the EZ Biocloud (Chunlab, Inc., Seoul, Republic of Korea).

7.3 Results and discussion

7.3.1 Influence of HRT in bioelectrochemical partial nitritation

The effluent concentration of NH_4-N in the bioelectrochemical reactor decreased exponentially after the start-up, and stabilized from 48^{th} day. However, the removal of NH_4-N varied depending on the HRT and the applied voltage performed in the bioelectrochemical reactors. When the bioelectrochemical reactors were operated at 1 day of HRT the ammonium removal rate was of 100mg/L under the electrostatic field of 0.24 V/cm and 150mg/L under the electrostatic field of 0.4 V/cm. When the HRT was increased to 2 days the removal efficiency of ammonium was 195mg/L under the electrostatic field of 0.24 V/cm and 230mg/L under the electrostatic field of 0.4 V/cm. As the longer HRT, the efficiency of ammonium removal rate in the BENRs were increased, so the HRT was increased of 4 days and it is noted that the ammonium oxidation in BENR1 under the electrostatic field of 0.24 V/cm was 280mg/L, BENR2 under the electrostatic field of 0.4 V/cm was 320mg/L. Under the electrostatic field of 0.4 V/cm, the nitrite was accumulated from 100 mg NO_2-N/L to 270 mg NO_2-N/L with the increase in HRT from 1 day to 4 days. However, the nitrite accumulation under the electrostatic field of 0.24 V/cm was less than those under the electrostatic field of 0.4 V/cm. Under the electrostatic field of 0.4 V/cm, the nitrate was slightly increased from 35 mg NO_3-N/L to 50 mg/L of NO_3-N with the increase in HRT from 1 day to 4 days, which was not significantly different from that under the electrostatic field of 0.4 V/cm. (Fig. 7.1). In Anammox process it is well known that nitrite was used as the only electron acceptor to oxidize ammonium, but partial nitritation to selectively produce nitrite only from ammonia-rich wastewater is one of the major obstacles that hinder the wide application of the Anammox process. In

bioelectrochemical reactors it was noted that ammonium oxidation was performed and in nitrification process the ammonium was converted into nitrite as by-product than nitrate which was a great drawback in anammox process. But further studies are needed on the removal of nitrate and nitrite to lower the total nitrogen level in the effluent.



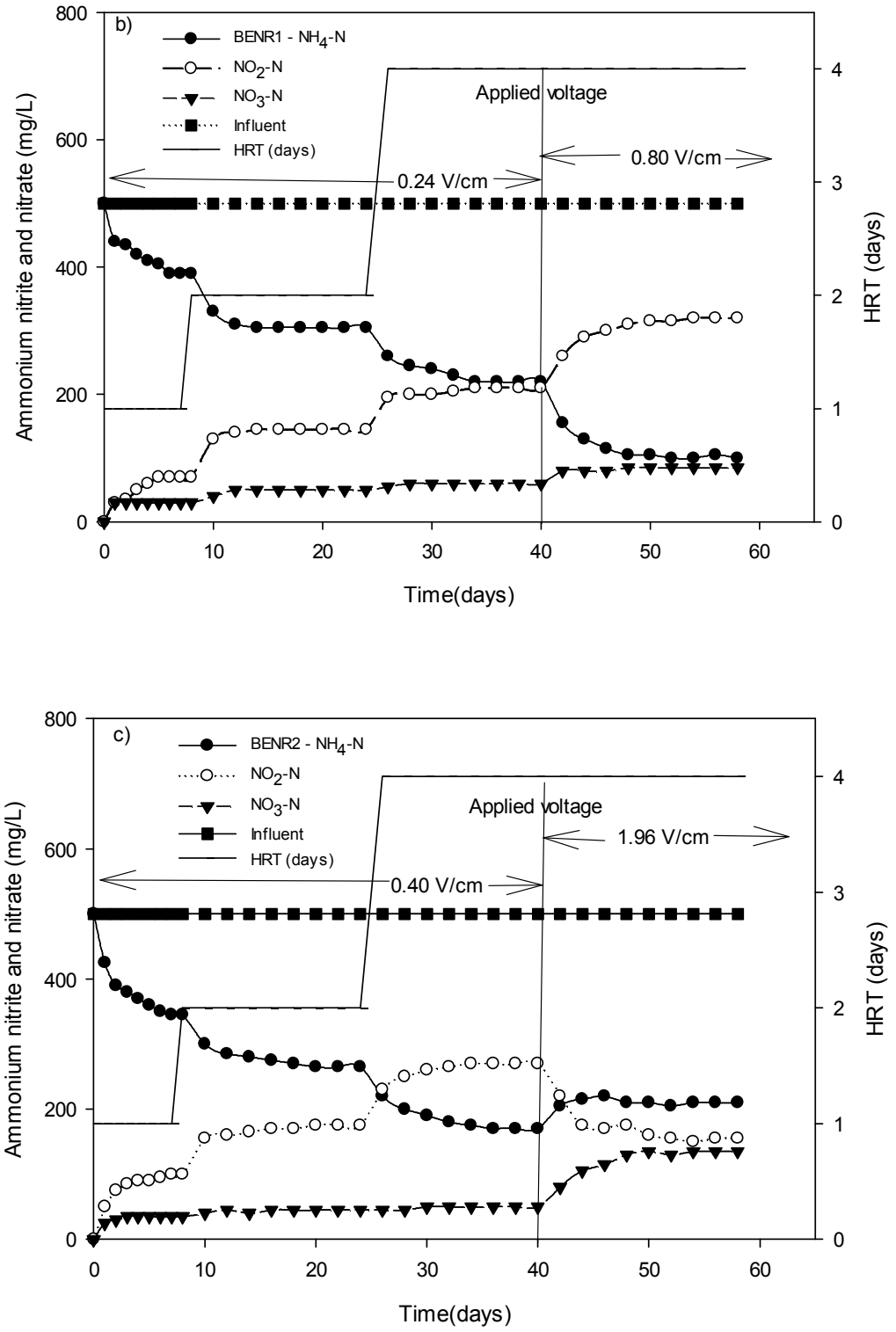


Fig 7.1. Effluent profiles in a) Control, b) BENR1 and c) BENR2

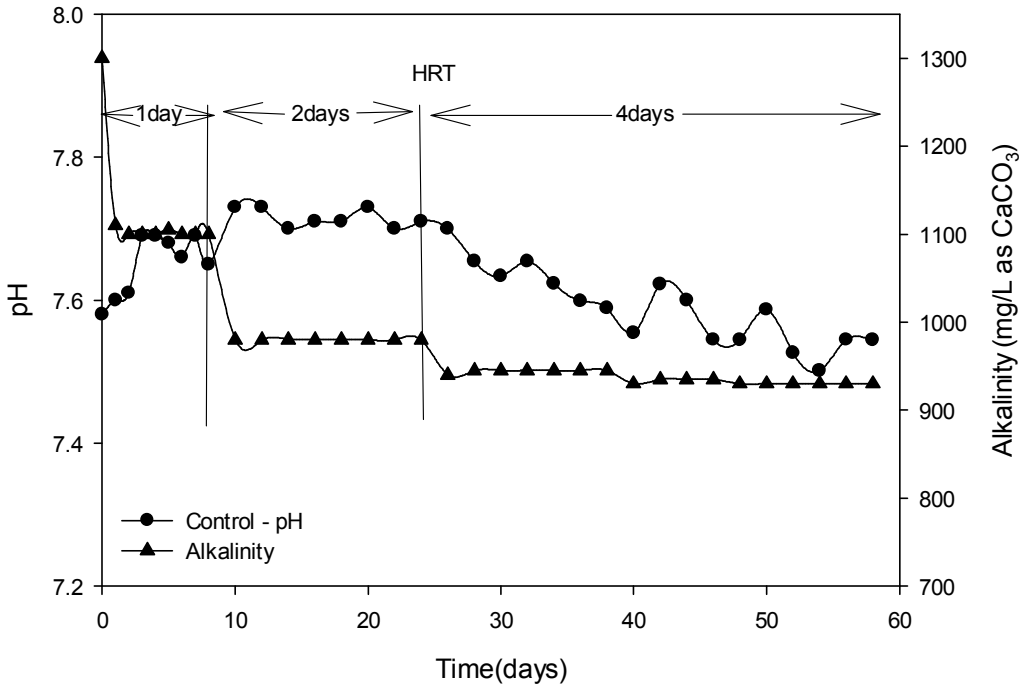
In the bioelectrochemical reactors, the potential routes for nitrogen removal are the assimilation to bacterial cell, the conventional nitrification and denitrification, and the bioelectrochemical reactions, such as anodic ammonia oxidation, anammox (Ma et al., 2016; Yang et al., 2009; Zhan et al., 2014). The growth yield of electroactive bacteria is generally smaller than the aerobic bacteria in conventional biological treatment process (Wilson and Kim, 2016). In the upflow bioelectrochemical reactor, the influent DO was low as3.0 - 3.7mg/L. It is probably that the influent DO is quickly consumed for the oxidation of organic matter in the lower part of the reactors. DO concentration enhances the free energy released from oxygen reduction hence leads to ammonium oxidation in the BENRs. Moreover, the available organic matter for heterotrophic denitrification also limited. This means that the nitrogen removal through the conventional nitrification and denitrification process is not significant. However, the ammonia nitrogen removal was improved by the conductive materials as activated carbon in the bioelectrochemical reactors (Fig.7.1). the conductive material as activated carbon is known to mediate DIET through electroactive microrganisms that enhances the ammonium removal in the continuous bioelectrochemical reactor. In the BENRs, the ammonium was oxidized into nitrite and nitrite gradually in the bulk solution, hence indicating that ammonium can be oxidized bioelectrochemically through activated carbon mediated DIET. The oxidation of ammonium was higher in the two BENR reactors in comparison with control where applied voltage was not supplied. The conductive materials including activated carbon are known to mediate the DIET between the polarized electrodes in the bulk solution. This implies that the electroactive ammonia oxidizing bacteria play an important role in nitrogen removal in the bioelectrochemical reactor.

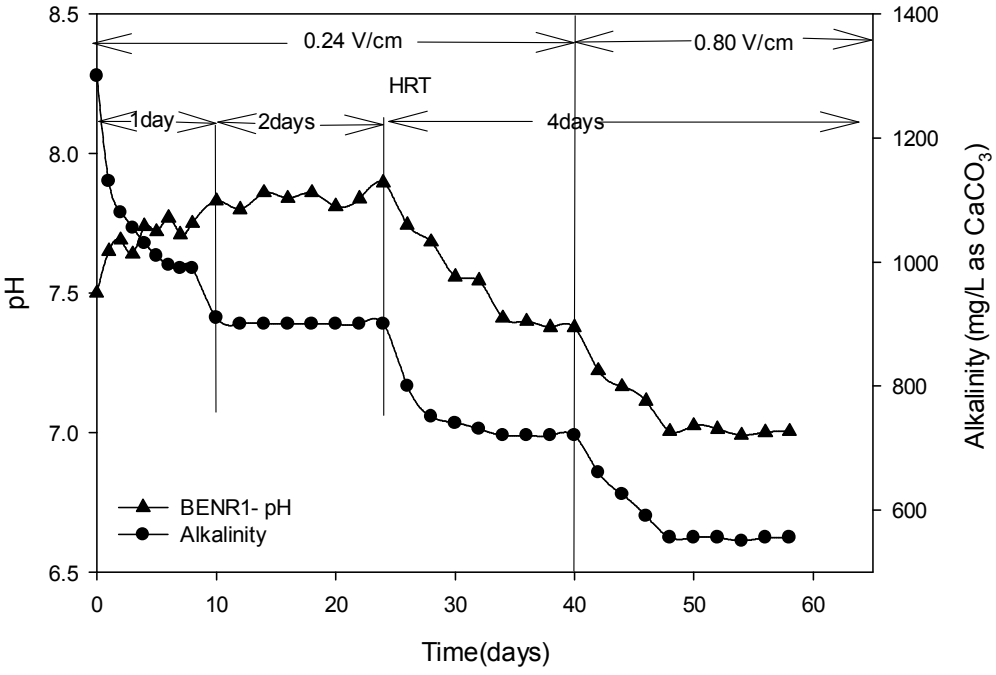
7.3.2 Effect of electrostatic field for bioelectrochemical nitritation and nitrification

NH_4-N removal was examined under different applied voltages ranging from 0.6 V to 5V to establish the electrostatic fields of 0.24 V/cm, 0.40 V/cm, 0.80 V/cm, 1.96 V/cm at HRT of 4

days when the NH_4-N was added about 500mg/L as the sole nitrogen source. The removal rates of ammonium nitrogen were gradually increased with the operation time of the BENRs. At BENR1 at 0.80 V/cm as electrostatic field between the dielectric electrodes showed higher ammonium oxidation of 395mg/L which was slightly higher than BENR2 with electrostatic field of 1.96 V/cm showed 290mg/L NH_4-N removal. In BENR1 where the applied voltage was 0.6V with electrostatic field of 0.24 V/cm, the ammonium removed was 280mg/L which was little less in comparison with 1.0V with electrostatic field of 0.40 V/cm, the ammonium removed was 320mg/L. Under the electrostatic field of 0.80 V/cm, the nitrite was further accumulated to 320 mg NO_2-N/L, but the nitrate was also slightly increased to 85 mg NO_3-N/L than the reactors where polarized to expose the bulk solution to an electrostatic field of 0.24 V/cm and 0.40 V/cm. However, under 1.96 V/cm, the nitrite was accumulated to 155 mg/L of NO_2-N, but the nitrate was significantly increased to 135 mg/L of NO_3-N. This indicates that nitrite can be accumulated from ammonium oxidation with small accumulation of nitrate under 0.8 V/cm at HRT of 4 days. Hence, it concludes that when the bioelectrochemical reactor was optimised by a proper combination of electrostatic field and HRT, bioelectrochemical partial nitritation of ammonium rich wasterwater was feasible (Fig.7.1). Normally in previous studies (Zhan et al., 2012; Qu et al., 2014; Joicy et al., 2019) the ammonium was oxidized at the anode surface by the AOE to release the electrons, and DNE reduced the nitrite to nitrogen gas at the cathode surface using the electrons. However, the electrode surface of BENR1 with electrostatic field of 0.80 V/cm was insulated with a dielectric polymer, and there was no electrode surface that DIET microorganisms could attach for the electron transfer. pH and alkalinity decreased simultaneously with the removal of ammonium in the reactors (Fig. 7.2). This indicates that the bicarbonate, a main component of the alkalinity, at neutral pH was used as the carbon source for the growths of activated carbon mediated and biological DIET in the bulk solution of bioelectrochemical reactors. From the reactors it was noted that

bioelectrochemical nitritation and nitrification are dependent on the intensity of the electrostatic field between the polarized electrodes as well as the HRT.





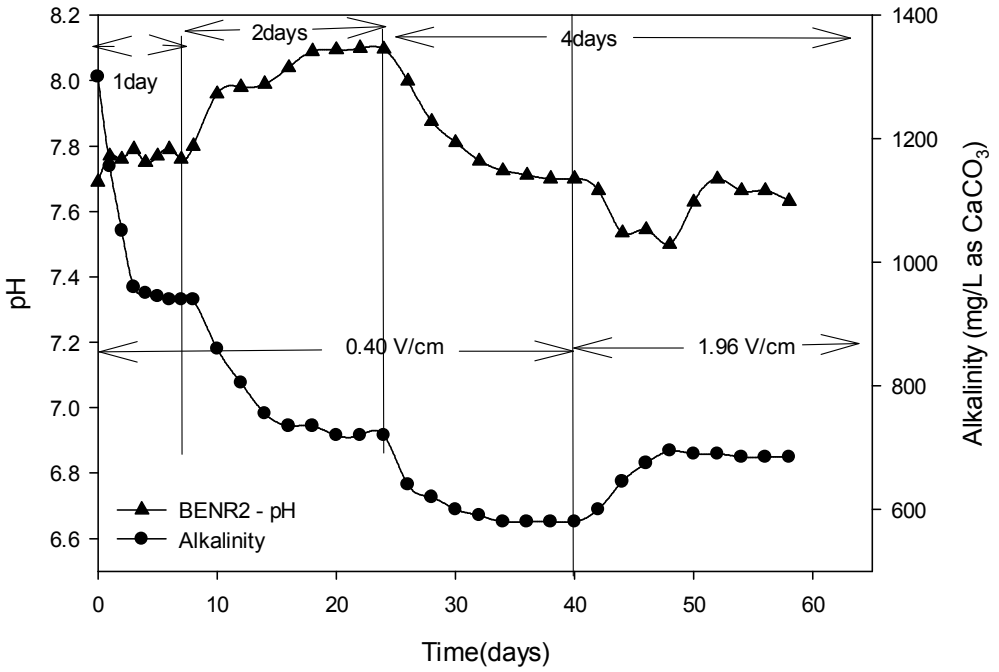


Fig 7.2. Effluent pH and Alkalinity in a) Control, b) BENR1 and c) BENR2

7.3.3 Electrochemical properties of biomass

In the bioelectrochemical reactors, the electrochemical activity of the bulk solution was dependent on the conductive and biological mediated DIET. The strength of the electric field exposed to the bulk solution affected the redox peaks in the voltammogram. In BENR1 with 0.80 V/cm as electrostatic field, the oxidation peak was 0.81 mA at 0.23V vs. Ag/AgCl, and the reduction peak was 0.54 mA at -0.69 V vs. Ag/AgCl. In BENR2 with 0.40 V/cm as electrostatic field, the oxidation peaks were 0.78 mA at 0.25V vs. Ag/AgCl and 0.2 mA at -0.49V, and the reduction peak was 0.41 mA at -0.78 V vs. Ag/AgCl (Fig.3). In the bioelectrochemical reactor, the redox peaks in the CV are occurred by the redox compounds as well as electroactive bacteria species such DIET (Newman and Kolter 2000; Canstein et al., 2008). There are several types of redox compounds, including flavin, quinone, and sulfur-based

compounds, phenazines and humic substances, which are originated from the endogenous decay of microorganisms or supplied from the outside of bioreactor (Joicy et al., 2019; Canstein et al., 2008; Richter et al., 2012; Wu et al., 2013). However, the fresh nitrogen-rich medium did not have any redox compounds. This indicate that the redox peaks in the CVs were mainly expressed by the electroactive bacteria in the BENRs rather than the redox compounds. Hence the electroactive bacteria suspended in the bulk solution contributed mainly to the nitrogen removal. BENR2 with 0.80V/cm significantly showed higher peak current comparatively with different electrostatic field in the range of 0.40 V/cm, 0.24 V/cm and 1.96 V/cm. In BENR1 with electrostatic field of 0.24 V/cm, an oxidation peak at 0.22V vs. Ag/AgCl was observed in the voltammogram, but there were two reduction peaks at -0.71 V vs. Ag/AgCl and –0.21 V vs. Ag/AgCl. In BENR2 with electrostatic field of 1.96 V/cm, two oxidation peaks at 0.21V vs. Ag/AgCl and -0.50 V vs. Ag/AgCl and two reduction peaks -0.69 V vs. Ag/AgCl and –0.23 V vs. Ag/AgCl, respectively. From the redox peak, it is likely that the DIET in the bulk solution contributed more in the BENR1 with electrostatic field of 0.80 V/cm to nitrogen removal than the other reactors.

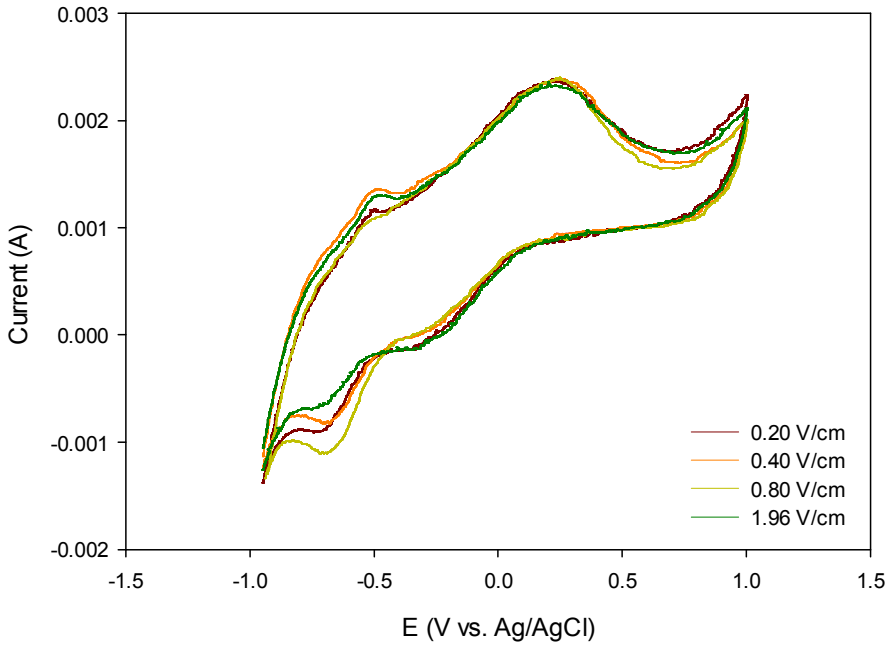


Fig 7.3. Cyclic voltammogram in the bulk solution of bioelectrochemical reactors

7.3.4 Microbial communities

The microbiome samples collected from the bulk solution were taxonomically profiled based on the NGS platform. In the BENRs the valid reads of the bacteria ranged from 26,527 to 35,670 and the OTUs ranged from 1325 to 1763 in which BENR2 with electrostatic field 1.96 V/cm showed less OUT and valid reads compared with other reactors. In the statistical estimates in the diversity indices, the richness (Ace, Chao1, Jack knife) of bacterial species was higher in the BENRs with electrostatic field 0.24 V/cm and 0.40 V/cm, but it was decreased in 0.80 V/cm and 1.96 V/cm. The evenness (Shannon, Simpson, NPShannon) of the bacterial species was higher in the BENRs with electrostatic field 0.80 V/cm, but slightly decreased in BENRs with electrostatic field 0.24 V/cm and 0.40 V/cm. It is likely that the microbial species were abounded by the activated carbon, and were selected by the electrostatic field.

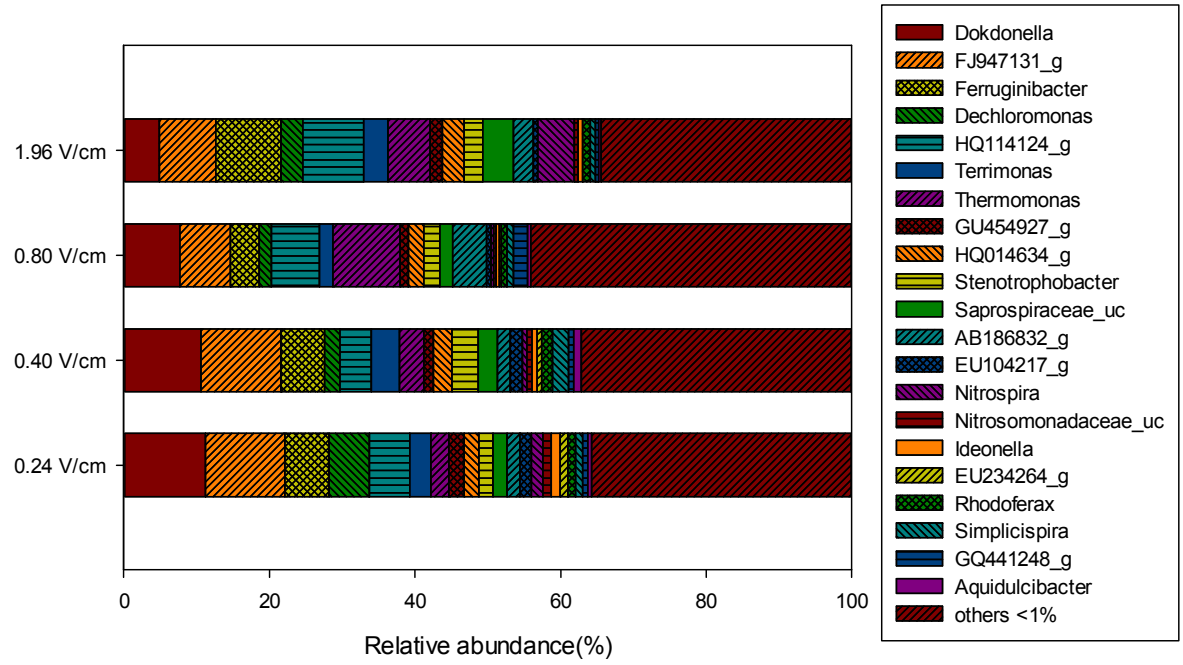


Fig 7.4. Microbial taxonomic profiling at genus level in bioelectrochemical reactors

Of the OTUs identified taxa, the four predominant phylum groups in bacteria were Proteobacteria, Bacteroidetes, Chlorobi and Acidobacteria in the BENRs. In BENRs with electrostatic field of 0.24 V/cm (47.23%), 0.40 V/cm (48.65%) and 0.80 V/cm (52.56%), the predominant phylum was Proteobacteria, but Bacteroidetes were the most dominant phylum in the BENR2 with electrostatic field of 1.96 V/cm (42.03%). It seems that the dominance of the phyla Proteobacteria and Bacteroidetes depends on the strength of the electrostatic field exposed to the bulk solution. At the class level, it is noted that Gammaproteobacteria were higher in BENR1 with electrostatic field of 0.80 V/cm (22.94%), compared with the BENR2 with electrostatic field of 0.40 V/cm (19.41%), BENR1 with electrostatic field of 0.24 V/cm (18.79%) and BENR2 with electrostatic field of 1.96 V/cm (13.86%). However, Sphingobacteriia was noted as the most dominant class in the BENRs with electrostatic field of 1.96 V/cm (29.52%), 0.24 V/cm (26.45%) and 0.40 V/cm (26.46%). At the genus level (Fig.4), the bacterial abundance was distinctly different depending on the electrostatic field in

the BENRs. In BENR1 with electrostatic field of 0.80 V/cm the most dominant genus was *Thermomonas* (9.30%), followed by *Dokdonella* (7.67%) and some uncultured genus *FJ947131_g* (7.01%), *HQ114124_g* (6.55%), *AB186832_g* (4.70%). In BENR2 with electrostatic field of 0.40 V/cm, the dominant genera are uncultured genus *FJ947131_g* (10.98%), *Dokdonella* (10.63%), *Ferrunginibacter* (5.99%) and *HQ114124_g* (4.30%). In BENR1 with electrostatic field of 0.24 V/cm, the dominant genera are *Dokdonella* (11.24%), *FJ947131_g* (10.89%), *Ferrunginibacter* (6.04%) and *Dechloromonas* (5.56%). In BENR2 with electrostatic field of 1.96 V/cm, the dominant genera are *Ferrunginibacter* (8.99%), *HQ114124_g* (8.29%), *FJ947131_g* (7.78%), *Thermomonas* (5.85%) and *Nitrospira* (4.97%). The features of the microbial community in the bulk sludge were more evident at the species level. Most of the abundant microorganisms were interestingly uncultured species except only a few species. The microbial species that are abundant more than 4% in all the BENRs were mostly uncultured species like *FJ947131_g FJ947131_s, Dokdonella FM213038_s, HQ114124_g fj529971_s, Ferrunginibacter FJ660602_s, Dechloromonas JF775627_s* and *Nitrospira defluvii group*.

7.4 Conclusion

Partial nitration of ammonium was feasible in bioelectrochemical reactors to economically remove nitrogen in biological nitrogen removal processes when the reactors were optimized by a proper combination of electrostatic field and HRT. In the bioelectrochemical reactor the electroactive nitrogen removal microorganisms could be enriched with higher nitrite and smaller nitrate accumulation from ammonium oxidation when the electrostatic field between the polarized electrodes were under 0.8 V/cm at HRT of 4 days. It can be concluded that the bioelectrochemical partial nitritation of ammonium rich wastewater could be further optimized by a proper combination of electrostatic field and HRT.

Chapter 8 – Conclusion and future study

8.1 Summary and conclusions

In this study, a bioelectrochemical nitrogen removal reactor was developed to remove nitrogen from nitrogen rich wastewater. In order to achieve that purpose, polarized electrodes were made to electrochemically induce the direct interspecies electron transfer through electrodes. Also, bioelectrochemical anaerobic nitrogen removal was demonstrated in an anaerobic sequential batch reactor after inoculating activated sludge/ anaerobic sludge and polarizing the electrodes to 0.6V to ensure the nitrogen removal performance through the inoculum. The electroactive microorganisms including ammonium oxidizing exoelectrogens (AOE) and denitritating electrotrophs (DNE) are enriched from activated sludge (based on the previous study) as inoculum in the ammonium and nitrite-rich medium that exposed to the electrostatic field. The autotrophic electroactive nitrogen removal species are enriched by the electrostatic field of 0.2 V/cm in the bulk solution containing nitrite and nitrate as the electron acceptors and ammonium as the only electron donor to prove the advantage that the stringent nitritation to selectively produce only nitrite from ammonium can be avoided when treating ammonium-rich wastewater in a bioelectrochemical reactor. These experiments were performed in batch reactors and based on the results obtained continuous upflow bioelectrochemical reactor was performed by addition of activated carbon which enriches the electroactive microorganisms and facilitates the cDIET for ammonium removal. In addition, the insulated polarized electrodes at different applied potentials enriches more of the electroactive microorganisms in the bioelectrochemical reactor containing activated carbon and removes ammonium nitrogen production by the contribution of the bDIET and the cDIET. Based on the study and results, the nitritation study was performed to ensure the nitrogen removal in the upflow bioelectrochemical anaerobic

reactors.

With anaerobic sludge as inoculum in bioelectrochemical anaerobic reactor, the requirements of nitrite and alkalinity for the removal of ammonium are around 0.58 mg NO_2-N/mg NH_4-N and 2.0 mg as $CaCO_3$/mg NH_4-N, respectively, and the bioelectrochemical nitrogen removal does not produce nitrate as a by-product. The bacterial groups involved in the bioelectrochemical nitrogen removal are the autotrophic AOE and DNE, which are enriched in the bulk solution from anaerobic sludge by the polarized bioelectrode. With activated sludge as inoculum in the bioelectrochemical anaerobic nitrogen removal by batch reactor by polarizing the electrodes to 0.6V. The requirements of nitrite and alkalinity for the removal of ammonia nitrogen are around 0.72 mg NO_2-N/mg NH_4-N and 1.73 mg as $CaCO_3$ /mg NH_4-N, respectively, and nitrate as a by-product was not produced from the bioelectrochemical ammonia oxidation, indicating that the bioelectrochemical nitrogen removal differ from the Anammox process. Bioelectrochemical anaerobic reactors were performed to demonstrate that the electrostatic field enriches the electroactive bacteria (AOE and DNE) in the anaerobic reactor and facilitates the nitrogen removal via the biological DIET between the electroactive bacteria. The exoelectrogenic and electrotrophic activities of the electroactive bacteria and it was noted that the specific ammonium removal rate in the BENR67 was 78.7 NH4-N/g VSS.d suggesting that the biological DIET for nitrogen removal between AOE and DNE for the nitrogen removal in the bulk solution is dependent on the strength of the electrostatic field. When studies undergone to demonstrate the nitrate, as well as nitrite, as an electron acceptor for the bioelectrochemical ammonium oxidation in an electrostatic field and to analyse whether ammonium oxidation was dependent on the fraction of nitrate of the electron acceptor in the bioelectrochemical nitrogen removal reactor. The specific removal rate of ammonium-based on the biomass is an indicator that shows the abilities of AOE and DNE to exchange the electrons for nitrogen removal in the BENR. The specific removal rate of ammonium was 48

NH_4-N/g VSS.d in the BENR1, which was higher than the BENR2 of 39 NH_4-N/g VSS.d and further the BENR3 of 24 NH_4-N/g VSS.d. Through the results it was noted that the use of nitrate as an electron acceptor is an excellent advantage in the BENR over Anammox. As per the studies it has been noted that DIET plays a major role in nitrogen removal. So, studies were undergone in continuous reactor and performing experiment of partial nitration in the bioelectrochemical reactors with different parameters of applying series of electrostatic field and HRT in the bulk solution with the addition of activated carbon which serve as the conduit for DIET between AOE and DNE, which is known as conductive material mediated DIET (cDIET). From the results compared with the series of electrostatic field and HRT, it suggests that nitrite can be accumulated from ammonium oxidation with small accumulation of nitrate under 0.8 V/cm at HRT of 4 days. It can be concluded that the bioelectrochemical partial nitritation of ammonium rich wastewater could be further optimized by a proper combination of electrostatic field and HRT.

As the result, the bioelectrochemical device with direct interspecies electron transfer in the bulk solution between the dielectric electrodes remove nitrogen from nitrogen rich wastewaters. The bioelectrochemical nitrogen removal (BENR) process is considered to be a technology that overcomes the disadvantages in the anammox process.

8.2 Suggestions for future study

In order to achieve high-rate nitrogen removal efficiency, further research should be conducted in the bioelectrochemical nitrogen removal reactor. The working mechanism, in particular, needs to be thoroughly investigated and understood. Thus, future studies on the current topic are recommended as follows:

1. The effects of DIET should be studied further for nitrogen removal in nitrogen rich wastewater. The working mechanism of DIET through AOE and DNE pathways for nitrogen removal need to be fully studied and perfected.
2. The biologically direct interspecies electron transfer (bDIET), which is highly efficient in transferring electrons through AOE and DNE, when compared to other electron transfer pathways, need to be further improved and also conductive materials mediated DIET need to be studied in depth to know the methods to enhance the ammonium removal rate.
3. The optimal operation conditions for the bioelectrochemical nitrogen removal was considerably unexplored for practical application in wastewater treatment. Therefore, the bioelectrochemical nitrogen removal reactor should be further studied and examined, in particular by scaling up for practical application.

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