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RESEARCH ARTICLE

Bioelectrochemical denitrification on biocathode buried in simulated aquifer saturated with nitrate-contaminated groundwater

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Abstract Nitrate contamination in aquifers has posed human health under high risk because people still rely on groundwater withdrawn from aquifers as drinking water and running water sources. These days, bioelectrochemical technologies have shown a great number of benefits for nitrate remediation via autotrophic denitrification in groundwater. This study tested the working possibility of a denitrifying biocathode when installed into a simulated aquifer. The reactors were filled with sand and synthetic groundwater at various ratios (10, 50, and 100 %) to clarify the effect of various biocathode states (not-buried, half-buried, and fully buried) on nitrate reduction rate and microbial communities. Decreases in specific nitrate reduction rates were found to be correlated with increases in sand/medium ratios. A specific nitrate reduction rate of 322.6 mg m^{-2} day^{-1} was obtained when the biocathode was fully buried in an aquifer. Microbial community analysis revealed slight differences in the microbial communities of biocathodes at various sand/medium ratios. Various coccus- and rod-shaped bacteria were found to contribute to bioelectrochemical denitrification including *Thiobacillus* spp. and *Paracoccus* spp. This study demonstrated that the denitrifying biocathode could work effectively in a saturated aquifer and confirmed the feasibility of in situ application of microbial electrochemical denitrification technology.

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Keywords Bioelectrochemical denitrification · Microbial communities · Bioremediation · Groundwater · Aquifer · Biocathode

Abbreviations

BES	Bioelectrochemical system
SHE	Standard hydrogen electrode
TN	Total nitrogen
CE	Coulombic efficiency
PCR-DGGE	Polymerase chain reaction-denaturing gradient gel electrophoresis
rRNA	Ribosomal ribonucleic acid
BLAST	Basic Local Alignment Search Tool
DNRA	Dissimilatory nitrate reduction to ammonia
SEM	Scanning electron microscopy

Introduction

Nitrate contamination in aquifers has attracted special attention from environmentalists worldwide because people still rely on groundwater withdrawn from aquifers as drinking water and running water sources. These days, biological technologies have shown many benefits for nitrate remediation via denitrification because of low costs and eco-friendly self-generating catalysts. Both heterotrophic and autotrophic denitrifications have been reported thus far (Rocca et al. 2007). In heterotrophic denitrification, organic substrates, such as acetates, lactates, and glucose, must be supplied into the subsurface of a contaminated site to serve as electron donors for nitrate reduction. However, the supply of organic substrates may activate the growth of other microorganisms in addition to denitrifying bacteria. Autotrophic denitrification with

H_2 gas as an electron donor has, therefore, been said to be more efficient than heterotrophic denitrification in this respect. As in situ denitrification in groundwater is taken into account, the delivery of H_2 gas into an aquifer is quite challenging owing to the low solubility of H_2 gas and high installation and operational costs (Ma et al. 2003; Roggy et al. 2004; Agarwal et al. 2005).

Denitrification with a polarized electrode serving as an electron donor has been considered to be more preeminent than conventional heterotrophic or autotrophic denitrification, which requires hydrogen or organic substrates as electron donors. This is because bioelectrochemical denitrification not only stimulates and controls microbial nitrate reduction reactions but also avoids other microbiological activities and growth, which may result in unpreventable and undesirable consequences within the contaminated site. Biocathodes of bioelectrochemical systems have recently been developed for nitrate removal from water sources (Ghafari et al. 2008; Mousavi et al. 2011; Mook et al. 2012; Feng et al. 2013; Huang et al. 2013; Pous et al. 2015). Denitrification carried out by autotrophic bacteria attached to a polarized cathode has been demonstrated widely with synthetic groundwater (Zhao et al. 2011; Zhao et al. 2012; Mousavi et al. 2012; Kondaveeti and Min 2013; Tong et al. 2013; Nguyen et al. 2014). With the aim of in situ application, nitrate removal in the biocathode of a bioelectrochemical system has been thoroughly investigated with continuous influence of real nitrate-contaminated groundwater (Pous et al. 2015). Some other studies have tried to fill the cathode chamber with rod graphite (Puig et al. 2011) or granular graphite (Pous et al. 2015) with an objective to increase conductivity. However, there have been no studies on bioelectrochemical denitrification in aquifers or in soils saturated with groundwater until now, according to our knowledge.

This study aimed to test the working possibility of a denitrifying biocathode polarized at −0.7 V vs. the standard hydrogen electrode (SHE) when inserted into a simulated aquifer saturated with synthetic nitrate-contaminated groundwater. The experiment was also designed to investigate the effect of the proportion of the biocathode buried in a simulated aquifer on denitrification rate and microbial communities. The interactions between electrode and saturated soil and the performance of microorganisms in a liquid phase and solid phase might be totally different. The approach proposed in this report would principally provide us a more clear understanding on how efficient the denitrifying biocathode performed at different depths of burial is. The response of denitrifying microbial communities enriched on bioelectrode to the changes of electrode condition would be also elucidated.

Materials and methods

Reactor configuration and operation

All experimental sets were carried out in duplicate at ambient temperature (25 °C) using a two-chamber bioelectrochemical system (BES) with a working volume of 350 mL per chamber (Supplementary Fig. S1). Both the working electrode and counter electrode were made of graphite felt (4 × 6 × 0.5 cm; GF-S6-06, Amherst, NY, USA) treated by autoclaving with 0.1 M HCl and washing with distilled water. Separators placed between anode and cathode chambers were proton exchange membranes (Nafion 117, DuPont Co., Wilmington, DE, USA) treated by boiling with H_2O_2 (30 % *v*/*v*) and distilled water. Every treatment procedure was repeated consecutively three times. Connection was done by using titanium wire (0.5 mm in diameter). The reference electrode in the cathode chamber was a Ag/AgCl electrode (assumed to be +0.197 V vs. SHE). Coarse sand with an average diameter of 0.58 mm was added to both chambers at four different sand/medium ratios (volume/volume) to simulate a saturated aquifer. The four different ratios including 0, 10, 50, and 100 % represented four conditions of operation involving good circulation, no circulation, half of an electrode submerged in an aquifer, and an electrode totally submerged in an aquifer, respectively. Particle distribution of applied coarse sand analyzed by an LS 13 320 Laser Diffraction Particle Size Analyzer (Beckman Coulter Inc., CA, USA) is shown in Supplementary Fig. S2. Porosity of this sand was determined to be 0.37. A cathode potential of −0.7 V vs. SHE was chosen based on one of our previous studies that suggested that denitrification efficiency was optimal at this cathode potential (Nguyen et al. 2016).

Nitrate-contaminated groundwater applied to BESs was synthesized by adding 50 mg L^{-1} of NO_3^--N to 50 mM phosphate buffer containing 3.9 g L^{-1} of $NaH_2PO_4{\cdot}2H_2O$ and 3.55 g L^{-1} of Na_2HPO_4. $NaHCO_3$ (2 g L^{-1}) was supplied as an inorganic carbon source for microorganisms. Anaerobic sludge collected from Suyoung Wastewater Treatment Plant (Suyoung, Busan, South Korea) was used as bacterial inoculum because it usually contains anaerobic consortia which can perform denitrification (Lee et al. 2013; Kondaveeti and Min 2013; Kondaveeti et al. 2014). An abiotic control (no inoculum addition) and a biotic control (open circuit between anode and cathode) were operated simultaneously to compare the results. The headspace of the reactors was purged with argon gas (99.9 %) to achieve the anoxic conditions. The experiment was carried out in batch mode and monitored until the denitrification process was complete. The cathode potential value reported throughout this paper is given in voltage vs. SHE, unless otherwise stated.

Analytical methods and calculations

Nitrate (NO_3^--N) and nitrite (NO_2^--N) concentrations were determined using an ICS-1000 Ion Chromatography System (Dionex, Sunnyvale, CA, USA) featuring a Dionex IonPac AS14 column, an electrochemical conductivity detector, and a suppressor. Column temperature was maintained at 30 °C. Total nitrogen (TN) and ammonium (NH_4^+-N) levels were determined using the Humas Kit (Humas Co., Ltd., Daejeon, Korea) according to standard methods for examination of water and wastewater (Franson et al. 1992). Gas-phase products, such as N_2, H_2, NO, and N_2O, that evolved in the headspace of the reactor were analyzed using a gas chromatography system (GC YL6500, Young Lin Instrument, Anyang, Korea) coupled with a thermal conductivity detector maintained at 150 °C.

The coulombic efficiency (CE) of bioreactors was calculated as a ratio between the number of cumulative reducing equivalents and cumulative consumed electric charge according to Eq. (1) as follows:

$$CE = \frac{eq_p}{eq_i} \times 100\% \quad (1)$$

where eq_p is the number of cumulative reducing equivalents that was obtained from Eq (2):

$$eq_p = \sum molar\ amount\ of\ products \times conversion\ factors \quad (2)$$

Products included NO_2^-, NH_4^+, N_2 gas, and H_2 gas, with conversion factors of 2, 8, 10, and 2 eq/mol, respectively. eq_i is the cumulative consumed electric charge, which is calculated by integrating current (I) over the period (t) of cathode polarization using Eq (3). In the case where N_2 gas was not totally recovered owing to its being captured by a saturated aquifer (at 50 and 100 %), all nitrate removal was considered to occur through transformation into N_2 gas.

$$eq_i = \frac{\int I \times t}{F} \quad (3)$$

where F is the Faraday constant (96485.4 C/mol electron) and data for I was obtained from a potentiostat.

Microbial community analyses

At the end of each experiment, cathode electrodes were taken out of simulated aquifers and the biofilm samples were collected. DNA extraction was performed using a PowerSoil™ DNA isolation kit (Mo Bio Lab., Carlsbad, CA, USA). Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was performed as described previously (Nguyen et al. 2014). The PCR primers and PCR conditions are summarized in Supplementary Table S1. Electrophoresis was carried out at 60 °C for 16 h. After electrophoresis was complete, the gel was stained in ethidium bromide for 30 min followed by washing with distilled water for 30 min. The DGGE profile was photographed with a digital camera (Olympus 720 UZ, Olympus Optical Co. Ltd., Japan). DNA bands excised from the DGGE profile were then amplified by PCR using the universal primers Eub 27F (5 -ACG GGC GGT GTG TAC AAG-3) and Eub 518R (5 -ATT ACC GCG GCT GCT GG-3). The partial 16S ribosomal ribonucleic acid (rRNA) sequences were then sequenced by Solgent Co., Daejeon, Korea. The phylogenetic identifications of obtained sequences were determined based on *16S* rRNA sequence homology by performing a nucleotide Basic Local Alignment Search Tool (BLAST) search at the website of the National Center for Biotechnology Information (Altschul et al. 1990).

Part of a biocathode electrode was pretreated with 2.5 % gluteraldehyde and 1 % osmium tetroxide followed by dehydration with 50, 70, 90, 95, and 100 % ethanol in succession. The pretreated specimens were dried in a desiccator overnight before observation under a field emission-scanning electron microscope (Zeiss FE-SEM SUPRA 25, Germany).

The partial 16S rRNA gene sequences (400–500 bp) obtained from PCR-DGGE analyses were deposited in the GenBank database under accession nos. KT932937–KT932947.

Results and discussion

Denitrification performance depending on the proportion of biocathode buried in simulated aquifer

The proportions of biocathodes buried in sand saturated with synthetic groundwater definitely affect bioelectrochemical denitrification performance (Fig. 1). Indeed, the time spent for complete denitrification varied with the sand/medium ratio in the reactors. Complete denitrification in the reactors with only a liquid phase (0 % sand) took only 15 days, whereas this time was extended to 25 days in the reactors containing 10 % sand (Fig. 1a, b). This may be because of the circulation conditions of the liquid phase in the cathode chamber. At a 0 % sand/medium ratio, the liquid phase was circulated well using a stirring magnetic bar, whereas addition of 10 % sand impeded its operation and the medium was not circulated as a consequence. In both of these cases, the biocathodes were still not buried in sand. When this ratio was increased to 50 % (50 % of biocathode buried in sand), the time for complete denitrification was reduced to 24 days. Only 20 days was required for total nitrate removal in the case where the biocathode was totally buried in sand (100 % sand/medium ratio). This reduction in time does not mean that the denitrification rate

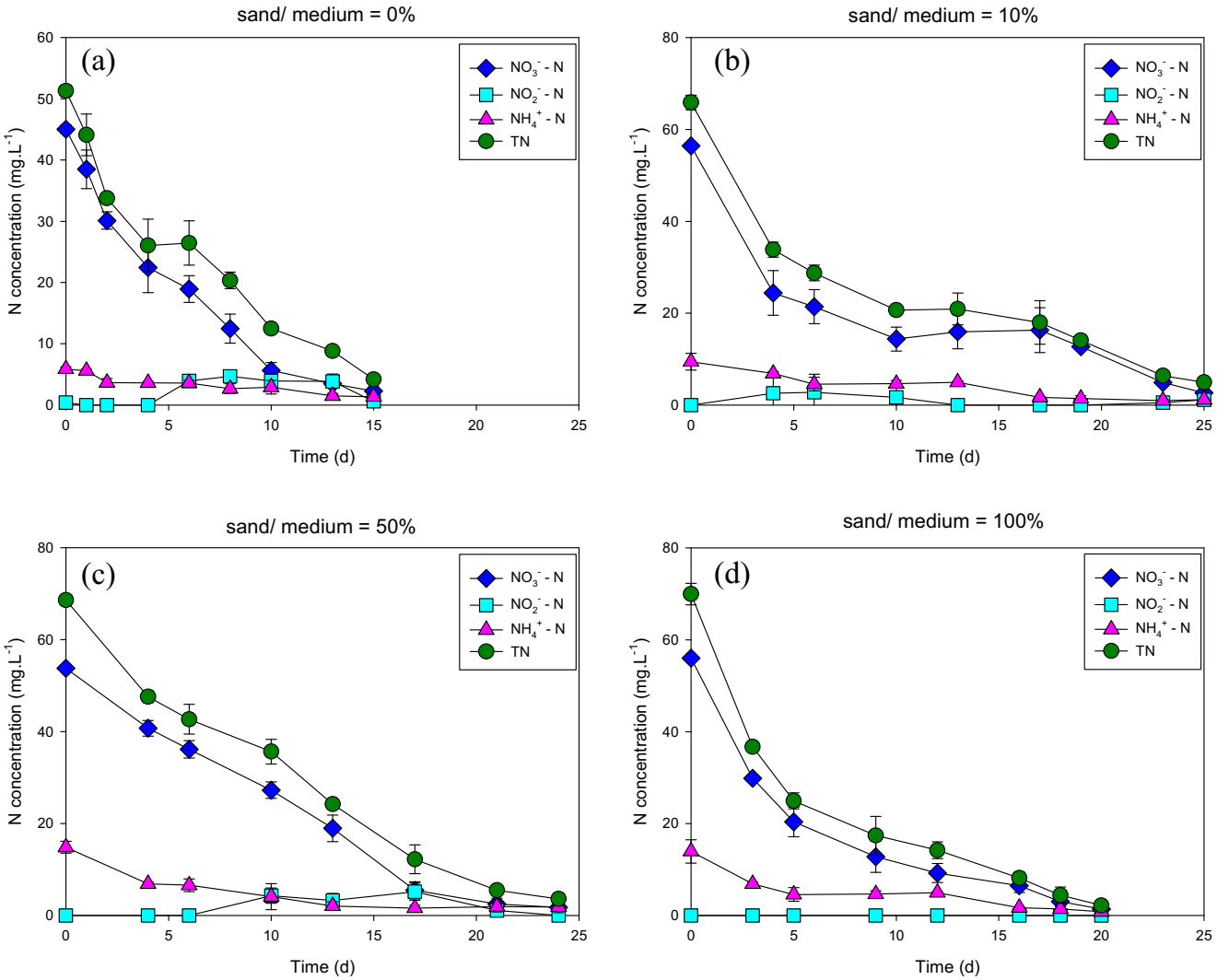

Fig. 1 Dependence of denitrification performance on the proportion of biocathode buried in simulated aquifer: **a** control with no sand addition, **b** not-buried biocathode with addition of 10 % (*v*/*v*) sand, **c** half-buried biocathode with addition of 50 % sand, and **d** fully buried biocathode with addition of 100 % sand

increased when these ratios increased from 0 to 100 % because the volume of medium decreased significantly upon increasing the sand/medium ratio.

It was interesting to find that nitrite that occurs as an intermediate of denitrification was detected in all cases except the case of the fully buried biocathode. The nitrite concentration detected during the denitrification varied from 1.1 to 4.7 mg L^{-1} NO_2^--N. The highest nitrate concentration was observed in the reactors with only a liquid phase where sand was not added. However, no nitrite accumulated until the end of the experiment in all of the cases. Ammonium (10–14 mg L^{-1} of NH_4^+-N) that initially occurred in the reactors is the consequence of inoculum addition. All ammonium ions were removed at the end of each batch test. The removal of ammonium was believed to be due to a non-bioelectrochemical process because the same amount of ammonium was also eliminated in the biotic controls, in which an open circuit between the anode and cathode was maintained (Supplementary Fig. S3). In the biotic controls, nitrate reduction of approximately 10.1–15.5 % by the heterotrophic process, which used the small amount of organic matter available in the inoculum, in addition to ammonium removal of 15.2–20.1 %, resulted in a TN removal efficiency of 26.1–34.6 %. No significant denitrification was recorded in all of the abiotic control reactors, as expressed by the stabilization of nitrate concentration during incubation (Supplementary Fig. S4), which indicated that electrochemical denitrification was not achieved at a cathode potential of −0.7 V. During the operation, when cathode potentials were maintained at −0.7 V, anode potentials fluctuated from 0.6 to 0.8 V (Supplementary Fig. S5).

It was noticeable that no significant amount of nitrite accumulated during denitrification in all reactors, which indicated

that direct transformation from nitrate to nitrogen gas was preferable in these cases. Denitrification without accumulation of nitrite was evaluated to be a strong point and a great advantage of this study. This is because nitrite in drinking water is known to be much more toxic than nitrate. The maximum accepted level of nitrite in potable water is 1 mg L^{-1} of NO_2^--N, whereas the allowance for nitrate is 10 mg L^{-1} of NO_3^--N according to the World Health Organization (WHO 2011). On the other hand, most of the previous studies on nitrate reduction in BESs indicated accumulation of nitrite at various levels (Virdis et al. 2009; Nguyen et al. 2014; Zhang et al. 2014; Pous et al. 2015).

Dissimilatory nitrate reduction to ammonium (DNRA) was not observed at the selected cathode potential (−0.7 V) in this study although some previous studies reported the occurrence of DNRA during denitrification at such low cathodic potentials (Su et al. 2012; Zhang et al. 2014; Sander et al. 2015; Yu et al. 2015). However, the result coincided well with that of a study on real nitrate-contaminated groundwater, which showed no ammonium production during biocathodic denitrification at −0.703 V (Pous et al. 2015).

The analysis data from the gas chromatograph of the headspace gas at the end of the experiment from the reactors with biocathodes half- or fully buried in sand showed the occurrence of only a small amount of nitrogen gas that was not appropriate to obtain a mass balance with the TN removal. This might be because the forming gases were captured in a saturated aquifer. Based on the present results, it could not be concluded that no gaseous products of nitrate reduction such as N_2O and NO were formed in both of these cases (50 and 100 % sand). However, the results from reactors where the biocathode was not buried in sand (0 and 10 % sand) asserted that no N_2O or NO gas was detected. On the other hand, our previous studies on the same electrode materials and reactor structure indicated denitrification without formation of these greenhouse gases (Nguyen et al. 2016). Total hydrogen evolutions at the end of the experiments with 0 and 10 % sand (not-buried biocathodes) were similar: 2.2 mmol for 0 % and 2.1 mmol for 10 %. The total hydrogen production in the reactor with the half-buried biocathode (50 % sand) was only 0.7 mmol. Hydrogen was not detected even in the headspace of the reactor with the fully buried biocathode (100 % sand). We cannot definitely assert that hydrogen was not produced in this case because hydrogen could have formed but was instantaneously consumed through hydrogenotrophic denitrification (Zhou et al. 2007; Karanasios et al. 2010; Feng et al. 2013) or possibly captured in a saturated aquifer. Furthermore, previous studies demonstrated that a biocathodic potential of −0.7 V could accelerate hydrogen production (Ra et al. 2008).

Specific nitrate reduction rate depending on the proportion of biocathode buried in simulated aquifer

The decrease in specific nitrate reduction rate was correlated with the increase in the proportion of biocathode buried in a simulated aquifer (Fig. 2). In this study, specific nitrate reduction rate was proposed to obtain an appropriate comparison between the denitrification performances of four different cases because the effective volume of the liquid phase would change with various sand/medium ratios. It was calculated based on the nitrate reduction rate per surface area of the cathodic electrode, which was constant in all reactors and was the main factor deciding denitrification efficiency. The highest specific nitrate reduction rate (831.5 mg m^{-2} day^{-1} of NO_3^--N) was achieved in the reactor with the 0 % sand/medium ratio, where the medium was thoroughly circulated. The specific nitrate reduction rate decreased approximately 30 % when 10 % sand was added to the reactors. In fact, under this condition, the cathodic electrode was not buried in sand and the added sand only impeded magnetic bar stirring. This revealed that the circulation plays a significant role in denitrification performance. Contrary to the expectation that an increase in nitrate reduction rate would be correlated with an increase in sand/medium ratio in the 10–100 % range owing to the reduction in overall denitrification time, specific nitrate reduction rate actually decreased when sand/medium ratio increased. A specific nitrate reduction rate of 322.1 mg m^{-2} day^{-1} of NO_3^--N was achieved in the 100 % sand/medium ratio, where the biocathode was fully buried in sand saturated with synthetic groundwater. This exhibited retention of 38.7 % denitrification performance in a simulated aquifer compared to the case of the biocathode working in only a liquid phase.

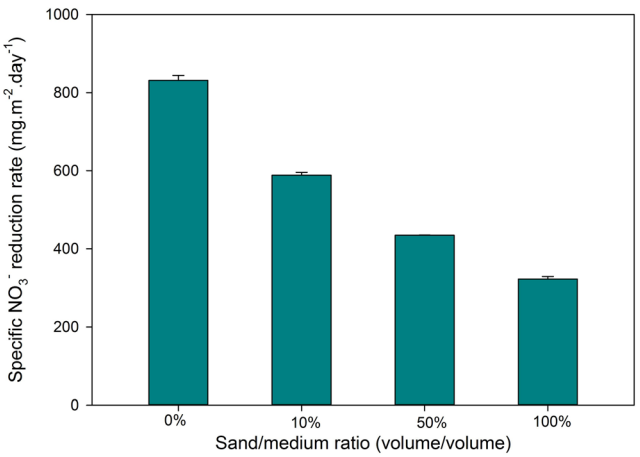

Fig. 2 Variation in specific nitrate reduction rate depending on the proportion of biocathode buried in simulated aquifer

Coulombic efficiency

In this study, total coulombic efficiency at the end of an experiment was one of the parameters used to evaluate the electron consumption performance. Total electron consumption efficiencies varied from 69 to 83 % (Fig. 3). The coulombic efficiencies achieved in this study were slightly lower than those obtained from some previous studies on bioelectrochemical denitrification in water environments (Su et al. 2012; Zhang et al. 2014; Xie et al. 2014), but higher than that from a study on denitrification at an extremely low cathode potential (−0.9 V) (Sander et al. 2015). In this study, electron consumption was not only for denitrification but also partially for hydrogen production. Hydrogen production could be considered as an electron sink during cathodic denitrification. However, hydrogen evolution at the biocathode of denitrification systems might have had a positive impact on nitrate reduction because many previous studies have suggested that H_2 would accelerate nitrate reduction through hydrogenotrophic denitrification (Zhou et al. 2007; Karanasios et al. 2010; Feng et al. 2013). Additionally, hydrogen evolution in the headspace of the reactors was believed to be produced biocatalytically because no hydrogen was detected under the same conditions of abiotic controls. Hydrogen production at the graphite felt electrode polarized at −0.7 V in this study coincided well with a previous study that focused on bioelectrochemical hydrogen production (Ra et al. 2008).

It seems that the efficacy of electron consumption in the reactors where the biocathode was not buried in sand (at sand ratios of 0 and 10 %) is higher than that where the cathode was inserted into sand (at sand ratios of 50 and 100 %) (Fig. 3). As a matter of fact, those lower coulombic efficiencies may be due to the loss of H_2, which might have been trapped in the simulated aquifer. However, coulombic efficiencies for H_2 production were only from 2.6 to 2.9 %, constituting 3.1 to 3.6 % of total coulombic efficiencies. This means that electron consumption for hydrogen production was not a substantial contributor to coulombic efficiency. Thus, in the cases of the half-buried and fully buried biocathodes (50 and 100 % of sand ratio), the calculated coulombic efficiencies reflect a high level of electron loss when the electrode worked in saturated aquifers. This might be due to the contact between the electrode and sand.

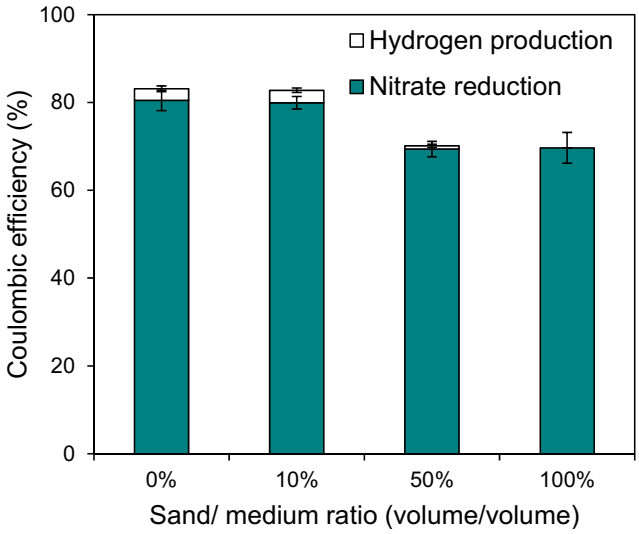

Fig. 3 Variation in coulombic efficiencies at different proportions of biocathode buried in simulated aquifer

Microbial community catalyzing denitrification

PCR-DGGE results proved that there are slight differences between microbial communities on not-buried and buried biocathodes (Fig. 4). The prominent 11 DNA bands observed on DGGE profiles were excised and sequenced to determine

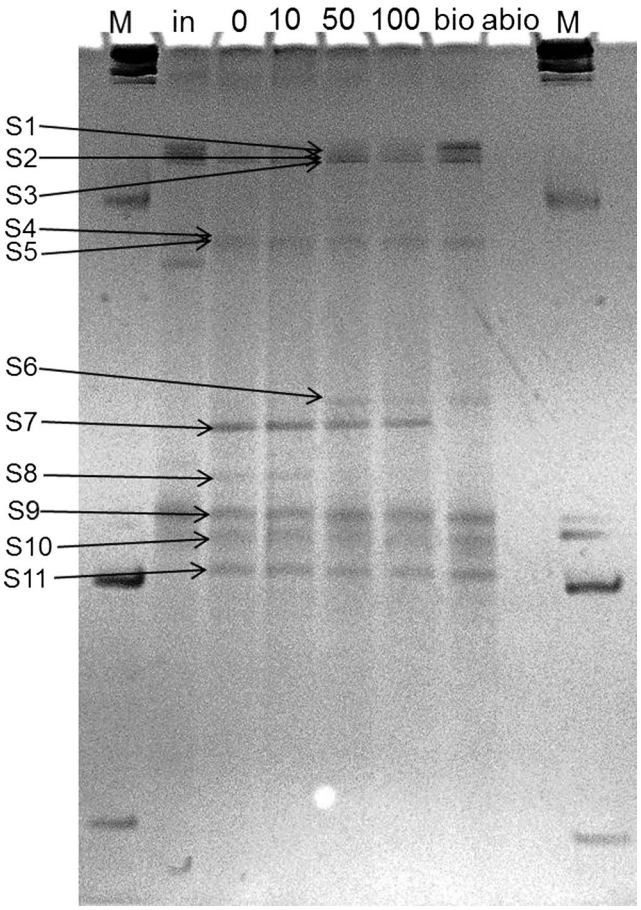

Fig. 4 Denaturing gradient gel electrophoresis profiles of microbial communities on the biocathodes with different proportions buried in simulated aquifer. *M* marker, *in* inoculum, *bio* biotic control, *abio* abiotic control. The *numbers* on the top of each lane indicate the sand/medium (*v*/*v*) ratio: 0 % represents the control with no sand addition, 10 % represents the not-buried biocathode, 50 % represents the half-buried biocathode, and 100 % represents the fully buried biocathode

the phylotypic levels. These obtained sequences were compared to the most similar sequences of cultured and uncultured microorganisms deposited in GenBank (Table 1). Phylotypic determination of these sequences indicated the dominance of *Betaproteobacteria* among bacterial communities (6/11 obtained sequences). The absence of bands in the case of abiotic control revealed that the reactors were successfully maintained in abiotic conditions.

Three bands including S7, S9, and S11 were observed to have the highest intensity, suggesting that the biocathodic denitrification was mainly attributed to *Thiobacillus denitrificans* (94 % similarity), *T. thioparus* (91 % similarity), and *Paracoccus denitrificans* (94 % similarity), respectively. Those bacteria are well known as autotrophic denitrifiers (Carlson and Ingraham 1983; Claus and Kutzner 1985) and were recently found to be the dominant denitrifiers in bioelectrochemical denitrification systems (Vilar-Sanz et al. 2013; Xie et al. 2014). Direct electron uptake by *T. denitrificans* has also been discovered (Yu et al. 2015). These findings are in good agreement with the scanning electron microscopic (SEM) observation of the cathode electrode (Fig. 5). SEM photographs revealed the attachment of some rod-shaped and coccus-shaped bacterial cells on the cathodic electrode.

It was interesting that band S8, which shared 91 % sequence similarity with an uncultured bacterium from cathode-suspended biomass of the bioelectrochemical denitrification system (Lee et al. 2013), was only detected on the biocathode suspended in medium (0 and 10 % of sand ratio). Alternatively, band S6, which shared 97 % sequence similarity with a betaproteobacterium, *Simplicispira* sp. RSG39, was only found on the electrode buried in sand. In addition, the

Table 1 Identification of DGGE bands

Band name	Closest uncultured sequence				Closest cultured sequence			
		Isolation source	Identity (%)	GenBank accession no.		Class	Identity (%)	GenBank accession no.
S1	Uncultured bacterium clone AcL_E12	MEC biocathode	99	HE583154	*Mesorhizobium* sp. ADC-19B	Alphaproteobacteria	99	KM210274
S2	Uncultured *Bacteroidetes* bacterium clone HKT_30B2	Sludge	98	JX170171	*Empedobacter* sp. C2-7	Flavobacteria	89	KC525956
S3	Uncultured *Pseudomonas* sp. clone E02rc	Sewage	92	KJ185105	*Pseudomonas* sp. SKU	Gammaproteobacteria	92	AY954288
S4	Uncultured bacterium clone W3056	sludge system for the ammonium removal	91	AM259163	*Thiobacillus thioparus* strain THI 115	Betaproteobacteria	88	HM535225
S5	Uncultured *Thiobacillus* sp. clone De188	leachate sediment	94	HQ183866	*Thiobacillus sajanensis* strain 4HG	Betaproteobacteria	91	NR_026130
S6	Uncultured *Simplicispira* sp. clone MFC-1-L9	anodic biofilm of MFC	99	JX944522	*Simplicispira* sp. RSG39	Betaproteobacteria	97	KC884002
S7	Uncultured bacterium clone N-130	waste water	97	JX040353	*Thiobacillus denitrificans* strain NCIMB 9548	Betaproteobacteria	94	NR_025358
S8	Uncultured bacterium clone MAB1_313	cathode suspended biomass	91	JQ983570	*Deinococcus deserti* strain VCD115	Deinococci	90	NR_043243
S9	Uncultured bacterium clone a-142	waste water	94	JX040406	*Thiobacillus thioparus* strain THI 111	Betaproteobacteria	91	NR_117864
S10	Uncultured bacterium clone W3070	sludge system for the ammonium removal	98	AM259154	*Zoogloea ramigera* strain NBRC 15342	Betaproteobacteria	93	NR_113749
S11	Uncultured bacterium clone 55-3	denitrifying reactor	94	KP641100	*Paracoccus denitrificans* strain SDT1S8	Alphaproteobacteria	94	JQ045827

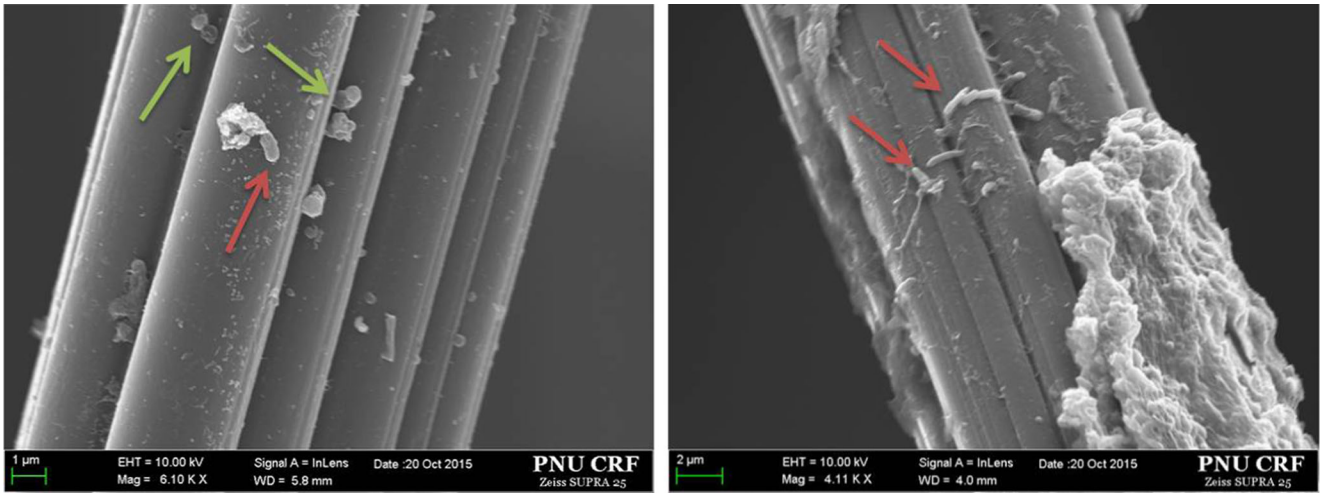

Fig. 5 Scanning electron microscopic photograph of graphite felt electrode showing the attachment of rod-shaped bacteria (*red arrow*) and coccus-shaped bacteria (*green arrow*)

sequence of this band also represented 99 % identity to an uncultured *Simplicispira* sp. clone, MFC-1-L9, that was found on the anodic biofilm of a microbial fuel cell (unpublished paper, GenBank accession no. JX944522), suggesting that this bacterium may possess electrochemically active properties.

Other bands, such as S1, S2, S3, S4, S5, and S10, were detected in all cases, even in the biotic control with lower band intensity. Band S1 shared 99 % similarity with an uncultured bacterium discovered in a microbial electrolysis cell biocathode (Croese et al. 2014). It also shared 99 % sequence similarity with an alphaproteobacterium, *Mesorhizobium* sp. ADC-19B. Bands S4 and S10 showed high sequence similarity to uncultured bacteria that were detected in sludge systems for ammonium removal via the combined nitrite nitrification and electrochemical denitrification process (Wang et al. 2014). These two bands may be assigned to the agent for ammonium removal in most reactors of this study.

Conclusion

This study showed that the first trial for testing denitrification performance of a biocathode buried in a simulated aquifer saturated with synthetic nitrate-contaminated groundwater was successful. Even though only 38.7 % of the specific nitrate reduction rate of the biocathode was retained in the case of the fully buried biocathode compared to that of the not-buried biocathode working with only a liquid phase, the results indicated the feasibility of in situ application of a graphite felt biocathode. Throughout the sand/medium ratio tests, the significant role of circulation was disclosed. A static state of the liquid phase could retard nitrate reduction by 30 % compared to the case with good circulation. In practical applications for groundwater remediation, this limitation could be attenuated by the natural dynamic flow of groundwater. The bioelectrochemical denitrifying microorganisms could grow and perform well in saturated aquifers. However, the state of the biocathode (not-buried or buried in aquifers) also impacts the growth of some specific bacteria slightly. More detailed studies on scaling up of this system should be taken into account before this technology is brought to field application.

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