Concurrent treatment of raw and aerated swine wastewater using an electrotrophic denitrification system

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Abstract

Enhanced nitrate removal in the cathode chamber of bioelectrochemical systems (BES) using aerated swine wastewater under high nitrate levels and low organic carbon was investigated in this study, focusing on the relationship between nitrogen and bacterial communities involved in denitrification pathways. BESs with the anion exchange membrane (AEM) under cathodic applied potentials of -0.6 V vs. AgCl/AgCl reference electrode showed a removal rate of 99 ± 2 mg L^{-1} d^{-1}. Moreover, organic compounds from the untreated full-strength wastewater were simultaneously eliminated in the anode chamber with a removal rate of 0.46 g COD L^{-1} d^{-1} with achieved efficiency of 61.4 ± 0.5% from an initial concentration of around 5 g of COD L^{-1}, measured over the course of 7 days. The highest microbial diversity was detected in BESs under potentials of -0.6 V, which include autotrophic denitrifiers such as *Syderoxidans, Gallionela* and *Thiobacillus*.

Keywords: biocathodic denitrification, wastewater treatment, bioelectrochemical systems, autotrophic microbial biofilm

1. Introduction

Sustainable wastewater treatment not only aims at reusing water and minimizing contamination, but also maximizing the recovery of valuable resources such as energy and nutrients (Verstraete et al., 2009). Agricultural wastewater is abundant in recyclable nutrients. Wastewater treatment is of pressing relevance for such places as Japan's Okinawa Islands, where intensive pig farming significantly increases the amount of wastewater, causing an accumulation of undesirable products that contribute to environmental pollution. This ammonium-rich wastewater from livestock farms is commonly treated by aeration system (Rosso et al., 2008). Nitrate is an ample and harmful inorganic contaminant commonly found in effluent from aeration tanks which are used to remove ammonium-laden wastewaters discharged from livestock farms. Nitrate contamination of wastewater has become a huge

concern because of its toxicity to human health and the environment (Powlson et al., 2008). When nitrate is ingested by people, it is converted to nitrite that binds further to the hemoglobin in the body, forming methemoglobin, which is unable to carry oxygen. Therefore excess levels of nitrate in drinking water can cause methemoglobinemia (also called as blue baby syndrome) (Majumdar and Gupta, 2000).

Biological denitrification, the reduction of oxidized nitrogen such as nitrate or nitrite to nitrogen gas, is traditionally achieved by heterotrophic facultative anaerobic microorganisms (Schmidt et al., 2003). While biological denitrification is a well-established technology, it often suffers from competition between aerobic and denitrifying microorganisms for available organics. This competition can result in sub-optimal denitrification due to insufficient substrate supply which often leads to a demand for additional carbon dosing to achieve complete denitrification in wastewater with a low concentration of organics.

Bioelectrochemical systems (BES), a cutting-edge environmental technology, may be able to address the limitations of anaerobic digestion and complement the aeration approach. BES couple the oxidation of an electron donor at the anode with the reduction of an electron acceptor at the cathode, using bacteria to catalyse one or both reactions (Clauwaert et al., 2007). Generally, in the anodic chamber of BES, electrogenic microbes oxidize organics and release electrons to an anode. Nitrogen-containing electron acceptors such as nitrate (NO_3^-), nitrite (NO_2^-) and even nitrous oxide (N_2O) can be reduced to nitrogen gas in the cathodic chamber of BES by electrotrophic denitrifiers. Compared to traditional biological nutrient removal techniques, denitrifying BES have obtained high nitrogen removal efficiency even under a low C/N ratio due to bacterial biofilms enriched on the cathodes (Zhang and He, 2012, Tian and Yu, 2020). Understanding the behavior of microbial communities in BES has been the recent focus of many research studies. A *Geobacter* sp. was found to use a graphite cathode directly as an electron donor source for reducing nitrate to nitrite in a potentiostat-

poised half-cell mode (Gregory et al., 2004). Another study using a similar system showed that nitrate is reduced completely to nitrogen gas by electrotrophic microorganisms, which consumed electrons directly from the cathode (Park et al., 2005). These electrotrophic denitrifying bacteria are autotrophs that are able to use the electrode as an electron donor and inorganic carbon (e.g. carbon dioxide and carbonates) as a carbon source. Therefore, biocathodes serve as a safe and endless source of electrons. Moreover, such microbial communities easily adapt to electrically stimulating environments, and can be enriched after the acclimatization period.

Recent advances in the development of biocathodic denitrification in BES have used synthetic wastewater (Park et al., 2017; N Pous et al., 2015). Therefore, the particular interest of the current study was to investigate whether autotrophic denitrification with cathodes can be achieved with swine wastewater, and to identify which conditions are optimal to stabilize such a system. To the best of our knowledge, this work represents the first study to achieve simultaneous treatment of full-strength raw swine wastewater in the anode chamber and the aerated swine wastewater in the cathode chamber. Overall performance and efficiency of carbon and malodor compounds removal in the anode chamber, together with the nitrate removal performance in the cathode chamber, were evaluated. Moreover, a long-term operational run of such a system was conducted.

Previous efforts have elucidated the bacterial communities responsible for autotrophic denitrification (Van Doan et al., 2013; Vilar-Sanz et al., 2013). However, much remains unknown about the long-term survival of these bacteria in swine wastewater. Therefore, we investigated variations in microbial communities on the cathodes using swine wastewater after aeration, together with the effect of applied cathodic potentials on these communities. In addition, functional analysis of these microbial communities was performed.

2. Material and methods

2.1 BES design and construction

Double-chambered BESs were fabricated using transparent poly-acrylic sheets. To provide high surface areas for bacterial growth, two carbon brush electrodes with carbon fiber ZOLTEK Panex 35 density 800K tips per 2.5 cm containing two pieces of stainless steel wire 3.5 mm in diameter (Hengshui Chiehwang Industry and Trade Co, China) with a length of 10 cm were used for both anodes and cathodes. Prior to initial use, the brushes were soaked in acetone overnight, heated at 450°C for 30 min in a muffle furnace (Feng et al., 2010) and washed three times with distilled water. The distance between the anode and cathode electrodes was 2 cm. A Nafion^{TM} 117 (Dupont, USA) membrane was used as a cation exchange membrane (CEM) between the anode and cathode chambers, and an AMI-7001 (Membranes International Inc., USA) was used as an anion exchange membrane (AEM). Two membrane frames were installed with a surface area of 48 cm^2. The electrodes were connected to a potentiostat (Uniscan PG580RM) using a titanium wire. All experiments were carried out in a three-electrode setup. The cathodic and anodic compartments were each 1 L. The system was run under a controlled temperature of 25 °C.

2.2 Inoculation, enrichment and operation of the system

Both swine wastewaters (raw and aerated) and activated sludge from the aeration tank were obtained from Okinawa Prefecture Livestock and Grassland Research Center, Japan. Both anode and cathode chambers were inoculated with sludge with an initial ratio to wastewater streams of 1:3. Following inoculation, the anode chamber was then filled with full-strength raw swine wastewater, whereas the cathode chamber was filled with wastewater after aeration treatment. The chemical compositions of the two types of wastewater in comparison with the same wastewater after treatment in BES are shown in Table 1. Before

being fed into the BES, wastewater was passed through a mesh with 1 mm pore size to remove any remaining sludge particles. The average initial pH for wastewater used in the anode chamber was 6.86 ± 0.26 with a conductivity of 263 ± 28 μS cm^{-1}. The average initial pH for wastewater after aeration used in the cathode chamber was 7.96 ± 0.34 with a conductivity of 315.5 ± 8.5 μS cm^{-1}. The nitrate-nitrogen level of the wastewater used in the cathode chamber was adjusted to 300 mg L^{-1} by sodium nitrate. During all the experiments both chambers were maintained under anaerobic conditions and were operated in a fed-batch mode.

2.3 BES operation

All experiments were carried out in a three-electrode setup, where the cathode was used as a working electrode controlled chronoamperometrically and the Ag/AgCl electrode was used as a reference electrode (0.197 V vs. standard hydrogen electrode, Radiometer XR300 Reference Electrode, Hach). After inoculation, BESs were pre-incubated under open circuit potential (OCP) to allow a bacterial biofilm to acclimatize to the environment. An overview of the three stages of the experimental runs tested in this study are shown in Table 2. In Stage I, nitrate removal and the obtained end-product were evaluated under the following conditions: different applied cathodic potentials (-0.2 V, -0.4 V and -0.6 V vs. Ag/AgCl reference electrode), open circuit potential (OCP), and reactors without inoculum with an applied potential of -0.6 V and reactors without electrodes. In Stage II and III, BESs were run under the applied potential of -0.6 V and OCP mode. All BESs were run in duplicates. For experiments in Stage III, BESs were operated for 180 days to examine their performance and to analyze how the microbial communities adapted over time. Cell voltages during the open circuit (OCP) mode were monitored with a data logger (GRAPHTEC Midi Logger GL240). Coulombic efficiency was calculated based on the following equation:

(1) $$CE = \frac{t \int I \, dt}{V F n \triangle NO_3}$$,

where F is a Faraday constant (F= 96485 C mol^{-1} e^-), V is the cathode liquid volume, n represents the number of electrons spent for this reaction (5 e^- for denitrification process) and $\triangle NO_3$ shows how much nitrate-nitrogen (NO_3^--N) was consumed in mmol N L^{-1} h^{-1}.

2.4 Chemical analysis

The concentrations of chemical oxygen demand (COD), volatile fatty acids (VFA), ammonium (NH_4^+ -N), nitrate (NO_3^--N) and nitrite (NO_2^--N) were analyzed using HACH test kits (USA). All samples prior to measurement (except COD) were filtered through 0.45 μm filters. The pH and conductivity were measured with a pH-meter (LAQUAtwin-pH-33, Horiba scientific, Japan) and EC-meter (LAQUAtwin-EC-33, Horiba Scientific, Japan).

N_2O in liquid phase was analyzed using gas chromatography mass spectrometry (PEGASUS 4D GCxGC-TOFMS, LECO, MI, USA) equipped with a PLOT column particle trap (0.25 mm x 2.5 m, GL Science, Tokyo, Japan) and RT-Q-BOND separation column (0.25 mm x 30 m, 8 μm, RESTEK, PA, USA).

Suspended solids (SS) was measured according to the Environmental Standards for Water Pollution method (Japan) Appendix 9.

2.5 Microbiological analysis

Biofilm samples were collected from both cathodic electrodes after seven days in Stage I, and after cycle #45 at day 180 in Stage III, when microbial communities stabilized. Genomic DNA was extracted from the solid samples using the Maxwell RSC DNA kit (Promega, USA). RNA was extracted using the Maxwell RSC RNA kit (Promega, USA). Quality of the extracted DNA and RNA was analysed using the 4200 TapeStation (Agilent, USA). For DNA shotgun sequencing NEBNext Ultra™ II FS DNA Library Prep Kit for

Illumina was used and sequencing was done on NovaSeq6000 (Illumina). For ribosomal RNA removal Ribo-Zero rRNA Removal Kit (Bacteria) was used. Libraries were prepared using a NEBNext Ultra II Directional RNA Library Prep Kit for Illumina and sequencing was done on NovaSeq6000 and HiSeq2500 Rapid (Illumina). Coliform bacteria was counted in accordance with the Japanese Industry Standard K 0350-20-10 : 2001 method.

For the scanning electron microscopy (SEM) analysis, small pieces of cathode electrodes with biofilm were taken from the BESs and immersed in 2.5% (w/v) glutaraldehyde in a 0.1 M cacodylate buffer at pH 7.4. Thereafter, samples were washed and dehydrated successively in an ethanol series. The fixed samples were dried with a critical-point drier and sputtered with a gold layer. The coated samples were examined with the SEM (JEOL JSM-7900F) at 15kV and the images were captured digitally.

2.6 Sequencing and data analysis

Combined taxonomic domain information analysis was conducted with the MG-RAST (Meta Genome Rapid Annotation using Subsystem Technology) server under the following conditions: taxonomy domain filters were set for Bacteria and Archaea, %-identity was set to 90%, length was set at 50; all other parameters were set as the default values.

The bar plot and heatmap illustrating genomic abundances were generated using the ggplot2 package (Wickham, 2016) within R (R Core Team, 2013). The data for the plot was exported as a tsv file using the RefSeq database within MG-RAST

3. Results and discussion

3.1 Overall performance of denitrifying BES for concurrent treatment

3.1.1 Biocathodic denitrification in BES under different electrochemical conditions (Stage I)

A 2 L biocathodic BES was constructed to treat raw full-strength wastewater containing high organic and volatile fatty acid levels in the anode chamber and treated wastewater after aeration in the cathode chamber. In the cathode chamber, where BOD can be less than 10 mg L^{-1}, nitrate was reduced to nitrite, nitrous oxide and to nitrogen gas by denitrification via the cathodic microbial community.

Stage I demonstrated the significance of the applied potential at the cathode for the rate of nitrate removal. Influence of the applied potentials on NO_3^--N removal in a fed-batch mode during the experimental period of seven days is shown in Fig. 1A. An initial NO_3^--N concentration of 300 mgL^{-1} was set based on the highest value at local farms. This initial concentration was almost completely removed in BESs under the applied potential of -0.6 V after seven days and amount of removed NO_3^--N was 282 ± 12 mg L^{-1}. The applied cathodic potential of -0.6 V showed the best removal compared to -0.4 V and -0.2 V (226 ± 21 mg $L^{-1}$and 213 ± 19 mg L^{-1} NO_3^--N under the applied potential of -0.4 V and -0.2 V, respectively). Also, the amount of removed nitrate under OCP was significantly lower in comparison with applied potential conditions (145 ± 13 mg L^{-1} NO_3^--N). Other control conditions with no electrodes BES and no inoculum BES also showed low removal ability (82 ± 7 mg $L^{-1}$and 87 ± 11 mg L^{-1} NO_3^--N, respectively). These results indicate the significance of applied potential, and presence of an inoculum, for faster denitrification. This is likely due to the enrichment of microbial communities under these conditions.

Fig. 1B demonstrates the current responses over time in reactors under different applied potentials (-0.6 V, -0.4 V and -0.2 V). A drop in the cathodic current indicated an active electrochemical reduction process, by which denitrification could proceed with the help of electrotrophic denitrifying bacteria, as reported previously (Chen et al., 2017). Fig. 1A and 1B show the clear correlation between nitrate removal and cathodic potentials: a lower cathodic potential leads to a higher nitrate removal rate. This correlation is in accord with a

previous report (Yu et al., 2015). When more nitrate was removed, it resulted in a rapid decrease of the cathodic current, which reflected the consumption of electrons donated for denitrification by the cathode. The subsequent increase of the cathodic current was caused by decreasing nitrate concentrations due to the limited amount of nitrate available in the fed-batch mode. The cathodic coulombic efficiency for nitrate reduction exceeded 100% under all applied potential conditions (data not shown). These high values indicate that heterotrophic denitrification using the organic substrate in the BES as the electron donor may also contribute to the rate of nitrate removal.

Nitrite as the intermediate product of denitrification was detected on day 1 of the experiment, where it increased until day 2, at which point it decreased as the enrichment time period progressed (Fig. 1C). This indicates that nitrate reduction to nitrite took place at the cathode at day 1 and 2, when the denitrification process has just started. Thereafter nitrite was bioelectrochemically reduced through the next steps of denitrification, i.e. NO, N_2O to N_2, by the cathodic microbial community which was consistent with a previous report (Puig et al., 2011). Moreover at day 1 and 2 ammonium flux from the anode chamber through the membrane to the cathode chamber was detected (Fig. 1D), which might be oxidized to nitrite during this time (See section 3.4.1). Under electrochemically stimulated conditions higher concentrations of nitrite were detected (25 – 46.7 mg L^{-1} NO_2^--N) in comparison with the control BESs (2 – 12 mg L^{-1} NO_2^--N) that is in line with the faster nitrate removal under applied potentials. GC/MS analysis revealed that only minor concentrations of N_2O, a potent greenhouse gas, were detected (data not shown), which suggests that the denitrification cycle likely completed with the release of nitrogen.

Consumption of protons by the denitrification reactions in BES is likely responsible for the increase in alkalinity to pH 8.0, which is still in the optimal pH range for conventional denitrification (Sun et al., 2020).

Generally, these results show the potential advantages of a biocathodic denitrification system using aerated wastewater coupled with the simultaneous treatment of raw wastewater with a controlled delivery of electrons. Moreover, this study demonstrates the importance of using sludge as an inoculum. In a previous study, Khilyas et al. (Khilyas et al., 2017) compared different types of sludge as an inoculum to treat swine wastewater and found that sludge taken from the same aeration tank performed better as a microbial fuel cell anodic inoculum than a brewery treatment sludge. In addition, high electron-utilization efficiency, low sludge production and easy handling are all promising features for nitrate removal using livestock wastewater for a large-scale reactor.

3.1.2 Treatment of a raw swine wastewater in the anode chamber

During these experiments, COD and VFA removal in the anode chamber were constantly monitored (Table 3). Under conditions with applied cathodic potentials and OCP mode, COD consumption rates were similar (around 0.87 ± 0.07 g COD L^{-1} d^{-1}), with the highest removal of 1.62 g L^{-1} d^{-1} ± 0.03 g L^{-1} d^{-1} at first day. The total average efficiency under these conditions was 58.5 ± 2.6%, where the highest was achieved at -0.4 V (61.4 ± 0.5%), although there was no statistically difference within BESs under applied potentials and OCP mode (data not shown). This may indicate that applied potential at cathode is not influencing COD removal rate significantly in the anode chamber in this system. Similar COD removal rate of 1.6 ± 0.7 g COD L^{-1} d^{-1} and 2.1 ± 0.5 g COD L^{-1} d^{-1} was reported previously (Vilajeliu-Pons et al., 2017), where swine manure was treated in six-stacked microbial fuel cells (MFC) with a continuous mode. These results show that denitrifying BES could achieve similar treatment rates as MFC, which is specifically design to treat organic matter. Meanwhile in reactors without inoculum (sludge) under the applied potential of -0.6 V, the COD removal rate was 0.53 g L^{-1} d^{-1} ± 0.21 g L^{-1} d^{-1} with an efficiency of 38.5 ± 2.1%. The

result indicated the importance of sludge from the aeration tank as an initial bacterial inoculum and contributed the faster enrichment of communities.

Best VFA removal was detected with applied potentials of -0.2 V and -0.4 V with an efficiency of 41.5 ± 8.3% and 39.7 ± 6.2% respectively. The highest removal rate of 486 ± 18.5 mg VFA L^{-1} d^{-1} was performed at -0.2 V. That indicates that activity of electrogenic community reached maximum at -0.2 V applied to cathode, because in this case cell voltage of BES stabilized at 0.13 ± 0.05 V (data not shown), meaning that anode potential was 0.33 ± 0.05 V. It was shown previously, that electrogenic community operated at maximum electron transfer rates when anode potentials were higher than 0.2 V vs. Ag/AgCl reference electrode (Prokhorova et al., 2017), leading to the higher rates of VFA removal. Under the other tested conditions potential at anodes did not exceed values of 0.2 V.

To investigate further the quality of the wastewater after BES treatment in the anode chamber, a coliform bacteria test, which is usually used as an indicator of the pathogenic or fecal contamination of the water, was performed. After seven days of the experimental run a more than 1000-fold reduction in coliform density was confirmed (data not shown). This suggests that treatment in BES could suppress pathogenic bacteria in wastewater. It is in a line with the previous findings that BES could disinfect wastewater enriched in Enterobacteriaceae (*Shigella, Yersinia, Vibrio*) (Vasieva et al., 2019). Further experiments are needed to understand whether different potentials at anodes influence reduction in coliform density. Moreover, suspended solids (SS) concentrations were also removed from the wastewater with an efficiency of 89% (Table 1).

3.2 Optimization of nitrate removal in BES (Stage II)

As a result of ion migration through the cation exchange membrane (CEM), the transport of ammonium from the anode chamber to the cathode chamber was detected with a

maximum concentration of 162.5 ± 7.5 mg NH_4^+-N L^{-1} (Fig. 1D). Accumulation of ammonium increased the total nitrogen concentration in the cathodic chamber. In order to overcome this limiting factor, the CEM was replaced with an anion exchange membrane (AEM) in Stage II of the experimental run. Total initial amount of 300 mg L^{-1} NO_3^--N was completely removed within 5 days (with maximum of 99 ± 2 mg NO_3^--N $L^{-1}d^{-1}$) in AEM reactors, while in CEM reactors it took around 8-9 days (with maximum of 34 mg $L^{-1}d^{-1}$± 7 mg L^{-1} d^{-1}), when -0.6 V was applied (Fig. 2A).

Overall, a BES with an AEM also achieved better organic removal in the anode chamber (0.8 g COD L^{-1} d^{-1}), where only a negligible amount of nitrate (~1.1 mg NO_3^--N L^{-1}) was detected. This indicated that either only a minor amount of diffusion could happen, or nitrate that migrated through the AEM was reduced to nitrogen gas by denitrifying bacteria in the anode chamber. This is consistent with previous research based on swine wastewater (Vilajeliu-Pons et al. 2015). In Stage II of the experimental run, there was only a slight increase of ammonium observed in the cathode chamber (~3.8 mg L^{-1} ± 2.2 mg NH_4^+-N L^{-1} d^{-1}).

3.3 Adaptability for a long-term run (Stage III)

At present, little is known about the long-term adaptation of electrotrophic denitrifying bacteria and the efficiency of denitrification over time using real livestock wastewater. In Stage III of the current study, long-term denitrification with a focus on stability and enrichment of denitrifying bacteria were investigated. BES with AEM were operated for 45 cycles, with each cycle having a hydraulic retention time (HRT) of three days. At every odd cycle number wastewater was changed in both chambers (the anode was filled with untreated wastewater and the cathode with aerated wastewater). At every even cycle number only the cathode chamber was filled with a new batch of aerated wastewater. BESs were run under the

applied potential of -0.6 V and the OCP mode, that was used as a control. The first 10 cycles of operation demonstrated high nitrate removal rates: 90 mg L^{-1} d^{-1} (under the applied potential conditions). As the absorption of nitrate by the anion exchange membrane was confirmed (data not shown), the initial high removal rate might be due to membrane absorption together with the denitrification process. After 10 cycles, the nitrate removal rate stabilized at an average value of 60 mg NO_3^--N L^{-1} d^{-1} (with the highest value being 78 mg NO_3^--N L^{-1} d^{-1}). These results show an increase in nitrate removal efficiency during long-term performance in comparison with previous research (Gregoire et al., 2014; Tang et al., 2017). Once stabilized, the removal efficiency of every odd cycle (both anodic and cathodic wastewater were changed) and even cycle (only cathodic wastewater was changed and run with lower organics in the anode chamber) were compared (Fig. 3). The average value of reduced nitrate in three days was significantly higher in the odd cycles (198 ± 51 mg L^{-1} under potential of -0.6 V and 160 ± 42 mg L^{-1} under OCP) compared to the even cycles (177.2 ± 58 mg L^{-1}, under potential of -0.6 V and 133.8 ± 56 mg L^{-1} under OCP), indicating the importance of anode organics as an electron source for faster nitrate removal.

Moreover, the significant reduction in nitrate concentration agreed with the distinct current consumption (Fig. 4). The current at initial cycles tended to have larger peak values on the first day, after the wastewater was changed, and then gradually decreased as nitrate was reduced (Fig. 4A, B). Following the longer operation time, the current was stabilized regardless of wastewater change (Fig. 4C). This effect might be attributed to the rapid consumption of electrons by denitrifying bacteria supplied by the cathode.

Taken together, our results suggest that cathodic denitrification in a BES with an AEM has a very promising removal rate using real wastewater. Our long-term experimental run promoted the growth of desired denitrifying bacteria that exhibited good electrochemical activity, leading to the higher nitrate reduction rates that were observed.

To enable the feasibility of scale-up for pig farms, we are developing reactors with less costly components, lower maintenance needs and proper microbial community stability over long-term operation.

3.4 Bacterial community structure

Taxonomic compositions of the microbial communities occupying cathodes from the experiments in Stage I with a CEM (under the applied potentials of -0.6 V and -0.2 V, OCP and no electrodes) and from the experiments in Stage III with an AEM (under the applied potential of -0.6 V and OCP mode after operating over six months) were evaluated in comparison with the original communities from an activated sludge using a shotgun metagenome sequencing approach (Fig. 5). In Stage I, the influence of the different electrochemical conditions on cathodic microbial community was examined. Later in Stage III, the main aim was to investigate the adaptation of the established electrotrophic and denitrifying microbial community on the cathodes after six months. About 800,000 sequences were obtained under each condition. Genera at relative abundances higher than 1% were considered to comprise the core community. Taxonomic distributions of samples were analyzed from the levels of genus to species.

3.4.1 Comparative analysis of the microbial community under different electrochemical conditions

Activated sludge, as an inoculum, represent the initial bacterial community and was mainly domintaed by *Thauera* (31.7%) and *Azoarcus* (4.3%) in the family *Zoogloeaceae* together with *Acidovorax* spp. (10.3%) in the family *Comamonadaceae* and *Mycobacterium* spp. (5.9%) in the family *Mycobacteriaceae*. Previously, two specific families, *Zoogloeaceae* and *Comamonadaceae*, were identified in activated sludge as being mainly involved in the

denitrification process (Khan et al., 2002). Also, *Thauera* and *Azoarcus* were shown to account for as much as 16% of all living bacteria in the activated sludge (Juretschko et al., 2002).

During the operation of the BES, the bacterial community had changed from a heterotrophic anaerobe-dominated community to an anaerobic community with diverse metabolic pathways, including both heterotrophs and autotrophs. In samples from the experiments in Stage I, microbial diversity and their functions varied depending on the applied electrochemical conditions. Significant change in taxonomic distribution was observed under conditions with the applied potential of -0.6 V, by which the fastest rate of denitrification was recorded. The most abundant bacteria belonged to the *Pseudomonas* genus (21.7%), which represent contains various denitrifying and exoelectrogenic bacteria (Deng et al., 2020; Vo et al., 2020). It was also shown by Deng et al. (2020) that in a MFC-granular sludge coupling system, denitrification was mainly carried out by the highly dominant *Pseudomonas* (14.79%) and *Thauera* (26.21%) spp.. Although *Thauera* had the highest relative abundance in the activated sludge samples, in reactors under the OCP mode (22.6%) and also under a potential of -0.2 V (26.6%), their abundance decreased to 9.8% under a potential of -0.6 V. This is a heterotrophic facultative anaerobic and obligate respiratory bacteria that can use nitrate and nitrite as an electron acceptor (Deng et al., 2020; Yang et al., 2019).

Instead the dominance of heterotrophic *Thauera*, the community was additionally enriched with the autotrophic denitrifying bacteria genera *Sideroxydans* (9.9%) and *Galionella* (8.6%), which have the potential ability to accept electrons from the electrode. Both are adapted for chemolithoautotrophy, including pathways for CO_2-fixation and electron transport pathways for growth on Fe(II) at low O_2-levels (Emerson et al., 2013). Their ability to oxidize extracellular Fe(II) is based on a specific pili structure and cytochrome sets that

allow these bacteria to accept electrons from the cathode and transport them to nitrate (Emerson et al., 2013). The main differences between these bacteria include the ability of *Sideroxydans* to grow on reduced S-compounds and fix nitrogen. On the other hand, *Galionella* is more tolerant to the presence of heavy metals (Fabisch et al., 2013), which may be common in livestock wastewater treatment environments (Irshad et al., 2013). Interestingly, the nitrite reductase/nitric oxide reductase operon of *Sideroxydans* is nearly identical to that of *Acidovorax*. Previous research confirmed that some *Acidovorax* spp. can grow by denitrification using inorganic electron donors such as Fe(II) (Chakraborty et al., 2011; Park et al., 2017), but our taxonomical composition analysis revealed that the abundance of *Acidovorax* decreased to 8.4% in reactors with the applied potential of -0.6 V. This might be due to their suppression by the dominant *Pseudomonas* spp.

Under the applied potential of -0.2 V, the core community was dominated by *Thauera* (26.6%), *Nitrosomonas* (12.4%), *Thiobacillus* (12%), *Acidovorax* (9.8%) and *Pseudomonas* (8.9%), all of which are involved in the nitrogen cycle. *Nitrosomonas* are the most well-known ammonia-oxidizing bacteria (Holmes et al., 2004) that may be electrochemically active and be able to accept electrons from the cathode electrode (Wang et al., 2013). In this study, where a CEM was used, the observed ammonium flux from the anode chamber to the cathode chamber (Fig. 1D) might have created the ideal conditions for the active growth of *Nitrosomonas*, which support the conversion of ammonium to nitrogen gas.

In the OCP mode, the core microbial community remained closely related to the original inoculum community, where the most dominant genera were: *Thauera* (22.6%), *Acidovorax* (11%) and *Azoarcus* (5.6%), but also with highly abundant *Geobacter* (19.9%). It was previously reported about the effective coexistence of exoelectrogenic *Geobacter* (6.5%) and denitrifying *Thauera* (59.9%) during a long-term operation in a single-chamber air cathode system with an external resistance of 1000 Ω (Huang et al., 2019). However, this syntrophic

relationship is still under-studied and needs further investigation. In the current study, we observed that *Geobacter* was more abundant under the OCP mode than any other conditions. These results imply that OCP conditions may be associated with the ferric reduction process.

All of the above indicate that the investigated biofilm that developed on the surface of the biocathode consisted of a very diverse microbial community, in which microorganisms with opposite functions (e.g., Fe^{3+} reducers/Fe^{2+} oxidizers) may coexist and interact on complementary processes. Although the relationship between species diversity and ecosystem functioning has been debated for decades, there is an emerging consensus that greater diversity enhances functional productivity and stability in communities of microorganisms (Tilman et al., 2014). The increased overall diversity of electrotrophic denitrifiers in reactors at -0.6 V relates to increased ecosystem function and stability in bacterial denitrifying communities with equivalent richness, thus improving BES performance for nitrate removal.

3.4.2 Cathodic microbial community after long-term adaptation

It was of interest to investigate how such a community had changed during a long-term run conducted in a fed-batch mode. In Stage III, adaptation of the microbial communities on the cathodes under the applied potential of -0.6 V and OCP mode over six months were examined. To the best of our knowledge, this current work is the first study to investigate the pre-grown denitrifying biofilm in a fed-batch system using real wastewater in a long-term operational run. The microbial community after six months under the applied potential of -0.6 V at the cathode was mainly enriched by *Thiobacillus* (60.7%) (Fig.5). These bacteria utilize the S from iron sulphide (FeS) as electron donor and oxygen as electron acceptor, with the S being oxidized to sulphate (SO_4^{2-}).Some species from this genus can oxidize Fe^{2+} and use nitrate as an electron acceptor (Straub et al., 1996). *Thiobacillus denitrificans* has been reported as an electroactive denitrifying bacteria that can directly utilize a solid electrode as a

sole electron donor and capable of enrichment on the cathode to promote denitrification (Pous et al., 2014; Yu et al., 2015). It was also reported previously that cathodic biofilms were dominated by *Thiobacillus* (75-80%) in BES, where a prior enriched inoculum was used (Pous et al., 2014). These results shows the importance of *Thiobacillus* for efficient and continuous denitrification process using a wastewater and its ability to create robust and stable biofilms on the electrodes.

Under the OCP mode, the major contributors were evenly distributed among the following bacteria: *Thauera* spp. (14.3%), *Nitrospira* (13.7%), *Mycobacterium* (10.6 %) and *Acidovorax* (8.2%). Interestingly, that *Acidovorax* (3.9%), *Mycobacterium* (2.9%) and *Nitrospira* (2.7%) were also found to be abundant next to *Thiobacillus* in the reactors under -0.6 V. *Mycobacterium* includes pathogens known to cause serious diseases in mammals and humans. This genus was previously found during autotrophic microbial denitrification (Broman et al., 2017). Decrease of their abundance might be likely associated with the ability of BES to disinfect as was previously reported by Vasieva *et* al. (Vasieva et al., 2019), but still require further investigation. *Nitrospira* is known to be a key player in nitrification as an aerobic chemolithoautotrophic nitrite-oxidizing bacterium (Mehrani et al., 2020). These results indicated that such bacteria are capable of developing physically stable and biologically active biofilms during long-term treatment, although under the electrically stimulated environment they are outcompeted by *Thiobacillus* as a major consumer of electrons on the electrode surface.

3.4.3 Cathodic microbial community at species level

Taxonomic distribution at the species level was further analyzed and the top 30 species were selected and a logarithmic scale heatmap was produced (Fig. 5B). In the short-term experiment (Stage I) two *Pseudomonas* species were found the most abundant under potential

of -0.6 V: *P. putida* and *P. aeruginosa.* Both are pathogenic bacteria and very closely related. But *P. aeruginosa* under anaerobic conditions can perform complete denitrification with the excessive production of nitrite (Arat et al., 2015), while heavy metal resistant *P.putida* can achieve simultaneous nitrification and aerobic denitrification (Zhang et al., 2019). Chemolithoautotrophic bacteria *Nitrosospira multiformis* oxidizes ammonia to nitrite and assimilates CO_2 as the major carbon source was found mainly in BES with CEM, where ammonium flux to the cathode chamber was detected. Abundant presence of *P. aeruginosa* and *N. multiformis* at -0.6 V may explain increased concentration of NO_2^- in comparison with the other conditions. In line with the analysis at genus level, two most abundant autotrophic denitrifiers (*Gallionella* and *Syderoxydans*) were identified as *Gallionella capsiferriformans* and *Sideroxydans lithotrophicus. Dechloromonas aromatica*, another known denitrifier, which is able for enhanced N_2O production under salt or stressed conditions (Han et al., 2019). In long-term experiment (Stage III) *Thiobacillus denitrificans* was the most abundant strain, that is in line with our study at genus level. It is interesting to note that the growth of such bacteria as *Sorangium cellulosum*, known for its ability to inhibit the growth of partners (Li et al., 2013), was reduced under long-term operation.

SEM analysis was used to visualize the microbial composition structure of the enriched biofilm on the cathodes at -0.6 V (Supplementary material).

3.5 Analysis of nitrogen metabolism in cathodic biofilms

Further nitrogen cycle related processes, denitrification, nitrification, ammonification and nitrogen fixation, in each reactor were analyzed. Overall, denitrification had the highest hits among the four processes. To demonstrate the expression of denitrification genes during Stage I, the metatranscriptome was analyzed from samples under applied potential of -0.6 V and OCP mode as a control. The expression of six representative genes, *napAB* and *narGHI*

for nitrate reductase, *nirS* and *nirK* for nitrite reductase, *norBC* for nitric oxide reductase and *nosZ* for nitrous oxide reductase, were investigated to analyze bacterial species involved in each step of the denitrification process (Fig.6). Considering the high microbial diversity, only gene abundance over 3% of total copies were counted. Depending on the different electrochemical conditions, bacteria involved in each step of the denitrification process varied. Among the two types of nitrate reductases, respiratory (NarGHI) and periplasmic (NapAB), the periplasmic nitrate reductase dominated the number of total reads per species.

High abundances of *napAB* genes in a strain most closely related to *Thauera* sp. MZ1T was only expressed under applied potential conditions (15.5%), whereas *Azoarcus* sp. BH72 (19.4% vs. 6.7% at OCP mode) and *Bordetella petrii* (12.4% vs. 6.7% at OCP mode) were found to be present in both conditions, but were significantly more abundant at -0.6 V. These results indicating on their ability to proceed the first step of denitrification electrotrophically. For respiratory nitrate reductase (NarGHI) *Aromatoleum aromaticum* was the most dominant species under applied potential (39%). Both *A. aromaticum* and *T. denitrificans* have enzymes to reduce all intermediates in denitrification process, although in the current study under some conditions the abundance of these strains were lower than 3%. *Thauera* sp., *Acidovorax* sp., *Alicycliphilus denitrificans* and *Dechloromonas aromatica* are also hosted all genes necessary for a complete denitrification and were captured in our study with the high abundance under applied potential conditions.

Two structurally different nitrite reductases are found among denitrifiers, although they both are never expressed in the same cell (Zumft, 1997): one contains copper (Cu-Nir) encoded by the nirK gene and one contains heme c and heme d1 (cd1-Nir) encoded by the nirS gene. *T. denitrificans* and *Burkholderia pseudomallei* are two major sources of the *nirSK* genes in the samples at -0.6V, while *A. aromaticum* and the *Thauera* sp. were dominant under the OCP mode. However, the abundances of *nirSK* genes hosted in *P. putida* (4%) and

S. lithotrophicus (6.1%) were identified only in conditions with applied potential. This is in line with our previous finding in section 3.4.1, where microbial community in BES at -0.6 V were dominated by these autotrophic bacteria. Also it was proved on a transcriptomical level the dominance of *Thauera* under OCP conditions: the expression of nitrite reductases (*nirSK*), nitric oxide reductases (*norBC*) and nitrous oxide reductases (*nosZ*) were clearly dominated by species closely related to *Thauera sp. MZ1T*.

Nitric oxide reductase has two subunits, NorC and NorB, where NorC as a c-type cytochrome receives electrons from a periplasmic donor and passes them to NorB, which contains two b-type hemes and a non-heme iron (Vaccaro et al., 2015). Potential electrotrophic denitrifiers were identified at this denitirification step in BES at -0.6 V that are closely related to the following species: *Maribacter sp. HTCC2170* (10.3%), *Methylococcus capsulatus* (8.1%), *Roseobacter denitrificans* (3.7%) and *Dechloromonas aromatica* (3.7%). However, further investigation of the enriched potential electrotrophs is needed. Meanwhile, under the OCP mode, *norBC* was mainly presented by the *Thauera* sp. (20.9% vs. 5.9% under the applied potential) and *T. denitrificans* (14% vs. 11% under the applied potential). The last steps of denitrification are completed by catalysis of a soluble periplasmic Cu-containing the N_2O reductase *nosZ*. Of note, bacterial composition with *norBC* and *nosZ* genes are highly diverse in samples under the applied potential and OCP. This indicates that such conditions are conducive to the last two steps of the denitrification process.

In summary, functional analysis of species involved in the denitrification process in this study shows high significant diversity at -0.6 V in comparison with OCP mode. Moreover the transcriptome data confirmed our metagenome analysis of potential electrotrophic denitrifying bacteria enriched on the cathode

4. Conclusions

The biocathodic denitrifying BES we developed is a promising technology for the treatment of two different types of swine wastewater: raw wastewater for organics, odors and pathogens removal and aerated wastewater for nitrate removal. Conditions when cathodes were poised to -0.6 V encouraged the growth of autotrophic denitrifying bacteria while simultaneously increasing the rate of nitrate removal. Electrotrophic *Syderoxydans lithotrophicus* and *Gallionella capsiferriformans* were the dominant species in short-term and *Thiobacillus denitrificans* in long-term operations. The results presented here shed light on the microbial communities involved in the denitrification pathway, and will help future optimization of autotrophic denitrification-based engineering applications.

CRediT authorship contribution statement

A.P.: Conceptualization, Methodology, Investigation, Writing – original draft, review & editing. R.H.: Investigation, Chemical analysis. M.K.: Concenpturalization, Funding acquisition, Methodology, Investigation, Wring – review & editing. S.B.: Bioinformatic analysis, Writing- review & editing. I.G.: Supervision.

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Analysis Section, and also the members of the Biological Systems Unit (Okinawa Institute of Science and Technology Graduate School).

Table 1

Chemical analysis of raw full-strength and aerated wastewater in comparison with wastewater after treatment in BES in Stage I.

	Anode chamber		Cathode chamber	
Parameter	Raw wastewater	Raw wastewater after BES	Aerated wastewater	Aerated wastewater after BES
pH	6.9 ± 0.3	7.0 ± 0.5	7.6 ± 0.3	8.0 ± 0.3
*EC (mS m^{-1})	263 ± 28	255 ± 25	316 ± 9	289 ± 31
CODcr (mg L^{-1})	5750 ± 1750	408 ± 92	275 ± 165	120 ± 20
NO_3^--N (mg L^{-1})	< 1.0	6.5 ± 2.0	245.0 ± 35.0	7.9 ± 4.8
NO_2^--N (mg L^{-1})	< 0.02	0.15 ± 0.02	0.24 ± 0.20	0.16 ± 0.03
NH_4^+-N (mg L^{-1})	287 ± 82	10 ± 1	2.2 ± 2	13 ± 9
SS (mg L^{-1})	2.30 ± 0.30 x 10^3	0.03 ± 0.02 x 10^3	0.04 ± 0.01 x 10^3	0.01 ± 0.01 x 10^3
Coliform (CFU cm^{-3})	2.5 ± 2 x 10^6	< 1.0	2.2 ± 1 x 10^3	< 1.0

Table 2

Overview of the stages of experimental runs.

Stage #	Applied potential (V)	Control conditions	Duration (days)	Type of membrane
Stage I	-0.6, -0.4, -0.2	OCP, no inoculum, no electrodes	7	Cation
Stage II	-0.6	OCP	11	Cation and anion
Stage III	-0.6	OCP	180	Anion

Table 3

COD and VFA removal under different electrochemical conditions in the anode chamber.

Conditions	COD degradation efficiency (%)	VFA degradation efficiency (%)
Applied -0.6 V	55.9 ± 3.5	32.1 ± 0.4
Applied -0.4 V	61.4 ± 0.5	39.7 ± 6.2
Applied -0.2 V	58.1 ± 3.1	41.5 ± 8.3
OCP	58.6 ± 1.5	21.8 ± 3.2
No inoculum (-0.6 V)	38.5 ± 2.1	18.0 ± 1.6

* Initial concentration of COD was 5.2 ± 0.2 g and initial concentration of VFA was 1.4 ± 0.2 g.

Fig. 1. Biocathodic denitrification performance in short-term experiment. A) NO_3^--N removal with initial concentration of 300 mg L^{-1}; B) cathodic current generation curves under applied potentials of -0.6 V, -0.4 V and -0.2 V; C) NO_2^--N concentration; D) NH_4^+-N concentration.

Fig. 2. Biocathodic denitrification performance with cation exchange membrane (CEM) vs anion (AEM) A) NO_3^--N removal with initial concentration of 300 mg L^{-1}, where BES with AEM were supplemented with additional 300 mg L^{-1} of nitrate-nitrogen due to its complete removal at day 6; B) cathodic current generation curves.

Fig. 3. Average cumulative concentrations of the removed NO_3^--N in long-term experiment. BES were run for 45 cycles each with HRT of 3 days using a fed-batch mode, where at every odd cycle number the anode chamber was filled with untreated wastewater and the cathode chamber with aerated wastewater, and at every even cycle number only the cathode chamber was filled with a new batch of aerated wastewater.

Fig.4. Current output and cumulative amount of the reduced nitrate at the last day of the cycle during the operation of A) 0-15 days; B) 15-35 days; C) 145-170 days. Blue rhombus: cumulative amount of the removed NO_3^--N at day 3.

Fig. 5. Taxonomic classification of the microbial communities on the cathodes. The relative abundances at A) genus level and B) species level (heatmap of the top 30 closest matching species. The color intensity indicates the value of relative abundance after a base-2 logarithmic transformation was applied). CEM: cation exchange membrane (short-term experiment), AEM: anion exchange membrane (long-term experiment).

Fig. 6. The abundance of dominant closest matching species involved in denitrification. Composition of denitrifying microbial communities in samples from Stage I under applied potential of -0.6 V and OCP shown with abundance higher than 3%.

Fig.1.

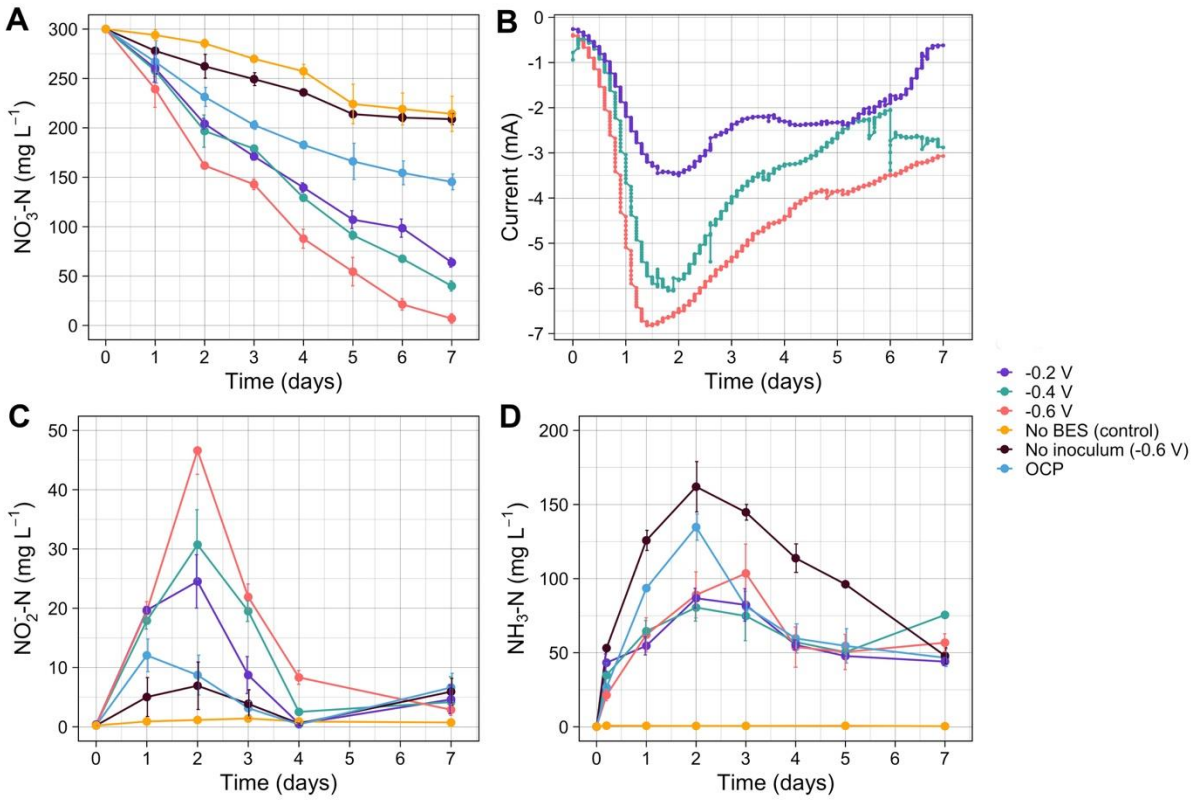

A
B
C
D
NO_3^--N (mg L^{-1})
Current (mA)
NO_2^--N (mg L^{-1})
NH_3-N (mg L^{-1})
Time (days)
-0.2 V
-0.4 V
-0.6 V
No BES (control)
No inoculum (-0.6 V)
OCP

Fig. 2.

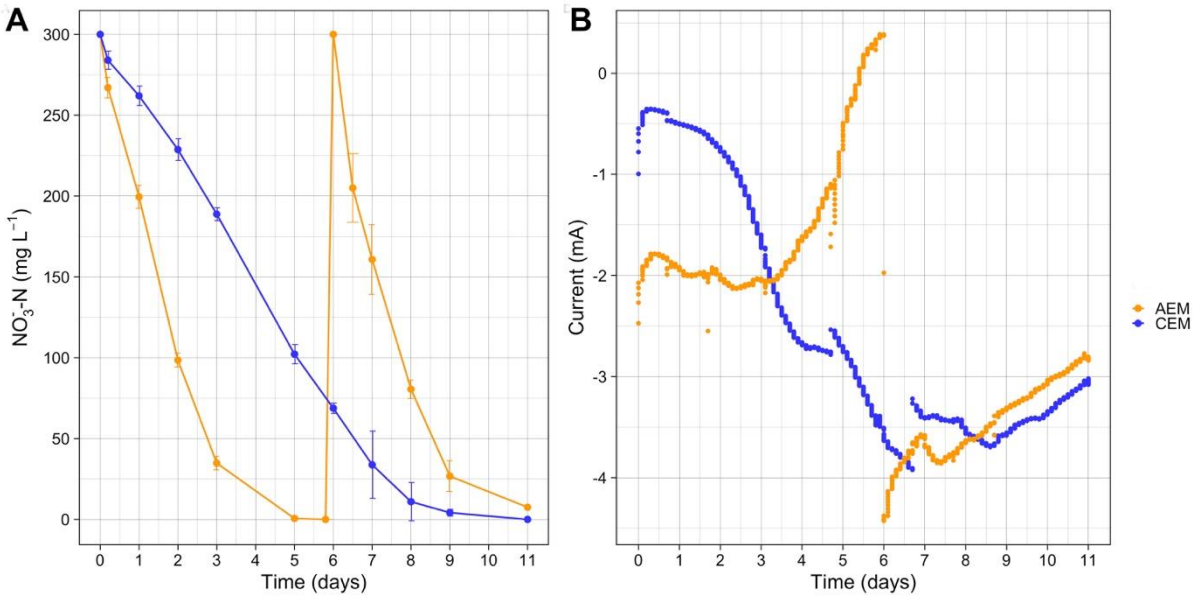


Fig. 3.

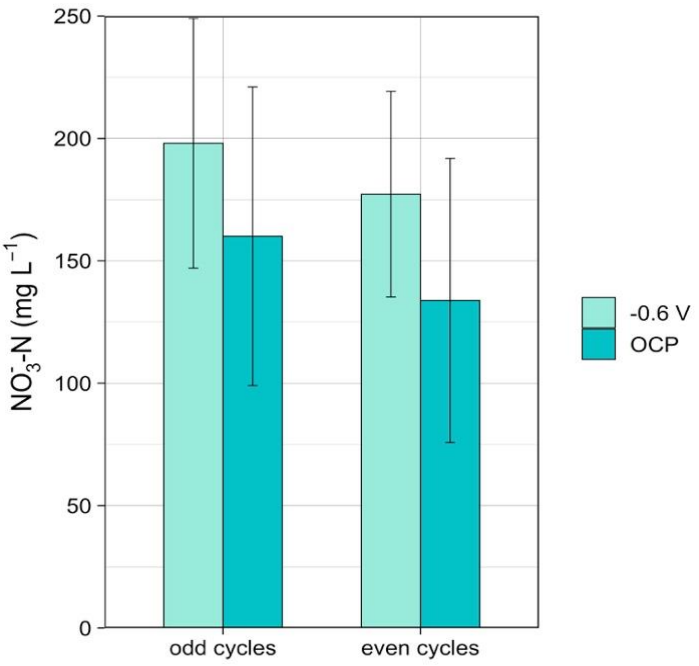


Fig. 4.

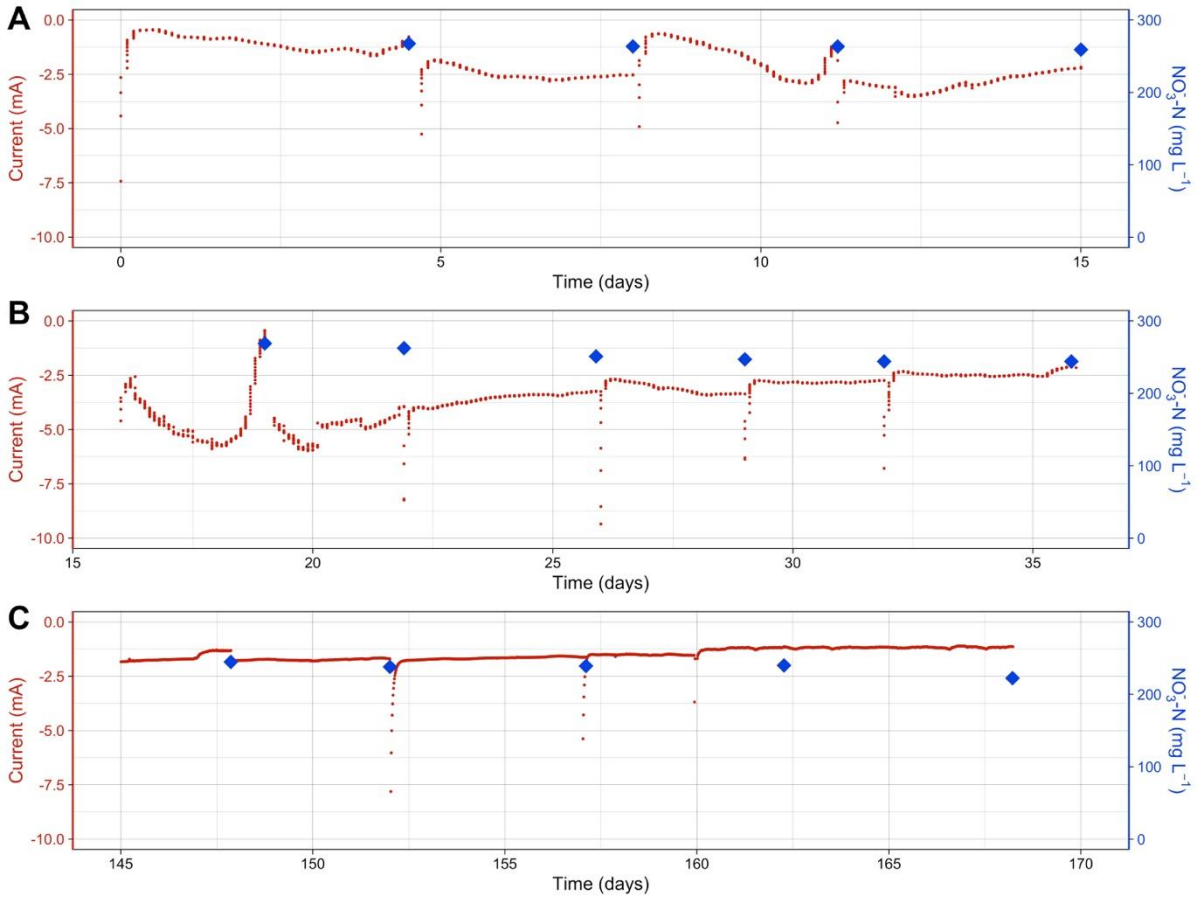

A
B
C
Current (mA)
NO_3^--N (mg L^{-1})
Time (days)
0.0
-2.5
-5.0
-7.5
-10.0
300
200
100
0
0
5
10
15
15
20
25
30
35
145
150
155
160
165
170

Fig. 5.

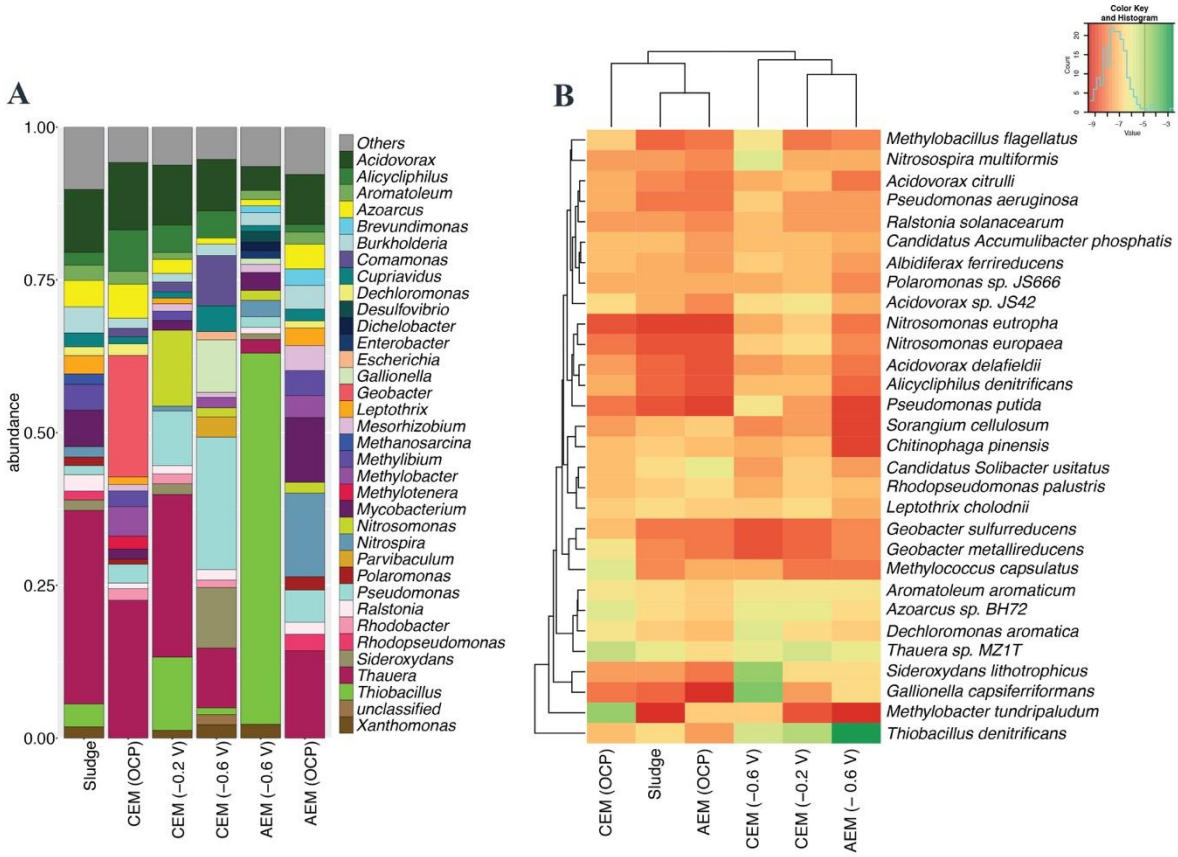
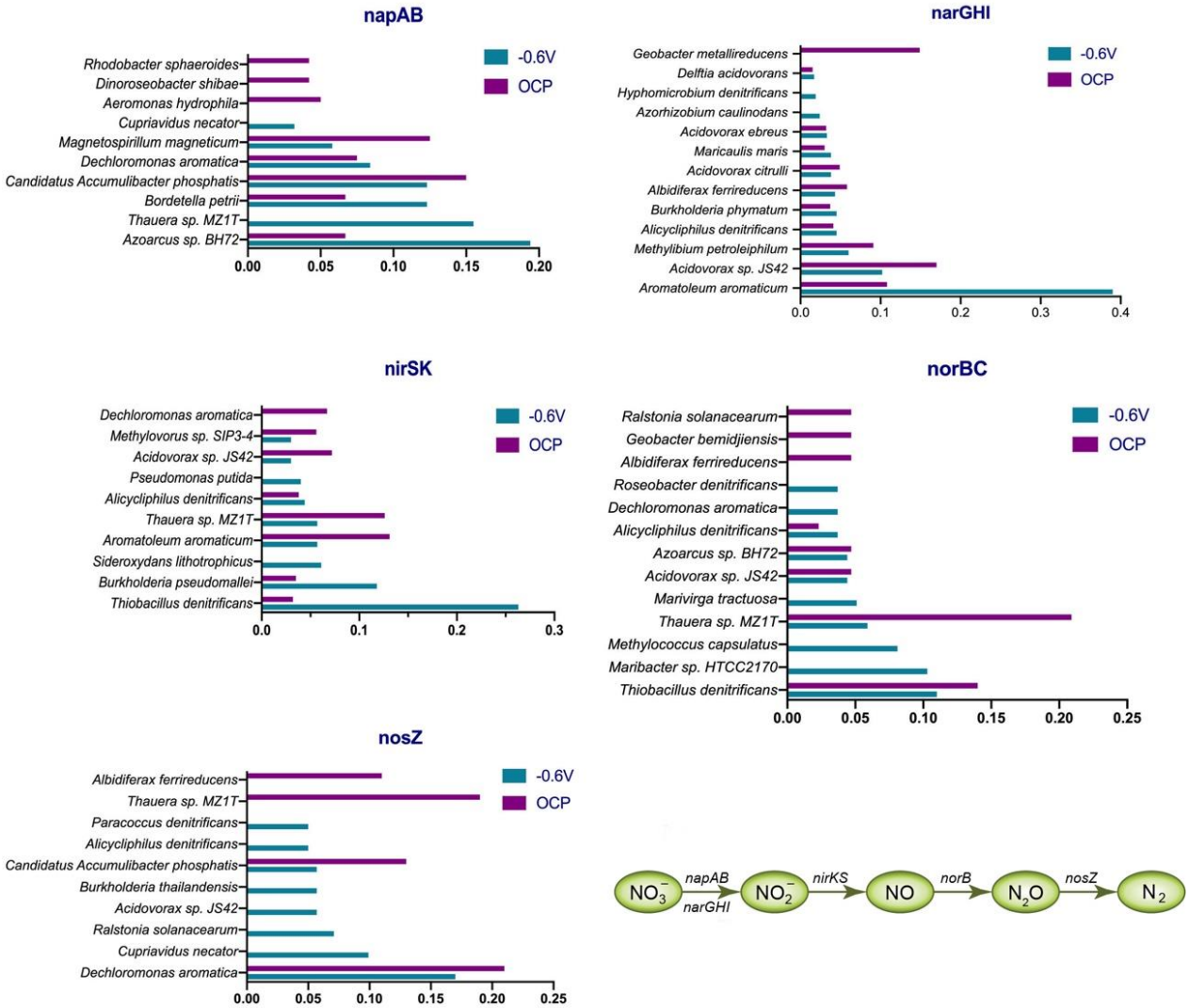
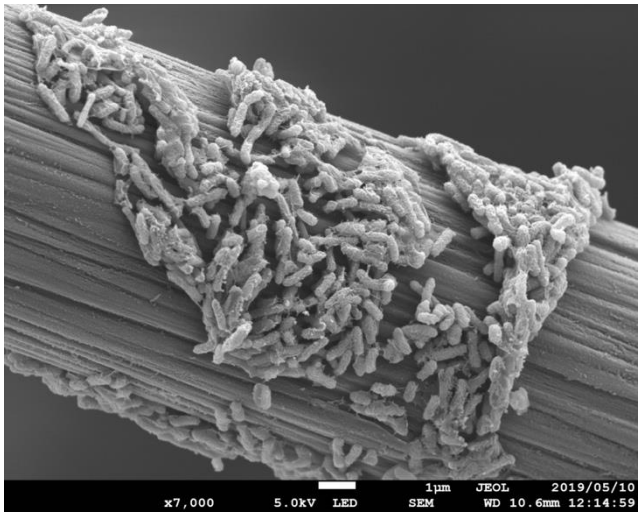

A
1.00
0.75
0.50
0.25
0.00
abundance
Sludge
CEM (OCP)
CEM (−0.2 V)
CEM (−0.6 V)
AEM (−0.6 V)
AEM (OCP)
Others
Acidovorax
Alicycliphilus
Aromatoleum
Azoarcus
Brevundimonas
Burkholderia
Comamonas
Cupriavidus
Dechloromonas
Desulfovibrio
Dichelobacter
Enterobacter
Escherichia
Gallionella
Geobacter
Leptothrix
Mesorhizobium
Methanosarcina
Methylibium
Methylobacter
Methylotenera
Mycobacterium
Nitrosomonas
Nitrospira
Parvibaculum
Polaromonas
Pseudomonas
Ralstonia
Rhodobacter
Rhodopseudomonas
Sideroxydans
Thauera
Thiobacillus
unclassified
Xanthomonas
B
Color Key and Histogram
Count
Value
CEM (OCP)
Sludge
AEM (OCP)
CEM (−0.6 V)
CEM (−0.2 V)
AEM (− 0.6 V)
Methylobacillus flagellatus
Nitrosospira multiformis
Acidovorax citrulli
Pseudomonas aeruginosa
Ralstonia solanacearum
Candidatus Accumulibacter phosphatis
Albidiferax ferrireducens
Polaromonas sp. JS666
Acidovorax sp. JS42
Nitrosomonas eutropha
Nitrosomonas europaea
Acidovorax delafieldii
Alicycliphilus denitrificans
Pseudomonas putida
Sorangium cellulosum
Chitinophaga pinensis
Candidatus Solibacter usitatus
Rhodopseudomonas palustris
Leptothrix cholodnii
Geobacter sulfurreducens
Geobacter metallireducens
Methylococcus capsulatus
Aromatoleum aromaticum
Azoarcus sp. BH72
Dechloromonas aromatica
Thauera sp. MZ1T
Sideroxydans lithotrophicus
Gallionella capsiferriformans
Methylobacter tundripaludum
Thiobacillus denitrificans

Fig. 6


napAB
-0.6V
OCP
Rhodobacter sphaeroides
Dinoroseobacter shibae
Aeromonas hydrophila
Cupriavidus necator
Magnetospirillum magneticum
Dechloromonas aromatica
Candidatus Accumulibacter phosphatis
Bordetella petrii
Thauera sp. MZ1T
Azoarcus sp. BH72
0.00 0.05 0.10 0.15 0.20
narGHI
-0.6V
OCP
Geobacter metallireducens
Delftia acidovorans
Hyphomicrobium denitrificans
Azorhizobium caulinodans
Acidovorax ebreus
Maricaulis maris
Acidovorax citrulli
Albidiferax ferrireducens
Burkholderia phymatum
Alicycliphilus denitrificans
Methylibium petroleiphilum
Acidovorax sp. JS42
Aromatoleum aromaticum
0.0 0.1 0.2 0.3 0.4
nirSK
-0.6V
OCP
Dechloromonas aromatica
Methylovorus sp. SIP3-4
Acidovorax sp. JS42
Pseudomonas putida
Alicycliphilus denitrificans
Thauera sp. MZ1T
Aromatoleum aromaticum
Sideroxydans lithotrophicus
Burkholderia pseudomallei
Thiobacillus denitrificans
0.0 0.1 0.2 0.3
norBC
-0.6V
OCP
Ralstonia solanacearum
Geobacter bemidjiensis
Albidiferax ferrireducens
Roseobacter denitrificans
Dechloromonas aromatica
Alicycliphilus denitrificans
Azoarcus sp. BH72
Acidovorax sp. JS42
Marivirga tractuosa
Thauera sp. MZ1T
Methylococcus capsulatus
Maribacter sp. HTCC2170
Thiobacillus denitrificans
0.00 0.05 0.10 0.15 0.20 0.25
nosZ
-0.6V
OCP
Albidiferax ferrireducens
Thauera sp. MZ1T
Paracoccus denitrificans
Alicycliphilus denitrificans
Candidatus Accumulibacter phosphatis
Burkholderia thailandensis
Acidovorax sp. JS42
Ralstonia solanacearum
Cupriavidus necator
Dechloromonas aromatica
0.00 0.05 0.10 0.15 0.20 0.25
NO3−
napAB
narGHI
NO2−
nirKS
NO
norB
N2O
nosZ
N2

Supplementary material.

Scanning electron microscopy (SEM) of the microbial community attached to the cathode at -0.6 V after long-term enrichment for 3 months.



References:

1. Broman, E., Jawad, A., Wu, X., Christel, S., Ni, G., Lopez-Fernandez, M., Sundkvist, J.E., Dopson, M., 2017. Low temperature, autotrophic microbial denitrification using thiosulfate or thiocyanate as electron donor. Biodegradation 28, 287–301. https://doi.org/10.1007/s10532-017-9796-7
2. Chakraborty, A., Roden, E.E., Schieber, J., Picardal, F., 2011. Enhanced growth of Acidovorax sp. strain 2AN during nitrate-dependent Fe(II) oxidation in batch and continuous-flow systems. Appl. Environ. Microbiol. 77, 8548–8556. https://doi.org/10.1128/AEM.06214-11
3. Chen, W., Wu, D., Wan, H., Tang, R., Li, C., Wang, G., Feng, C., 2017. Carbon-based cathode as an electron donor driving direct bioelectrochemical denitri fi cation in bio fi lm-electrode reactors : Role of oxygen functional groups 118, 310–318. https://doi.org/10.1016/j.carbon.2017.03.062
4. Clauwaert, P., Rabaey, K., Aelterman, P., De Schamphelaire, L., Pham, T.H., Boeckx, P., Boon, N., Verstraete, W., 2007. Biological denitrification in microbial fuel cells. Environ. Sci. Technol. 41, 3354–3360. https://doi.org/10.1021/es062580r
5. Deng, Q., Su, C., Lu, X., Chen, W., Guan, X., Chen, S., Chen, M., 2020. Performance and functional microbial communities of denitrification process of a novel MFC-granular sludge coupling system. Bioresour. Technol. 306, 123173. https://doi.org/10.1016/j.biortech.2020.123173
6. Emerson, D., Field, E.K., Chertkov, O., Davenport, K.W., Goodwin, L., Munk, C., Nolan, M., Woyke, T., 2013. Comparative genomics of freshwater Fe-oxidizing bacteria : implications for physiology , ecology , and systematics 4, 1–17. https://doi.org/10.3389/fmicb.2013.00254
7. Fabisch, M., Beulig, F., Akob, D.M., Küsel, K., 2013. Surprising abundance of Gallionella-related iron oxidizers in creek sediments at pH 4.4 or at high heavy metal concentrations. Front. Microbiol. 4, 1–12. https://doi.org/10.3389/fmicb.2013.00390
8. Feng, Y., Yang, Q., Wang, X., Logan, B.E., 2010. Treatment of carbon fiber brush anodes for improving power generation in air–cathode microbial fuel cells. J. Power Sources 195, 1841–1844. https://doi.org/10.1016/j.jpowsour.2009.10.030
9. Gregoire, K.P., Glaven, S.M., Hervey, J., Lin, B., Tender, L.M., 2014. Enrichment of a High-Current Density Denitrifying Microbial Biocathode. J. Electrochem. Soc. 161, H3049–H3057. https://doi.org/10.1149/2.0101413jes
10. Gregory, K.B., Bond, D.R., Lovley, D.R., 2004. Graphite electrodes as electron donors for anaerobic respiration. Environ. Microbiol. 6, 596–604. https://doi.org/10.1111/j.1462-2920.2004.00593.x
11. Holmes, D.E., Bond, D.R., O'Neil, R.A., Reimers, C.E., Tender, L.R., Lovley, D.R., 2004. Microbial communities associated with electrodes harvesting electricity from a variety of aquatic sediments. Microb. Ecol. 48, 178–190. https://doi.org/10.1007/s00248-003-0004-4
12. Huang, H., Cheng, S., Li, F., Mao, Z., Lin, Z., Cen, K., 2019. Enhancement of the denitrification activity by exoelectrogens in single-chamber air cathode microbial fuel cells. Chemosphere 225, 548–556. https://doi.org/10.1016/j.chemosphere.2019.03.052
13. Irshad, M., Malik, A.H., Shaukat, S., Mushtaq, S., Ashraf, M., 2013. Characterization of Heavy Metals in Livestock Manures 22, 1257–1262.
14. Juretschko, S., Loy, A., Lehner, A., Wagner, M., 2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. Syst. Appl. Microbiol. 25, 84–99. https://doi.org/10.1078/0723-2020-00093
15. Khan, S.T., Horiba, Y., Yamamoto, M., Hiraishi, A., 2002. Members of the Family Comamonadaceae as Primary Denitrifiers in Activated Sludge as Revealed by a Polyphasic Approach. Appl. Environ. Microbiol. 68, 3206–3214. https://doi.org/10.1128/AEM.68.7.3206
16. Khilyas, I. V, Sorokin, A.A., Kiseleva, L., Simpson, D.J.W., Fedorovich, V., Sharipova, M.R., Kainuma, M., Cohen, M.F., Goryanin, I., 2017. Comparative Metagenomic Analysis of Electrogenic Microbial Communities in Differentially Inoculated Swine Wastewater-Fed Microbial Fuel Cells. Scientifica (Cairo). 2017, 7616359. https://doi.org/10.1155/2017/7616359
17. Li, P.F., Li, S.G., Li, Z.F., Zhao, L., Wang, T., Pan, H.W., Liu, H., Wu, Z.H., Li, Y.Z., 2013. Co-cultivation of Sorangium cellulosum strains affects cellular growth and biosynthesis of secondary metabolite epothilones. FEMS Microbiol. Ecol. 85, 358–368. https://doi.org/10.1111/1574-6941.12125
18. Liu, B., Mao, Y., Bergaust, L., Bakken, L.R., Frostegård, Å., 2013. Strains in the genus Thauera exhibit remarkably different denitrification regulatory phenotypes 15, 2816–2828. https://doi.org/10.1111/1462-2920.12142
19. Mehrani, M.J., Sobotka, D., Kowal, P., Ciesielski, S., Makinia, J., 2020. The occurrence and role of Nitrospira in nitrogen removal systems. Bioresour. Technol. 303. https://doi.org/10.1016/j.biortech.2020.122936
20. Ming-Ju Chen, Kreuter, J.Y.-T.K., 1996. Kinetics of Pure Cultures ofHydrogen-Oxidizing Denitrifying Bacteria and Modeling of the Interactions AmongThem in Mixed Cultures. J. Anat. 189 (Pt 3, 503–

505. https://doi.org/10.1002/bit
21. Park, H. Il, Kim, D.K., Choi, Y.J., Pak, D., 2005. Nitrate reduction using an electrode as direct electron donor in a biofilm-electrode reactor. Process Biochem. 40, 3383–3388. https://doi.org/10.1016/j.procbio.2005.03.017
22. Park, Y., Park, S., Nguyen, V.K., Yu, J., Torres, C.I., Rittmann, B.E., Lee, T., 2017. Complete nitrogen removal by simultaneous nitrification and denitrification in flat-panel air-cathode microbial fuel cells treating domestic wastewater. Chem. Eng. J. 316, 673–679. https://doi.org/10.1016/j.cej.2017.02.005
23. Pous, N., Koch, C., Colprim, J., Puig, S., Harnisch, F., 2014. Extracellular electron transfer of biocathodes: Revealing the potentials for nitrate and nitrite reduction of denitrifying microbiomes dominated by Thiobacillus sp. Electrochem. commun. 49, 93–97. https://doi.org/10.1016/j.elecom.2014.10.011
24. Pous, N, Koch, C., Viì A-Rovira, A., Balaguer, M.D., Colprim, J., Harnisch, F., Puig, S., 2015. Monitoring and engineering reactor microbiomes of denitrifying bioelectrochemical systems †. https://doi.org/10.1039/c5ra12113b
25. Pous, Narcis, Puig, S., Balaguer, M.D., Colprim, J., 2015. Cathode potential and anode electron donor evaluation for a suitable treatment of nitrate-contaminated groundwater in bioelectrochemical systems. Chem. Eng. J. 263, 151–159. https://doi.org/10.1016/j.cej.2014.11.002
26. Powlson, D.S., Addiscott, T.M., Benjamin, N., Cassman, K.G., de Kok, T.M., van Grinsven, H., L'hirondel, J.-L., Avery, A.A., van Kessel, C., 2008. When Does Nitrate Become a Risk for Humans? J. Environ. Qual. 37, 291–295. https://doi.org/10.2134/jeq2007.0177
27. Prokhorova, K. Sturm-Richter, A. Doetsch, J.G., 2017. Resilience, Dynamics, and Interactions within a Model Multispecies Exoelectrogenic-Biofilm Community. J of Appl. and Environ. Microbiol, 83, 1–15.
28. Puig, S., Serra, M., Vilar-Sanz, A., Cabré, M., Bañeras, L., Colprim, J., Balaguer, M.D., 2011. Autotrophic nitrite removal in the cathode of microbial fuel cells. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2010.12.100
29. Rosso, D., Larson, L.E., Stenstrom, M.K., 2008. Aeration of large-scale municipal wastewater treatment plants: State of the art. Water Sci. Technol. 57, 973–978. https://doi.org/10.2166/wst.2008.218
30. Schmidt, I., Sliekers, O., Schmid, M., Bock, E., Fuerst, J., Kuenen, J.G., Jetten, M.S.M., Strous, M., 2003. New concepts of microbial treatment processes for the nitrogen removal in wastewater. FEMS Microbiol. Rev. 27, 481–492. https://doi.org/10.1016/S0168-6445(03)00039-1
31. Shrestha, N.K., Hadano, S., Kamachi, T., Okura, I., 2001. Conversion of ammonia to dinitrogen in wastewater by Nitrosomonas europaea. Appl. Biochem. Biotechnol. - Part A Enzym. Eng. Biotechnol. 90, 221–232. https://doi.org/10.1385/ABAB:90:3:221
32. Sun, J., Cao, H., Wang, Z., 2020. Progress in nitrogen removal in bioelectrochemical systems. Processes 8. https://doi.org/10.3390/pr8070831
33. Tang, R., Wu, D., Chen, W., Feng, C., Wei, C., 2017. Biocathode denitrification of coke wastewater effluent from an industrial aeration tank: Effect of long-term adaptation. Biochem. Eng. J. 125, 151–160. https://doi.org/10.1016/j.bej.2017.05.022
34. Tian, T., Yu, H.Q., 2020. Denitrification with non-organic electron donor for treating low C/N ratio wastewaters. Bioresour. Technol. 299, 122686. https://doi.org/10.1016/j.biortech.2019.122686
35. Tilman, D., Isbell, F., Cowles, M., 2014. Biodiversity and Ecosystem Functioning. Annu. Rev. Ecol. Evol. Syst. 45, 471–493. https://doi.org/10.1146/annurev-ecolsys-120213-091917
36. Van Doan, T., Lee, T.K., Shukla, S.K., Tiedje, J.M., Park, J., 2013. Increased nitrous oxide accumulation by bioelectrochemical denitrification under autotrophic conditions: Kinetics and expression ofdenitrification pathway genes. Water Res. 47, 7087–7097. https://doi.org/10.1016/j.watres.2013.08.041
37. Vasieva, O., Sorokin, A., Szydlowski, L., Goryanin, I., 2019. Do Microbial Fuel Cells have Antipathogenic Properties ? Computer Science & Systems Biology Do Microbial Fuel Cells have Antipathogenic Properties ? J. Comput. Sci. Syst. Biol. 12, 57–70. https://doi.org/10.4172/0974-7230.1000301
38. Verstraete, W., Van de Caveye, P., Diamantis, V., 2009. Maximum use of resources present in domestic "used water." Bioresour. Technol. 100, 5537–5545. https://doi.org/10.1016/j.biortech.2009.05.047
39. Vilajeliu-Pons, A., Puig, S., Pous, N., Salcedo-Dávila, I., Bañeras, L., Balaguer, M.D., Colprim, J., 2015. Microbiome characterization of MFCs used for the treatment of swine manure. J. Hazard. Mater. 288, 60–68. https://doi.org/10.1016/j.jhazmat.2015.02.014
40. Vilajeliu-Pons, A., Puig, S., Salcedo-Dávila, I., Balaguer, M.D., Colprim, J., 2017. Long-term assessment of six-stacked scaled-up MFCs treating swine manure with different electrode materials.

Environ. Sci. Water Res. Technol. 3, 947–959. https://doi.org/10.1039/c7ew00079k
41. Vilar-Sanz, A., Puig, S., García-Lledó, A., Trias, R., Balaguer, M.D., 2013. Denitrifying Bacterial Communities Affect Current Production and Nitrous Oxide Accumulation in a Microbial Fuel Cell) Denitrifying Bacterial Communities Affect Current Production and Nitrous Oxide Accumulation in a Microbial Fuel. Cell. PLoS ONE 8, 63460. https://doi.org/10.1371/journal.pone.0063460
42. Vo, C.-D.-T., Michaud, J., Elsen, S., Faivre, B., Bouveret, E., Barras, F., Fontecave, M., Pierrel, F., Lombard, M., Pelosi, L., 2020. The O 2 -independent pathway of ubiquinone biosynthesis is essential for denitrification in Pseudomonas aeruginosa . J. Biol. Chem. jbc.RA120.013748. https://doi.org/10.1074/jbc.ra120.013748
43. Wang, H., Jiang, S.C., Wang, Y., Xiao, B., 2013. Substrate removal and electricity generation in a membrane-less microbial fuel cell for biological treatment of wastewater. Bioresour. Technol. 138, 109–116. https://doi.org/10.1016/j.biortech.2013.03.172
44. Yang, N., Zhan, G., Li, D., Wang, X., He, X., Liu, H., 2019. Complete nitrogen removal and electricity production in Thauera-dominated air-cathode single chambered microbial fuel cell. Chem. Eng. J. 356, 506–515. https://doi.org/10.1016/j.cej.2018.08.161
45. Yu, L., Yuan, Y., Chen, S., Zhuang, L., Zhou, S., 2015. Direct uptake of electrode electrons for autotrophic denitri fi cation by Thiobacillus denitrificans 60, 126–130. https://doi.org/10.1016/j.elecom.2015.08.025
46. Zhang, N., Chen, H., Lyu, Y., Wang, Y., 2019. Nitrogen removal by a metal-resistant bacterium, Pseudomonas putida ZN1, capable of heterotrophic nitrification–aerobic denitrification. J. Chem. Technol. Biotechnol. 94, 1165–1175. https://doi.org/10.1002/jctb.5863

CRediT authorship contribution statement

A.P.: Conceptualization, Methodology, Investigation, Writing – original draft, review & editing. R.H.: Investigation, Chemical analysis. M.K.: Concenpturalization, Funding acquisition, Methodology, Investigation, Wring – review & editing. S.B.: Bioinformatic analysis, Writing-review & editing. I.G.: Supervision.

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
