Enrichment of a High-CURRENT Density Denitrifying Microbial Biocathode

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The pairing of a nitrate reducing microbial biocathode with an organic matter oxidizing microbial bioanode represents a potential high value wastewater treatment methodology. While such bioanodes are relatively optimized, such biocathodes suffer from relatively low current densities and low operating potentials. Here we present enrichment and characterization of a denitrifying microbial biocathode that generates more than 10-fold greater current density per unit geometric surface area and operates at nearly 0.2 V higher than those previously reported. A mixture of aquatic sediments and denitrifying biomass was first enriched for microbes that reduce nitrate with electrons supplied by oxidation of Fe(II), then enriched for microbes that reduce nitrate with electrons supplied by a graphite electrode. The resulting biocathode exhibited a Nemstian current-potential dependency (onset of current at = −0.125 V vs. Ag/AgCl, limiting current density = −3.2 A/m²). Non-turnover voltammetry exhibited current peaks that scale with the square root of scan rate, consistent with diffusive electron hopping to microbes acting as nitrate reduction catalysts from the electrode surface via endogenous redox cofactors. In non-optimized electrochemical reactors, the biocathode removed 14–40% of influent NO₃⁻ without significant production of ammonia-nitrogen (NH3-N), suggesting that reduction of nitrate to nitric or nitrous oxide gas is occurring and that reduction of NO₃⁻ to NH₃ is not a metabolic pathway. Results of 16S rDNA sequencing revealed a predominance of Betaproteobacteria, including Rhodocyclaceae and Burkholderiales, known environmental nitrogen cyclers, as the potential microbial cathode catalysts.

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Certain microbial biofilms formed on cathode surfaces can perform reduction reactions using electrons supplied by cathodes through microbial extracellular electron transfer (EET) processes.1−7 Such biocathodes have thus far demonstrated possible high value reactions if proven scalable including dechlorination,8 denitrification,9 H₂ generation,2 and carbon fixation (i.e., electrosynthesis of organic compounds including fuel precursors from carbon dioxide).5,6,10−12 Little is known about the underlying EET mechanisms of biocathodes because cultivation of cathode microorganisms is challenging and typically requires complicating modifications to nutrients and reactor conditions to enable biofilm formation and catalysis that are not required by microbial bioanodes.13 Only recently has an organism been identified (Mariprofundus ferrooxydans) that can grow on a cathode without such manipulation from pure culture,15 potentially providing a model system for study analogous to Geobacter sulfurreducens for microbial bioanodes.14−20

In this study, we describe formation and preliminary characterization of a relatively high current density denitrifying microbial biocathode that operates at relatively high potential. Motivation for this research is energy efficient treatment of wastewater using microbial fuel cells (MFCs).21−22 MFCs use microbial bioanodes to oxidize wastewater organic matter—the primary function of a wastewater treatment—liberating energy when coupled to a cathodic half-reaction that can achieve appreciable current at potentials higher than −0.2 V vs. Ag/AgCl. The vast majority of wastewater-fed MFCs studied to date have relied on abiological oxygen reduction at the cathode, owing to its high theoretical operating potential (i.e. +0.59 V vs. Ag/AgCl, pH7) and availability of oxygen as a sustainable oxidant. It is increasingly recognized however that oxygen reduction is not a viable MFC cathode reaction due to low oxygen solubility in water and the need for expensive metal catalysts.5,23 Moreover, the circumneutral pH and ambient temperature conditions of MFCs required by bioanodes are ill suited for abiotic oxygen reduction, resulting in relatively low operating potentials.

A denitrifying biocathode represents one possible alternative. Ammonia, present in wastewater, can be converted to nitrate through aerobic microbiological pathways.28 Denitrification is a desired reaction with respect to treating wastewater. Moreover, the orders of magnitude greater solubility of nitrate compared to oxygen is expected to enable higher cathodic current densities under mass transport limiting conditions, potentially enabling higher rates of organic matter oxidation at MFC bioanodes. Realization of denitrifying biocathodes useful for wastewater treatment requires however development of biocathodes that can reduce nitrate at higher rates and at higher potentials than those previously reported. Enrichment of microorganisms from the environment under these conditions is one potential strategy for achieving this goal.

A potential indicator for the ability of microorganisms to act as cathode catalysts is ability to oxidize Fe(II) compounds which are often insoluble in the environment, requiring the microbes to perform EET in order to obtain electrons as when using a cathode as an electron source.5,13,25 Nitrate-reducing, iron-oxidizing bacteria have been identified in aquatic sediments, as well as marine, brackish, fresh and wastewaters.30−32 Moreover, previous studies on microbial biocathode denitrification have demonstrated plausibility of enriching such bacteria from such environmental samples.29,33,34 These studies however have resulted in biocathodes operating at electrode potentials that are too low for use with MFCs (ca. −0.5 V vs. Ag/AgCl).23,34−36 Here we present a general enrichment strategy for denitrifying biocathodes with a relatively high current density and a midpoint potential of −0.250 V vs. Ag/AgCl, sufficiently positive to be possibly coupled with a wastewater-fed MFC bioanode operated at closed circuit. We present a systematic characterization of the catalytic and non-turnover properties (e.g. voltammetry, limiting current, mid-point potential), nitrate removal efficiency, and preliminary analysis of biofilm microbial composition using 16s rDNA sequencing.

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Materials and Methods

Environmental samples.— Environmental inocula were collected from three sites: (1) freshwater sediment from the Potomac River (Rock Creek tributary) in Washington, DC, USA at approximately +38° 55′ 49.18″, −77° 3′ 39.66″; (2) freshwater sediment from Paint Branch Creek in College Park, MD, USA at approximately +38° 59′ 37.94″, −76° 56′ 14.20″; and (3) denitrifying biomass from a sewage treatment plant in Washington, DC, USA (District of Columbia Water and Sewer Authority, Blue Plains Advanced Wastewater Treatment Plant). Denitrifying biomass was collected from a sampling point in the biomass (i.e. ‘solids’) recycle line that feeds sequential nitrification and denitrification tank reactors. Enrichment for denitrifying microorganisms in the second tank is accomplished through maintenance of anoxic conditions and addition of an auxiliary carbon source (e.g. methanol) to wastewater that has already received primary and secondary treatment (i.e. organic matter removal). Denitrifying biomass was collected in 300–1,000 mL plastic, screw-top containers with no headspace. At freshwater sites, anaerobic sediments were collected in 500 mL plastic containers approximately half-filled with sediment and at least 200 mL of overlying water. Sediment samples were collected using anaerobic techniques, but no attempts were made to preserve any naturally occurring redox gradients. In all cases, the samples were stored on ice and used as inoculants within six hours of sampling.

Planktonic enrichment for iron(II)-Dependent denitrifying microorganisms.—Enrichment for (II)-oxidizing, nitrate-reducing microorganisms was performed at room temperature using methods modified from Benz et al. (1998). A freshwater, bicarbonate buffered media was used containing (per L): 2.52 g NaHCO₃, 0.3 g NH₄Cl, 0.05 g MgSO₄·7H₂O, 0.4 g MgCl₂·6H₂O, 0.6 g KH₂PO₄, 0.1 g CaCl₂·2H₂O, 1 mL SL9 Mineral Solution and 2 mL Vitamin Solution. In addition, 0.606 g KNO₃ (per L) was added to enrich for nitrate reducing bacteria. One half of all enrichments contained acetate (0.15 g/L NaCH₃COOH) to evaluate dependency on organic carbon for cell growth. The pH of the medium was adjusted to 7.2 and filter sterilized (0.22 μm). Anoxic stock solutions of ferrous chloride (60 g/L FeCl₂) and ferrous sulfate (200 g/L FeSO₄) were prepared in deionized water while continually flushing with N₂ to remove dissolved oxygen. Ferrous iron was added via sterile filtration from an anoxic stock solution to achieve a final concentration of 6 mM and 200 μM after 24 hours in batch mode that exceeded background levels (< -4 μA; -5 mA/m²), after which the reactor was placed on flow mode whereby sterile anaerobic media was continuously supplied using a peristaltic pump. For initial experiments, the flow rate was adjusted periodically to evaluate the effect of dilution rate (0.048–0.189 hr⁻¹), hydraulic retention time (HRT): 318–1250 min) on current production and nitrate removal.

Biofilm scappings were taken from the surface of DN250 after it reached a stable, maximum current density of approximately -650 μA/m². Scaping was performed in-situ maintaining anaerobic conditions using a sterile cannula needle and syringe to remove 10 mL of electrode scappings plus media. The scappings were used to inoculate a subsequent electrochemical reactor, identical to the first one (referred to as bioanode DN250-2 for DN250 2nd generation) to further enrich the microbial consortia for microbes able to use an electrode as a metabolic electron donor.

Biofilm scappings taken from the surface of DN250-2 were taken after >10 days of continuous current production >600 mA/m². In this case the electrochemical reactor was removed from potentiostatic control and all steps of the transfer were performed in an anaerobic glove box (Coy brand) using a sterile blade and syringe. Electrode scappings plus media were preserved in three 10-mL syringes for <10 min before being used to inoculate triplicate electrochemical reactors. The resulting bioanodes (referred to as DN250-3a, DN250-3b, and DN250-3c) were identical to those described above, except that a relatively large carbon cloth flag (2.54 cm by 2.54 cm; total geometric length) which served as the working electrode and the counter electrode. The graphite rods were sterilized via autoclave in partially assembled cells prior to start-up. Reference electrodes were sterilized immediately prior to use without any bleach in media (v/v).

Two electrochemical reactors were filled with approximately 175 mL of medium (same as above but with ferrous iron and vitamins omitted to ensure that the electrode is the only viable metabolic electron donor) and were continuously purged with the anaerobic gas mix (N₂/CO₂ 80:20 v/v). The reactors were maintained on stir plates at a rate of 3′ (VWR Brand), and temperature was controlled at 30 °C with a circulating water bath. The reactors were wrapped in black cloth to inhibit growth of photosynthetic organisms. Cultures from Paint Branch Creek and DC Water Blue Plains facility amended with 2.5 mM NaCH₃COO⁻ during planktonic enrichment were used as the inoculum for two identical reactors. Each reactor was inoculated via syringe at a rate of 10% (v/v) using equal volumes from the two enrichments (total inoculation volume of 16 mL) and initially maintained in batch mode (i.e., media not flowing in or out of the reactor) at open circuit for 48 h. Inoculation was performed with anaerobic, asceptic techniques. A potential of 0.00 V was applied to the working electrode of one reactor and −0.250 to the other, referred to as bioanodes DN0 and DN250, respectively. Any appreciable current was observed for DN0 after approximately 600 hours in batch mode. In contrast, cathodic current was observed for DN250 after 24 hours in batch mode that exceeded background levels (< ±4 μA; ±5 mA/m²) after which the reactor was placed on flow mode whereby sterile anaerobic media was continuously supplied using a peristaltic pump. For initial experiments, the flow rate was adjusted periodically to evaluate the effect of dilution rate (0.048–0.189 hr⁻¹), hydraulic retention time (HRT): 318–1250 min) on current production and nitrate removal.

Electrochemical methods.—All electrochemical reactors were maintained on a Solartron 1470E multi-channel potentiostat (Solartron Analytical) and Multistat software program (Scribner Assoc.). Slow-scan cyclic voltammetry (CV) was performed at: (1) prior to inoculation under abiotic conditions; (2) immediately following inoculation; (3) at the onset of cathodic current generation; and (4) at maximum cathodic current production. CVs were performed in-situ from +0.550 V to −0.500 V and back to +0.550 V at 1, 2, and 5 mV/sec. Once bioanodes reached a steady-state, maximum limiting current at poised potential, voltammetry was performed at 0.2, 2, 10, 20, 100 and 200 mV/s. All current measurements were normalized by the cathode...
Biocathodes were preserved in sterile polypropylene containers after maximum, steady-state current production was maintained for 10 days of continuous, stable current production (>0.6 A/m²). Electrode scraping was performed in an anaerobic glove box (Coy) using a sterile blade and syringe. Electrode scrapings and media were preserved in three 10-mL syringes for 10 min before being used to inoculate triplicate electrochemical reactors. The resulting biocathodes (referred to as DN250-3a, DN250-3b, and DN250-3c for 3rd generation DN250 1, 2, and 3 of 3) were identical to those described in above, except that a relatively large carbon cloth flag (CC6, Fuel Cell Earth) with an area of 1,29 cm² was mounted into a single-welled chambered cover glass slide (Lab Tek) with several microliters of mounting fluid (Prolong Gold Antifade, Invitrogen). Imaging was performed with a Nikon TE-2000e inverted confocal microscope (Nikon), with a Nikon CFI Apo TIRF 100x (numerical aperture, 1.49) oil objective. Two wavelengths, 488 nm and 514 nm, were used to excite the fluorescent stains. Image processing was performed using Nikon software.

16S rDNA sequencing and phylogenetic analysis.— Scrapings of the DN250-2 cathode biofilm were taken after >10 days of continuous, stable current production (ranging from 0.6 to 1.25 A/m²). Electrode scraping was performed using an anaerobic glove box (Coy) using a sterile blade and syringe. Electrode scrapings and media were preserved in three 10-mL syringes for <10 min before being used to inoculate triplicate electrochemical reactors. The resulting biocathodes (referred to as DN250-3a, DN250-3b, and DN250-3c for 3rd generation DN250 1, 2, and 3 of 3) were identical to those described in above, except that a relatively large carbon cloth flag (CC6, Fuel Cell Earth) with a titanium wire lead was used as the working electrode in each reactor. A titanium wire lead was used as the working electrode in each reactor (2.54 cm by 2.54 cm; total geometric surface area, 12.9 cm²). These biocathodes were removed from the electrochemical reactors after maximum, steady-state current production was maintained for >4 d. Biocathodes were preserved in sterile polypropylene containers at −20°C until DNA extraction. Total DNA was extracted from electrodes using a soil DNA extraction kit according to the manufacturer’s instructions (UltraClean Soil DNA Isolation Kit, MoBio). The V4 region of the 16S rDNA gene was amplified using previously published primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Amplification was carried out in 25 μL standard PCR reactions using 1ng genomic DNA as PCR template with initial denaturation at 94°C for 3 minutes; followed by 35 cycles of 94°C, 45 sec., 50°C, 60 sec, 72°C, 90 sec; then final extension at 72°C, for 10 minutes. Microbial 16S rDNA sequences of the variable 4 (V4) region of the 16S rDNA gene were acquired on a MiSEQ instrument under automated software control (version 2.2.0, Illumina, San Diego, CA). Raw FASTQ-formatted DNA sequences were processed with the mothur software package (version 1.31.1) according to the author’s procedure for MiSEQ data [http://www.mothur.org/wiki/MiSeq_SOP]. From the three biological replicates, mothur processed a total of 1,402,642 sequences, filtering 16S sequences to total of 10,180 sequence reads of high-quality (e.g. non-ambiguous, >95% percentile in quality, and ≥197 base-pairs in length), then aligned the 10,180 sequences to the Silva bacterial reference database [http://arb-silva.de/] which was optimized for mothur compatibility as previously described [http://blog.mothur.org/2014/08/08/SILVA-v119-reference-files/]. This alignment was used to classify operational taxonomic units (OTUs) and phylotypes present in the isolated microbial consortia. Raw FASTQ-formatted sequencing reads of the V4 region acquired on the MiSEQ instrument have been assigned sample accession number SRS679278 in the National Center for Biotechnology’s (NCBI) Sequence Read Archive (SRA) and are accessible via BioProject accession PRJNA244670 [http://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA244670].

Results and Discussion

Planktonic enrichment for iron(II)-dependent denitrifying microorganisms.— Two of the environmental samples (Potomac sediment and denitrifying biomass) were initially inoculated in media containing 10 mM ferrous sulfate as the sole electron donor and 6 mM nitrate as the intended electron acceptor to enrich for Fe(II)-oxidizing, nitrate-reducing bacteria. This apparently resulted in unintentional enrichment of Fe(II)-oxidizing, sulfate reducing bacteria, qualitatively evidenced by darkening of cultures and production of odorous compounds consistent with sulphide formation 1–2 weeks after inoculation. Transfers of the two cultures and inoculation of the Paint Branch sediment were subsequently performed into fresh media containing 10 mM ferrous chloride instead of ferrous sulfate. In all cases, a vivid, rust-colored precipitate developed within five days of incubation, consistent with oxidation of Fe(II). Sediments may have been able to oxidize ferrous carbonates at a faster rate, based on the presence of rust-colored precipitates within 2 to 3 days of inoculation.

Electrode enrichment of cathode-dependent denitrifying microorganisms.— Equal volumes of the planktonic enrichments grown on ferrous chloride were used to inoculate the electrochemical reactors. Following inoculation, 48 h of open circuit conditions were maintained in batch mode. For both DN0 and DN250, the initial open circuit potential was approximately −0.06 V. Subsequently, DN0 and DN250 were poised at 0.00 V and −0.250 V, respectively. These potentials were chosen based on oxidation potentials of various iron containing compounds found in sediments and to test dependency of electrode enrichment on the potential applied to the electrode, where a lower potential may thermodynamically benefit microbial metabolism, but also impart a larger overpotential needed to drive the desired catalytic reaction. After approximately 600 hours at a fixed potential in batch mode, no appreciable catalytic current could be detected from DN0 (Figure 1). In contrast, DN250 developed an appreciable cathodic catalytic current density (−5 mA/cm²) within 48 hours, after which the reactor was placed in flow mode (dilution rate: 0.09 hr⁻¹, flow rate (Q): 0.275 mL/min), and the onset of an exponential current increase was observed after approximately 220 hours (Figure 1). Such an increase in current over time for an
electrochemical reactor inoculated with a bacterial culture is consistent with growth of a catalytic bacterial biofilm on the electrode, for which the electrode acts as a metabolic electron acceptor in the case of a bioanode,17,48 or as a metabolic electron donor as in the case here for a biocathode.6,13 Moreover, since the only viable metabolic electron acceptor in the electrochemical reactor was nitrate, it is consistent with growth of a biofilm that catalyzes electrode reduction of nitrate (vide infra). Switching to flow mode once current is observed is a strategy intended to clear out planktonic cells from the reactor in order to promote biofilm growth from proliferation of the subset of microbial species in the planktonic inoculum that adhered to the electrode and used it as a metabolic electron acceptor (i.e., electrochemical enrichment). After switching to flow mode, DN250 reached a maximum cathodic current density of −905 mA/m² after approximately 650 hours (Figure 1). The inability to enrich a biocathode at 0.00 V (DN0) is attributed here to thermodynamics of the planktonic enrichments on ferrous carbonates, which are energetically more favorable for iron-oxidizing bacteria than solid iron oxide. The significant lag period (time between inoculation and onset of exponential current growth), approximately 220 hours, of the DN250 biocathode is consistent with this hypothesis, whereby metabolic adaptation may have been required by cells to shift from growth on the semi-soluble ferrous carbonates (vide infra) to an insoluble electrode poised at −0.250 V. Alternatively, a very small portion of microbes in the inoculum may have been able to accept electrons from the electrode, requiring sufficient time to proliferate and generate detectable current over background.

A second-generation electrochemical enrichment was performed by inoculating an identical electrochemical reactor with scrapings taken from the biocathode of DN250, resulting in formation of biocathode DN250-2. DN250-2 exhibited exponential current growth within 48 h of inoculation and achieved a stable current density, comparable to that of DN250, within 135 hours (Figure 2). The shortened lag period is consistent with further enrichment of the inoculum of DN250-2, taken from the cathode surface of DN250, in bacteria able to use a cathode graphite electrode as a metabolic electron donor. A similar effect has been observed for a biocathode originally enriched from seawater that catalyzes oxygen reduction.6 After maintaining stable current production for >300 hours, the potential of DN250-2 was shifted from −0.25 V to −0.35 V, which resulted in a dramatic increase in current production to approximately −3,200 mA/m² (Figure 2B), which is the highest current density reported to date from a nitrate-reducing biocathode.

Replicate electrochemical enrichments from DN250-2 were subsequently performed onto carbon cloth electrodes (12.9 cm² geometric area), resulting in 3rd generation biocathodes (DN250-3a, b, c). All three replicates of DN250-3 exhibited exponential current growth almost immediately after inoculation (Figure 3), consistent with yet further electrode enrichment, and achieved a stable current density of approximately −2,150 mA/m² at −0.25 V. This is more than double the current density achieved by the graphite rod per unit geometric surface area, attributed in part to the higher ratio of electrochemically active to geometric surface area resulting from the cloth’s weave, as previously observed for bioanodes.31

**Nitrate dependency.**— The essential role of nitrate in current production was confirmed for DN250 and subsequent biocathodes enriched from DN250 by replacing media containing nitrate with identical media lacking nitrate. In all cases, current production sharply declined to background levels (±4 μA) within 24 hours, and stable current production resumed 2–3 hours after re-introducing nitrate (Figure 4). Increasing the flow rate (Q: 0.550 mL/min) did not increase current (not shown), nor did doubling the nitrate concentration (from 6 to 12 mM) by spiking with a degassed concentrated, de-oxygenated solution of KNO₃ (not shown). Slightly increasing the stir rate resulted in a modest (5–10%) increase in current (not shown) whereas further increasing the stir rate did not increase current (not shown). Eliminating stirring entirely resulted in decreased current by 12–50% (data not shown). Collectively, these results are consistent with current production by DN250 that is directly dependent on nitrate availability, and also with current production during electrode enrichment of DN250 that was not limited by nitrate concentration; nor appreciably by diffusion of nitrate, reaction products (e.g. OH⁻), nutrients, or ions in bulk solution to or from cells in the biofilm.

**Turnover voltammetry.**— Slow-scan cyclic voltammetry (CV) was performed immediately before and after inoculation of the DN0
Figure 4. Chronoamperometric plot (E_WE = −0.25 V vs. Ag/AgCl) illustrating the decline in current to background levels within 24 h of flowing media without nitrate as an electron acceptor to the DN250-2 biocathode. Stable current production resumed within 2–3 hours of re-introducing NO_3 to media feed.

Figure 5. Voltammograms captured immediately before and after inoculation with the planktonic enrichments, well before catalytic current was observed, exhibited defined peaks, which we attribute to abiotic oxidation and reduction, respectively, of ferrous and ferric iron by the graphite electrodes (Figure 5). The addition of ferrous chloride to anaerobic, bicarbonate buffered media presumably generated a FeCO_3 precipitate, consistent with the FeCO_3/Fe(OH)_3 redox couple. The cathodic scan (initial sweep from +0.500 to −0.500 V) revealed a reduction peak (occurring between −0.310 V and −0.390 V vs. Ag/AgCl), consistent with abiotic reduction (i.e., directly by the electrode) of ferric iron precipitates. Similarly, the subsequent anodic scan (from −0.500 V to +0.500 V) yields a smaller oxidation peak, presumably corresponding to the re-oxidation of soluble ferrous iron generated during the cathodic scan. Averaging the peak potentials indicates that the abiological mid-point potential is approximately −0.260 V.

The production of exponential current growth commenced around day 9, and evidence of a Nernst-Monod current-potential dependency (i.e., a sigmoid-shaped voltammetric feature) analogous to that observed for microbial bioanodes and wired enzyme electrodes was observed in all subsequent CVs (Figure 7). During early growth, the onset of catalysis occurred at approximately −0.130 V and the mid-point of the catalytic wave centered at approximately −0.180 V, indicated by the first derivative of the CV; (Figure 8). Cathodic current saturated at potentials below −0.300 V. CV repeated at

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faster rates (1, 2, 5, 10, 20, 100, and 200 mV/s) exhibited increasing distortion, as expected due to kinetics of one or more steps involved in the catalytic process. A clear catalytic mid-point could not be observed at v > 20 mV/s (Figure 9).

CV was also performed periodically after current production stabilized for each biocathode. In all instances, the mid-point potentials of CVs were observed to shift by approximately −0.050 to −0.070 V after prolonged operation at a fixed potential of −0.250 V (Figure 10). This was confirmed with a first derivative analysis of the voltammograms (Figure 8). Importantly, the shift in CVs coincided with increased saturation current. Key features of CV (mid-point potential; saturation current; catalytic onset) were highly reproducible with increased saturation current. Key features of CV (mid-point potential; saturation current; catalytic onset) were highly reproducible with increased saturation current.

Previous Nernst-Monod models for bio-catalytic behavior have theorized a 5-step mechanism for bioanode catalyzed acetate oxidation. Here, we propose an analogous five-step mechanism for biocathode catalyzed nitrate reduction reported here:

1. Electron transfer from the cathode surface to immobile redox cofactors adjacent to the cathode surface (i.e., across the cathode/biofilm interface), resulting in current production. In the case of G. sulfurreducens bioanodes these redox cofactors are proposed to be outer membrane and/or extracellular cytochromes. In the case of the biocathode described here, the identity of the redox cofactors are yet to be proposed.
2. Electron transport through the biofilm via incoherent multi-step electron hopping, also referred to as redox conduction, occurring by a sequence of electron transfer reactions through a network of immobile redox cofactors (vide supra).
3. Transfer of electrons from redox cofactors to cells within the biofilm (i.e., across the biofilm/cell interface).
4. Binding and reduction of substrate (NO₃⁻) in cells consuming electrons and yielding products.
5. Diffusion of cellular substrate into (e.g., nitrate from media into biofilm) and products out (e.g., NO, N₂O, N₂, OH⁻ out of the biofilm).

Drawing on a limiting case analysis performed on the analogous 5-step model for G sulfurreducens bioanode oxidation of acetate following directly from that of enzyme-modified electrodes, the sigmoid shape of the slow-scan voltammetry reported in this study for biocathode reduction of nitrate indicates that the rate of Step 1 is fast and reversible, and that neither Step 1 nor Step 5 is the rate-limiting step in the catalytic process.

Non-turnover CV.— Non-turnover CV was recorded after the cessation of appreciable current production (±4 µA; 4.8 mA/m²) when anaerobic media lacking nitrate was flushed into reactors containing DN250 and DN250-2. Figure 11 depicts representative non-turnover CV at 0.2, 2, 5, and 10 mV/s for the original biocathode DN250. Distinct redox peaks are observed which attribute to the biofilm redox cofactors accessible by the electrode via steps 1 and 2 above. In the absence of an electron acceptor (i.e., nitrate), redox cofactors within the biofilm involved in EET (i.e., Steps 1–3 above) can be oxidized and reduced, respectively, when the cathode potential is driven very negative or very positive vs. the redox cofactor formal potential during voltammetry in the same manner, it is thought, as occurs for a redox polymer modified electrode or wired enzyme electrode in the absence of enzyme substrate. In the case of DN250 and DN250-2, two sets of redox peaks near equal in size were observed in all scans from 0.2 to 20 mV/s. The first set of peaks (A and B in Figure 11) are centered at approximately −0.240 V; nearly identical to the mid-point potential of the turnover CV (Figure 5). A second set of peaks (C and D) was centered at −0.450 V similar in size to A and B. This suggests that there are two distinct types of redox cofactors with different formal potentials involved Steps 1,
Figure 11. (A) Representative non-turnover CV from original biocathode DN250, captured at $i < 4 \mu A$ in the absence of NO$_3^-$ as electron acceptor. Scans were recorded at 2 mV/s (solid black line), 5 mV/s (dashed black line), and 10 mV/s (dashed gray line). (B-C) Relationship between height of visible redox peaks (A, B, C, D in Figure 11) and the square root of scan rate during non-turnover voltammetry. Linear regression lines are overlaid with correlation coefficient. Baseline correction was accounted for in calculating peak height.

2, and 3. Alternatively, a single type of cofactor is involved that can be reversibly reduced by two electrons, where electron repulsion inside the molecule makes it thermodynamically more challenging to perform the second reduction reaction. It therefore requires a more negative potential ($-0.210 \text{ V}$) to drive the second electron into the molecule.57 Regardless, drawing on the same limiting case analysis applied above,2 alignment of the A/B voltammetric peaks with the mid-point potential of catalytic CVs depicted in Figure 8 suggests that electrons associated with generation of peaks A/B ($E^o = -0.240 \text{ V}$) are the ones used by the cells in Step 4. If so, then Step 2 or 3 of the 5-step mechanism proposed above would be the rate limiting step for the biocathode catalyzed nitrate reduction reaction reported here. The fact that voltammetric peaks A/B align with the abiotic voltammetric peaks observed initially following inoculation of the electrochemical reactors proposed to be associated with the FeCO$_3$/Fe(OH)$_3$ redox couple suggests that cofactors used by microbes to accept electrons from ferrous carbonates in the planktonic enrichments are similar to or the same cofactors used to accept electrons from electrodes. Alternatively, it may be the case that electrons associated with generation of voltammetric peaks C/D ($E^o = -0.450 \text{ V}$) are the ones used by the cells in Step 4. If this is the case, then Step 4 would be the rate limiting step.

Sweep-rate analysis was also performed on non-turnover CVs to evaluate the effect of scan rate on peak height and area. With baseline subtraction, the net height of redox peaks was evaluated at 0.2, 1, 2, 5, 10, 20, 50, 100, and 150 mV/s. A linear dependency ($R^2, \geq 89\%$) was found between peak height and the square root of scan rate (from 0.2 to 50 mV/s) for each redox peak (Figure 11). This is consistent with diffusive electron transport trough the biofilm expected for redox conduction involving immobile redox cofactors.17,41,54,55

Confocal imaging.— Imaging of DNA at the electrode surface was performed after stable current generation on carbon cloth squares was maintained for approximately 4 days. Confocal images revealed a relatively dense biofilm, with cells extending up to 60 $\mu m$ from the electrode surface covering the majority of the electrode surface. A representative three-dimensional image is shown in Figure 12 indicating biofilm growth around individual carbon fibers.

16S rDNA sequencing and phylogenetic analysis.— The 16S rDNA sequencing revealed a biocathode community highly enriched in Betaproteobacteria. At the conclusion of the experiment, DNA was extracted from DN250-3a, -3b, and 3C for 16S rDNA sequencing in order to determine the taxonomic profile of the biocathode microbial community. The biocathode community was overwhelmingly enriched in Proteobacteria (93%), a phylum known to be associated with nitrogen cycling and denitrification.58 Betaproteobacteria made up 78% of all Proteobacteria and consisted of sequences identified to belong to the orders Rhodocyclales and Burkholderiales, with Rhodocyclales making up 46% of all bacterial sequences and Burkholderiales making up 15%. Betaproteobacteria are known to carry out nitrate-dependent iron oxidation59 and members of both Rhodocyclales and Burkholderiales have previously been identified in acetate-amended, denitrifying microbial communities60 and we hypothesize that they are the primer drivers of denitrification in the biocathode community described here. Alpha- (15%) and Gamma- (6%) were also identified. The majority of Gammaproteobacteria belong to the family Xanthomonadaceae (96% of Gamma-), members of which have been
show to carry out denitrification (Kostka et al. J. Bacteriol. August 2012 vol. 194 no. 16 4461-4462). Alphaproteobacteria were divided between the Rhodobacterales and Rhizobiiales, subsurface associated taxa both known to harbor,21 Phyla other than Proteobacteria made up 3% or less of the total 16S rDNA sequences and may also participate in nitrogen cycling or biofilm maintenance.

Treatment efficiencies.— The influent and effluent of each reactor containing the denitrifying biocathode was sampled under conditions of maximum, stable current production and assayed for nitrate (NO3-N), nitrite (NO2-N), and ammonia (NH3-N) to assess possible nitrate reduction pathways and products. A significant influent/effluent nutrient composition and associated removal rates for each generation of biocathode is provided in Tables I and II. In all cases, there was significant removal of NO3-N (p < 0.05) by the biocathode (avg. removal, 14.2–36.9%), with associated daily removal rates of 2.20–11.4 g NO3-N/Nm3/day (based on electrode surface area), or 10–54 g NO3-N/Nm3/day (based on aqueous reactor volume). Sampled were also assayed for a number of iterations of reactor set-up and operation to assess possible effects of electrode surface area or hydraulic retention time on NO3-N removal rates (see Table I); however, no correlation was observed between electrode surface area or hydraulic retention time, and percent nitrate removal.

A small increase in the concentration of NH3-N was observed (Δ[NO3-N] = 0.71 mg N/L); however, this represents <7% of the net removal of NO3- + NO2-N, suggesting that the bio-cathode was at a minimum, reducing nitrate to nitric or nitrous oxide (Table II). Ammonium concentrations (NH3-N) increased by 4.51 mg/L; however, this was insignificant relative to the starting concentration ([NH3-N] = 83.5 mg/L), indicating that reduction of NO3- to NH3 was not a metabolic pathway of the biofilm.

The removal rates presented here serve as a conservative estimation of the NO3- removal capacity of the biocathode for a number of reasons. 1) Influent nitrate concentrations were based on sampling and analysis (64.8 mg NO3-N/L, equivalent to a loading rate of 0.147 kg NO3-N/Nm3/d). 2) As previously noted, reactors were operated with a very small electrode surface area-to-volume ratio. 3) Reactors were operated under conditions of continuous flow of media, such that the concentration of electron acceptor (NO3-) was non-limiting for cell growth and for current production. In this way, we conservatively estimate the removal efficiencies from this biocathode under the condition of maximum catalytic activity (i.e. maximum current production, minimum substrate depletion). To better estimate the potential rate of NO3 removal by the biocathode, a reactor design that maximizes the surface area-to-volume ratio should be employed, as in fixed-film wastewater processes (e.g. trickling filter; anaerobic filter).

Table I. Nitrate-Nitrogen Removal Rates from Denitrifying Cathode (values presented as mean ± stdev, n).

<table>
<thead>
<tr>
<th>Electrode Material</th>
<th>Surface Area (cm²)</th>
<th>HRT (min)</th>
<th>[NO3-N] Influent (mg/L)</th>
<th>[NO3-N] Effluent (mg/L)</th>
<th>% Removal NO3-N</th>
<th>Loading Rate (g NO3-N/Nm³/day)</th>
<th>Removable Rate (g NO3-N/Nm³/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graphite Rod</td>
<td>8.30</td>
<td>636</td>
<td>64.8±4.59</td>
<td>41.15±12.4</td>
<td>36.9%</td>
<td>147</td>
<td>30.95</td>
</tr>
<tr>
<td>Graphite Rod</td>
<td>8.30</td>
<td>1250</td>
<td>64.8±4.59</td>
<td>54.36±2.16</td>
<td>14.2%</td>
<td>75</td>
<td>15.75</td>
</tr>
<tr>
<td>Graphite Rod</td>
<td>8.30</td>
<td>2134</td>
<td>64.8±4.59</td>
<td>49.56±7.09</td>
<td>23.8%</td>
<td>44</td>
<td>9.22</td>
</tr>
<tr>
<td>Carbon Cloth</td>
<td>12.90</td>
<td>1250</td>
<td>64.8±4.59</td>
<td>45.11±2.33</td>
<td>30.6%</td>
<td>75</td>
<td>10.12</td>
</tr>
</tbody>
</table>

1A negative value denotes an increase in concentration.

Table II. Nitrite- and Ammonia-Nitrogen Removal Rates from Denitrifying Cathode (values presented as mean ± stdev, n).

<table>
<thead>
<tr>
<th></th>
<th>Influent (mg/L)</th>
<th>Effluent (mg/L)</th>
<th>% Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO2-N</td>
<td>0.20 ± 0.29</td>
<td>0.91 ± 1.27</td>
<td>-357%</td>
</tr>
<tr>
<td>NH3-N</td>
<td>83.5 ± 5.99</td>
<td>79.0 ± 1.24</td>
<td>5%</td>
</tr>
</tbody>
</table>

References
