Bacterial community structure corresponds to performance during cathodic nitrate reduction

Kelly C. Wrighton[1], Bernardino Virdis[2,3], Peter Clauwaert[4], Suzanne Read[2], Rebecca A. Daly[1], Nico Boon[4], Yvette Piceno[5], Gary L. Andersen[5], John D Coates[1], Korneel Rabaey[2]

[1]Department of Plant and Microbial Biology, University of California, Berkeley, USA

[2]Advanced Water Management Centre, The University of Queensland, Brisbane QLD4072, Australia

[3]DIGITA, University of Cagliari, Piazza d'Armi, 09123 Cagliari, Italy

[4]Laboratory of Microbial Ecology and Technology (LabMET), University of Belgium, Belgium, 9000 Belgium

[5]Earth Sciences Division, Ernest Orlando Lawrence Berkeley National Laboratory, Berkeley, CA 94720

Correspondence should be addressed to: Korneel Rabaey, Advanced Water Management Centre, The University of Queensland, Brisbane QLD4072, Tel. +61 7 3365 7519; Fax. +61 7 3365 4726; k.rabaey@uq.edu.au ; http://awmc.uq.edu.au

Running title: Cathodic denitrifying biofilms

Key words: microbial fuel cell, bioelectrochemical system, nitrate, chemolithotrophic, PhyloChip

Subject Category: Microbial ecology and functional diversity of natural habitats

Abstract

Microbial fuel cells (MFCs) have applications beyond electricity production, including the capacity to power desirable reactions in the cathode chamber. However, current knowledge of the microbial ecology and physiology of biocathodes is minimal, and as a result more research dedicated to understanding the microbial communities active in cathode biofilms is required. Here we characterize the microbiology of denitrifying bacterial communities stimulated by reducing equivalents generated from the anodic oxidation of acetate. We analysed biofilms isolated from two types of cathodic denitrification systems: (1) a loop format where the effluent from the carbon oxidation step in the anode is subjected to a nitrifying reactor which is fed to the cathode chamber and (2) an alternative non-loop format where anodic and cathodic feed streams are separated. Our results indicate the superior performance of the loop reactor in terms of enhanced current production and nitrate removal rates. We hypothesized that phylogenetic or structural features of the microbial communities could explain the increased performance of the loop reactor and used PhyloChip with 16S rRNA (cDNA) and Fluorescent *In situ* hybridization (FISH) to characterize the active bacterial communities. Our results reveal a greater richness, as well as an increased phylogenetic diversity, active in denitrifying biofilms than was previously identified in cathodic systems. Specifically, we identified Proteobacteria, Firmicutes and Chloroflexi members that were dominant in denitrifying cathodes. Additionally, our results indicate that it is the structural component, in terms of bacterial richness and evenness, rather than the phylogenetic affiliation of dominant bacteria, that best corresponds to cathode performance.

Introduction

Bioelectrochemical Systems (BESs) use microorganisms to catalyze oxidation or reduction reactions at an electrode (Rabaey, 2007). When net electrical energy is obtained from a BES, the system is referred to as a Microbial Fuel Cell (MFC). This technology includes anodic reactions where electron donors such as organic compounds and sulfide are oxidized, and cathodic reactions where electron acceptors such as oxygen, nitrate, nitrite or perchlorate are reduced (Clauwaert et al, 2007; Rabaey et al, 2008; Thrash et al, 2007; Virdis et al, 2008). Anodic and cathodic reactions can be coupled so that the anodic oxidation reaction generates sufficient power for cathodic reduction reactions to occur. Combining anodic and cathodic reactions in a single BES holds promise for wastewater treatment because carbon and nitrogen can be removed simultaneously regardless of the C:N ratios in the waste streams (Clauwaert et al, 2007; Virdis et al, 2008; Virdis et al, 2009).

In two previous papers we have demonstrated that microbial anodic acetate oxidation can power microbial cathodic nitrate reduction (Clauwaert et al, 2007; Virdis et al, 2008). To investigate differences in engineering and operation of these systems, we utilized two reactor configurations in loop and non-loop formats (Figure 1). In the loop configuration, effluent from the acetate-supplied MFC anode chamber was directed to an external aerobic stage where nitrification was stimulated. This stream was subsequently fed into the cathode chamber for denitrification. The non-loop format differs by keeping the anodic and cathodic feed streams separate (without routing the anodic stream to an external nitrifying reactor). We demonstrated that the performance of the loop reactor was notably superior to the non-loop reactors in terms of current production and rates of nitrate removal. However, biological denitrification was achieved in the cathode chamber regardless of operation format. (Virdis et al, 2008)

Cathodic nitrate reduction is dependent upon the activity of denitrifying microbes. Denitrifying bacteria are phylogenetically diverse; relevant taxa belong to over 60 genera including representatives from the *Proteobacteria, Firmicutes* and *Bacteroidetes* (Demaneche et al, 2009). While denitrifying bacteria are generally considered to be heterotrophic, some can reduce nitrate with sulfide, iron(II), or hydrogen as the electron donor (Weber et al, 2006). In the case of cathodic denitrification, researchers have considered using cathodes to generate hydrogen electrolytically or as a direct source of reducing equivalents for microbial respiration (Thrash et al, 2008). In previous studies of denitrifying cathodes, potentials at the cathode were measured above 0 V versus standard hydrogen electrode (SHE) (Virdis et al, 2008; Clauwaert et al, 2007). At these potentials, the hydrogen partial pressure would theoretically be below 10^{-14} atm (at pH 7), well below the known bacterial affinities for hydrogen (Virdis et al, 2008). Consequently, it is likely that bacteria reducing nitrate at cathodes powered by anodic reactions access electrons from the cathode and not via hydrogen. Our current knowledge of bacterial denitrification reactions relevant to BES cathodes is based on pure-culture studies of chemolithotrophic denitrification coupled to inorganic electron donors (Fernández et al, 2008; Weber et al, 2006) and cathodes as electron donors for anaerobic respiration (Gregory et al, 2004; Gregory and Lovley, 2005; Thrash et al, 2007; Strycharz et al, 2008; Thrash and Coates, 2008). Presently, only two studies examined microbial biofilm communities in denitrifying BESs (Gregory et al, 2004; Park et al, 2006).

Currently, knowledge of bacterial communities contributing to cathodic denitrification is limited, we collected biofilm samples from four cathodic denitrifying reactors- one operated in loop format and three in non-loop format- with two primary objectives. Our first aim was to expand the known diversity of bacteria active in cathodic denitrifying biofilms. Our second aim was to investigate whether features of the microbial community could explain the increased performance of the loop reactor. We hypothesized that both differences in the phylogenetic affiliation of dominant bacterial members and the community structure contributed to the enhanced current production and nitrate removal rates in the loop-format reactor.

We used a high-density, phylogenetic microarray (PhyloChip) to characterize the bacterial communities (Brodie et al, 2006; Brodie et al, 2007). Application of the PhyloChip to cathode biofilm communities offered a higher-resolution analysis of the microbial community composition than previously reported methods (DeSantis et al, 2007; Wrighton et al, 2008), and thus the possibility of uncovering more diversity than was previously observed in these systems. We restricted our analysis to the active members of the communities by monitoring 16S rRNA (rather than the 16S rRNA gene), which is a more responsive biomarker and a better surrogate for microbial activity in bacterial communities (Lueders et al, 2004). Populations identified as dominant by PhyloChip were verified with Fluorescence *In Situ* Hybridization (FISH). This study represents an in-depth analysis of cathodic microbial communities and we leveraged this data to examine the relative importance of phylogenetic affiliation and community structure in MFC cathodic functionality.

Methods

Microbial fuel cell (MFC) design and operation. The validation of this technology has been previously demonstrated (Virdis et al, 2008; Clauwaert et al., 2007). Our goal was to complement the previous functional characterization by examining the bacterial community of four nitrate-amended cathodes powered by MFCs operated either in non-loop format (BNL1, BNL2, ANL) or loop format (AL). The Australian (A) reactors (Virdis et al, 2008) and Belgium (B) reactors (Clauwaert et al, 2007) were designed as denoted in respective references. The anode and cathode compartments were filled with granular graphite (diameter 1.5-6mm) contacted by graphite rods to the electrical circuit. The liquid volume between the graphite granules, the net cathodic compartment volume (NCC), was 182 mL (A) and 62 mL (B). Both anodic and cathodic solutions were recirculated at a rate of approximately 200 $mL{\cdot}min^{-1}$ (A) and

7 mL·min^{-1} (B) to maintain well-mixed conditions and avoid concentration gradients and clogging of the granular matrix.

To account for differences in reactor design and operation, we sampled the bacterial communities after current stabilization and approximately twenty-five days later upon renewed functional stabilization after sampling. For these experiments the AL reactor was operated for 260 days, the ANL 127 days, the BNL reactors for 74.5 days, during which the current in all gradually increased and reached a plateau. For this experiment, all anodes were inoculated with biomass from previously running MFCs amended with acetate. Anodes were fed with same modified M9 medium amended with acetate as previously described (Rabaey et al, 2005). Cathodes were fed with a modified M9 medium lacking NH_4Cl, with nitrate as the sole electron acceptor and carbonate as the exogenous carbon source. Cathodes were inoculated with a mixed denitrifying sludge treating wastewater from a sequencing batch reactor (A) or a mixture of aerobic and anaerobic sludge and sediment (B).

Analysis and electrochemical calculations. The voltage over the MFCs was monitored using a data acquisition unit (Agilent 34970A) every 60s. Calculations were performed according to previous reports (Clauwaert et al, 2007; Virdis et al 2008). The cathodic half-cell potentials of the A reactors were measured by placing an Ag/AgCl reference electrode (R201, BioAnalytical Systems) in the cathode compartment of each MFC. The potential of this reference electrode was assumed to be +197 mV versus (SHE). Polarization curves were performed for the whole MFCs using a PAR VMP-3 Potentiostat (Princeton Applied Research, USA), at a scan rate of 0.1 mV.s^{-1} and a prior open circuit potential period of 3 h. To identify differences between loop and non-loop operation, the denitrification process of the A cathodes was monitored by batch tests. Samples obtained from the liquid phase were immediately filtered with a 0.22 μm sterile filter. NO_2^- and NO_3^- were determined using a Lachat Quik Chem8000 Flow Injection Analyzer (FIA). N_2O was measured with a N_2O microsensor (Unisense A/S, Denmark). The total coulombs produced during batch tests were evaluated as the area beneath the current profile. Coulombic efficiency was calculated as the ratio of the coulombs produced and the coulombs injected as nitrate assuming complete reduction to dinitrogen (i.e., 5 e^- mol per mol NO_3^- to N_2).

RNA extraction and cDNA preparation. Graphite granules were removed from the reactors when current and denitrification rates stabilized. The graphite biofilms were extracted as described with the exception that Trizol was used in the place of CTAB extraction buffer (Wrighton et al, 2008). RNA samples were DNase treated using Ambion's Turbo DNase (Ambion, Texas). To confirm the purity of RNA, and lack of DNA contamination, PCR amplifications were performed using non-reverse transcribed DNAse-treated RNA as a control. Only samples demonstrating negative results (no amplification) were reverse transcribed to cDNA using Superscript II reverse transcriptase per the manufacturer's protocol (Invitrogen, California). Bacterial 16S rRNA genes were amplified from cDNA using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')n. PCR amplifications and PhyloChip hybridization, staining, and detection were performed as described previously (Wrighton et al, 2008).

PhyloChip analysis. Rank abundance curves were constructed using PhyloChip hybridization data to graphically illustrate richness and evenness of each sample. We define bacterial operational taxonomic units (OTUs) as a collection of closely related organisms (>97%) by full-length 16S rRNA gene similarity. Richness, or the presence of an OTU in a sample, is denoted when more than 90% the assigned probe pairs for the corresponding probe set were positive. Simpson's measure of evenness ($E_{1/D}$) was calculated for each sample using the statistical program R (R Development Core Team, 2008). This measure ranges from 0 to 1, with 0 representing complete dominance and 1 representing an evenly structured community. To visualize the similarity in bacterial communities associated with each reactor sample, Bray-Curtis non-metric multidimensional scaling (NMDS) and accompanying stress tests were performed on PhyloChip hybridization data using the statistical programs R and Primer V, with 20 iterations each. Hierarchical cluster analysis with average weight and ANOSIM in Primer V statistically confirmed NMDS clustering (Clarke et al, 1993). ANOSIM generates a test statistic, R, which specifies the amount of separation between groups. An R-value of 1.0 indicates complete separation of groups while an R-value of 0 indicates little or no separation. To identify OTUs enriched in each cluster, the hybridization intensities for reactors within each cluster were averaged and normalized. Identification of bacteria from the subtractive analysis was confirmed by using the similarity percentages (SIMPER) routine in Primer V, which detected the relative contribution of individual OTUs towards the dissimilarity between clusters. Comparisons of species scores on the NMDS plot further verified the contribution of Bacterial OTUs to the dissimilarity between cluster I and II.

Fluorescent *In situ* hybridization (FISH). FISH was performed directly on graphite granules with sample fixation, hybridization and washing performed as described previously (Amann et al, 1995). Anti-fade agent DABCO® was used to mount slides which were visualised using a Confocal laser scanning microscope (Zeiss LSM510). FISH probes for the discriminating taxa are listed (SI Table 1). For each sample the reported value is based on average of 3 samples with 3-5 fields of view per sample. The intensity of each FISH probe was normalized to the Bacterial domain probe to give accurate relative abundance of the probe to bacterial biomass. Given that FISH was intended to support the trends identified by PhyloChip, the relative abundance was averaged and this value is summarized by the following designations low (1-25%), medium (40-65%), and high (75-100%).

Results

Operation of cathodic denitrification

For the loop reactor, the current stabilized 131 ± 24 A m^{-3} NCC over the last forty days of operation, while the non-loop reactor produced a current of 69 ± 7 A m^{-3} NCC during the same time frame (Figure 2a). Both B reactors (BNL1 and BNL2), operated in a non-loop mode showed a similar profile as the ANL reactor, reaching a current of 67 ± 18 and 54 ± 20 A m^{-3} NCC over the active period of the systems (SI Figure 1). Despite any differences in design, operation, or inocula, the current densities between non-loop reactors operated in Belgium (BNL1 & 2) and Australia (ANL) are not statistically discernable. In contrast, the current density during continuous operation of the loop reactor (AL) was statistically superior to all three non-loop reactors. Regardless of operation type, the amount of current matched the expected electron flow for complete reduction of nitrate to dinitrogen gas.

Nitrogen removal and end products

The cathodic denitrification activities of the AL (Figure 3a) and ANL (Figure 3b) reactors were examined using batch tests performed during the last 40 days of reactor maintenance. The experiments consisted of a pulse injection of nitrate (target concentrations ~10 and ~20 mg NO_3^--N L^{-1}, respectively) and regular sampling for NOx analysis with on-line measurements of current and N_2O. The current generation of both systems was comparable during these batch tests, which eliminates current-dependent biases that could directly correlate to kinetic limitations. Polarization curves depict the voltage as a function of the current density whereas power curves represent the power as a function of the current density. Polarization and power curves represent an important tool for the characterization of the electrochemical performance of fuel cells (Logan et al, 2006). Figures 3c and 3d illustrate polarization and power curves for the AL and ANL reactors performed during the same period as the batch tests, respectively. The comparison between the curves obtained for the two types of configuration (loop and non-loop) also confirmed the superiority of the loop-reactor compared with the non-loop configuration. While AL reached up to 304.9 A·m^{-3} NCC, ANL achieved no more than 135.3 A·m^{-3} NCC. Higher power was produced by AL during polarization measurements (42.4 W·m^{-3} NCC at a current of 176.7 A·m^{-3} NCC) when compared to ANL (11.1 W·m^{-3} NCC at a current of 69.3 A·m^{-3} NCC).

At the time of sampling, N_2O production was detected in both the reactors, however the proportion of nitrous oxide to the nitrate injected was much greater in the AL (17.7%) versus the non-loop reactor (7.8%). This finding is consistent with replicated studies performed on the Australian reactors (Virdis, personal communication), which observed statistically higher N2O production on the loop-reactor than on the non-loop. Based on the nitrate consumption profiles shown in Figure 3, the A loop-reactor volumetric consumption rate was 0.198 kg N L^{-1} d^{-1}, while the non-loop reactor reached 0.139 kg N L^{-1} d^{-1}. These findings are consistent with nitrogen removal rates reported for loop and non-loop reactors (Virdis et al, 2009), which varied between 0.086 and 0.104 kg N L^{-1} d^{-1} for a MFC operating in non-loop configuration.

Bacterial community similarity

RNA was isolated from the four denitrifying cathodes (AL, ANL, BNL1, and BNL2). Two samples were selected from each reactor approximately 25 days apart (denoted T1 and T2), resulting in a total of eight samples. Purified RNA was converted to cDNA, PCR amplified, and communities analysed by PhyloChip. NMDS analysis visualized differences in the active bacterial communities between the samples (Figure 4). Samples that are spatially closer on the NMDS plot have more similar communities, while samples further apart are more dissimilar. The low stress value (0.01) indicates that the two-dimensional plot accurately represents the relationships between samples. The results from a hierarchical cluster analysis are superimposed on the NMDS to quantify the similarity in community composition between the reactors with two statistically significant ($R=0.887$, $p=0.018$; $p<0.05$) clusters discernable.

The differences between the clusters is best summarized along NMDS axis one with cluster I samples located at values less than 0 and cluster II samples located at values greater than 0.2. Cluster I contains five of the six non-loop reactor sample, indicating a shared overall bacterial community composition and distribution despite differences in reactor design, inoculum, or temporal sampling. Cluster II contained the AL reactor samples (AL_T1 and AL_T2) and time point two sample from ANL (ANL_T2). Interestingly, over time the ANL reactor shifted along axis 1 from cluster 1 (-0.03) to cluster II (0.28) (Figure 4, black arrow). This temporal crossover of ANL between the two clusters was indicative of a strong shift within the microbial community, which did not correspond to an observable difference in cathodic function.

Bacterial phylogenetic identification

To confirm the NMDS results and subsequently identify the phylogenetic diversity of these systems, we determined which bacteria most contributed to the clustering observed in Figure 4. A total of 79 OTUs most contributed to the dissimilarity between the clusters and are hereafter referred to as discriminating bacteria (Figure 5, SI Table 2), as these where most enriched in one cluster and constituted a minor portion of the other. FISH probes corresponding to the 16S rRNA gene sequences of these bacteria confirmed the PhyloChip identity and relative abundance trends (Table 2, SI Figure 2).

PhyloChip analysis revealed that Proteobacteria were enriched in cluster I while Firmicutes and Chloroflexi were enriched in cluster II. Strikingly, each of these phyla accounted for less than 5% of the OTUs in the alternative cluster (Figure 5a). The class level identification of the discriminating Proteobacteria and Firmicutes 16S rRNA sequences in each cluster is illustrated (Figure 5b). Proteobacteria account for 80% of the cluster I discriminating bacteria (63 of 79 taxa) with members of the Gammaproteobacteria (26 of 79 taxa, 33%), Alphaproteobacteria (17 of 79 taxa, 22%), and Betaproteobacteria (14 of 79, 18%) enriched relative to cluster II. Firmicutes account for 60% of the Cluster II discriminating taxa (47 of 79 taxa) with the Clostridia (27 of 79 taxa, 34%) and Bacilli (10 of 79 taxa, 13%) constituting the most enriched Firmicutes classes.

The overall phylogenetic breadth is greater in cluster II than cluster I. Members of the phyla Chloroflexi, Chlorobi, and Lentisphaerae were exclusively enriched in cluster II, with Chloroflexi accounting for a sizable portion of the community (11%, 9 of 79). Chloroflexi OTUs enriched in cluster II belong to the following families: group I or Anaerolineae (3 OTUs), group II or Dehalococcoides (2 OTUs) group IV (1 OTU), Thermomicrobia (1 OTU), and unclassified (2 OTUs). Contrasting to the discrete phylum-level clustering of the Chloroflexi, Firmicutes and Proteobacteria, other phyla were enriched equally in both clusters. Members of the Actinobacteria and Bacteriodetes had members enriched in both clusters, suggesting these phyla contain bacterial populations active in autotrophic denitrifying biofilms regardless of reactor operation method or inoculum.

A detailed identification of the 16S rRNA sequences that are most enriched in each cluster is provided in Table 1, with each OTU increased by at least one log in relative abundance relative to the other cluster. Members of the Gamma, Alpha, and Beta classes of the Proteobacteria are most enriched in cluster I. The 16S rRNA identification most closely related to the enriched PhyloChip OTU indicates an enrichment of previously identified bacterial denitrifying genre in cluster I samples. Specifically, *Rhizobium* (X67234), *Sphingopyxis* (AY554010), and *Zoogloea* (X74066) are enriched in cluster I. Cluster II was dominated by Firmicutes with 16S rRNA sequences most closely related to Clostridiaceae clones (AB089032, AB088983, AB100488, AB089035) from termite gut homogenate.

We performed FISH using probes designed for the discriminating bacteria, to confirm dominance of the PhyloChip identified populations in the clusters (Figure 5, Table 1), as well as validate the shift in community composition identified in the ANL reactor with time (Figure 4, black arrow). The FISH results agreed with the trends identified by PhyloChip, with cluster I (ANL_T1) dominated by Betaproteobacteria and Gammaproteobacteria and cluster II (ANL_T2) by Firmicutes and Chloroflexi. According to the FISH data, Betaproteobacteria represented approximately 45% of the bacterial community in T1 and decreased to 15% in T2 (SI Figure 2). Together the PhyloChip and FISH results demonstrate that members of the Proteobacteria, Firmicutes and Chloroflexi represent the most dominant members in the cathode biofilms with selective enrichment of Proteobacteria in cluster I and Firmicutes and Chloroflexi in cluster II.

Cluster II samples (ANL_T2, AL_T1, and AL_T2) shared similar dominant members, yet there was a significant functional difference between the performance of these reactors (see discussion), with the loop reactor having a higher current and a greater proportion of nitrate converted to nitrous oxide than the non-loop reactor. Therefore, we wanted to identify bacterial OTUs within cluster I that could be associated with the increased formation of nitrous oxide in the loop reactor and were not enriched in ANL_T2 sample. The production of nitrous oxide results from several microbial processes including incomplete denitrification by denitrifying bacteria, normal nitrifier-nitrification producing small quantities, or high levels by nitrifying populations via partial-denitrification processes under oxygen-limited conditions (Colliver and Stephenson 2000; Schmidt et al, 2004). The latter pathway, known as nitrifier-denitrification, has been demonstrated in environments similar to the cathode chamber, characterized by low oxygen and organic carbon concentrations.

Unlike the 16S rRNA of denitrifying bacteria, chemolithotrophic nitrifying Bacteria belong to coherent phylogenetic and functional groups (Kowalchuk and Stephen, 2001) and thus could be assessed with this molecular analysis. To ascertain the presence and abundance of autotrophic nitrifiers in the loop reactor, a subtractive analysis was performed. PhyloChip analyses revealed that populations of ammonia (both aerobic and anaerobic) and nitrite oxidizing populations were enriched in the loop reactor relative to the non-loop reactor at time 2

(SI Figure 3). Bacteria enriched in the loop system included members of the Nitrosomonadaceae, Nitrospiraceae, Nitrospira, and Annamoxales, with members of the Nitrosomonadacaeae demonstrating a significant enrichment (>1 log increase) in the loop reactors.

Community structure of denitrifying cathodic biofilms

Our second hypothesis was that differences in community structure corresponded to differences in reactor function. This included assessment of richness (number of bacterial OTUs) and evenness (distribution of bacterial abundances) for each sample. While a common practice in ecological studies for visualizing community structure and diversity, our analysis represents the first evaluation of rank abundance curves for electrode-associated communities. Rank abundance curves were created by plotting hybridization intensity data (arbitrary units) for each bacterial OTU, ranked from highest to lowest hybridization intensity, corresponding to decreasing relative abundance. The richness, or the number of bacterial OTUs detected in each sample is denoted in Figure 6 as the number of ranked OTUs on the x-axis. Evenness is accounted for in the initial slope of the curve, with a more uneven (dominant) community reflected by exponential decrease in shape while an even community is represented by more linear sloped line.

Of the 8743 resolvable OTUs on the PhyloChip, we detected 1614 in at least one of the eight samples. Our data show that when compared to non-loop reactors, the loop reactor is capable of maintaining average of 1143 bacteria at the OTU level, nearly 48% more bacterial OTUs than the non-loop reactors (Figure 6, x-axis). Despite the lower richness in the non-loop reactor samples, the number of active OTUs maintained at the final time point was well replicated (286±43, n=3) within the non-loop reactors irrespective of differences in reactor design, location, or the initial inoculum in the non-loop reactors.

In addition to conveying differences in sample richness, rank abundance curves illustrate changes in the distribution of bacterial OTUs in the reactor samples over time. Similar to the richness data, the slope of the non-loop reactors (BNL1, BNL2, ANL) is more similar to each other at time point 2 than the AL reactor, indicating a temporal convergence in community structure between reactors operated in a similar fashion. In addition to decreased richness in the non-loop reactors, the slope of the rank abundance curve is greater than the loop reactor samples, indicating a community with increase presence of dominant OTUs. To confirm the trends observed by visual interpretation of the curves, for each sample we calculated Simpson's measure of evenness. The AL reactor had the greatest overall evenness (0.91, T1 and 0.89, T2), while the non-loop reactors resulted in more dominantly structured communities (0.81±0.02, n=6). Together, our results demonstrate that the loop reactor has an increased number of active bacterial OTUs accompanied by a greater evenness relative to the non-loop reactors (Figure 6), suggesting a greater overall diversity in these systems.

Discussion

Identification of bacteria active and dominant in denitrifying biofilms

The first objective of our study was to characterize the bacterial phylogenetic membership of denitrifying biocathode biofilms. PhyloChip and FISH results confirmed the dominance of Proteobacteria, Firmicutes and Chloroflexi in our reactors, signifying that future research dedicated to the functional importance of these bacteria is warranted. The enrichment of Gammaproteobacteria, Betaproteobacteria and Firmicutes has been detected in previous studies characterizing denitrification from waste treatment systems (Knowles et al, 1982). More significant, the dominance of the Proteobacteria in five of the six non-loop reactors is consistent with identification in the two previously published non-loop denitrifying biocathodes studies (Park et al, 2006; Gregory et al, 2005). While 16S rRNA does not indicate physiological function, the congruence between multiple community data sets from different inoculum, sampling regimes, and reactor designs suggests a functional role for members of the Gammaproteobacteria and Betaproteobacteria in cathodic denitrifying biofilms.

Broad scale phylogenetic analysis revealed a shared enrichment of Proteobacteria in non-loop operated denitrification cathodes, however, higher resolution analysis at the OTU level revealed differences in these systems. With the exception of ANL_T2, our non-loop reactors samples were enriched in members of the order Burkholderiales of the Betaproteobacteria (10 of 14 taxa, 71%). Park et al (2006) also identified a member of the Burkholderiales as dominant in denitrifying cathodes. The former study used 16S rRNA gene analysis with DGGE and FISH to speculate that members from the family Burkholderiaceae most closely related (97%) to *Burkholderia symbiont* of *Asellus aquaticus* were abundant in denitrifying biofilms. In our study, Burkholderiaceae represented 16% of the most enriched Betaproteobacteria sequences in cluster I, while members of the Comamonadaceae were the most dominant (50%). Interestingly members of the Comamonadaceae have been demonstrated to denitrify chemolithotrophically, by oxidizing iron minerals coupled to the reduction of nitrate (Straub et al, 2004; Kappler et al, 2005; Kappler and Straub, 2005).

In addition to the Betaproteobacteria, our non-loop cathodes were also enriched in Gammaproteobacteria and to a lesser extent Deltaproteobacteria. This finding is similar to clone library results conducted on denitrifying biocathodes inoculated with marine sediment (Gregory et al, 2004), which revealed the enrichment of 16S rRNA sequences related to *Geobacter* (Deltaproteobacteria) and *Thermomonas* (Gammaproteobacteria) *species*. It was also demonstrated in this study that a pure culture of *Geobacter metallireducens* used the cathode as an electron donor for nitrate reduction. While PhyloChip has been shown to accurately monitor the relative abundance of Geobacteraceae populations (Wan et al, 2005), *Geobacter* species were not significantly enriched during our study (1%, 1 of 79 discriminating OTUs).

Compared to the Proteobacteria, much less is known regarding the role of Firmicutes or Chloroflexi in biocathode systems. Part of this discrepancy could be attributed to the fact that these bacteria were enriched mainly in the loop-operated cathode and this study represents the first characterization of cathodes powered by anode current. In our reactor system, members of the Chloroflexi accounted for 11% of the dominant community in the AL samples and ANL_T2. Chloroflexi sequences have been identified in studies from wastewater systems and even chemolithotrophic denitrification (Fernández et al, 2008). Despite their abundance in molecular surveys, knowledge about Chloroflexi physiology is scarce (Krangelund, 2007), yet isolated members of the Chloroflexi have been demonstrated to reduce nitrate to nitrite (Kohno, 2002).

In our study, PhyloChip and FISH results show the enrichment of the Firmicutes in cluster II samples. Furthermore, Clostridiaceae constituted the most dominant members in cluster II, with sequences most closely related (>97%) to *Clostridium leptae* being significantly enriched. However, given the concerns regarding the current taxonomic structure of the traditional genus *Clostridium* and the family Clostridiaceae in general (Wiegel et al, 2006), the identity of these sequences to genus level must be taken with some caution.

Despite the fact that Firmicutes are known to denitrify heterotrophically (Knowles, 1982), little is known about their role in chemolithotrophic denitrification processes. A recent 16S rRNA gene based analysis from a denitrifying reactor with sulfur as an electron donor noted that Firmicutes accounted for 13% of the clone library diversity (Fernández et al, 2008), with approximately 11% of the community composed of *Clostridium species.* Likewise, a 16S rRNA gene DGGE community analysis of denitrifying biofilm reactor communities with hydrogen as an electron donor also noted the significant dominance of *Clostridium spp*. (Park et al, 2005). Additionally, Firmicutes 16S rRNA has been detected in community analyses of denitrifying biocathodes. Sequences similar to *Bacillus vedderi* were identified as dominant member of biocathode communities by Park et al (2006), while Gregory et al (2004) noted that Grampositive bacteria were enriched in denitrifying biocathode communities and not in no-current controls, unfortunately taxonomic identification of these bacteria was not provided.

The role of Firmicutes on biocathodes deserves further consideration, as members of the Firmicutes, most notably members of the genus *Clostridium* (senso stricto), are generally considered obligate fermenters. Yet, nitrate reduction has been demonstrated by multiple *Clostridium* species (Hass and Hall, 1977; Caskey et al, 1979; Caskey et al, 1980; Keith et al, 1983). As such it is possible that *Clostridium spp*. are active in the denitrification process in our reactor systems. Given the consistency to other autotrophic denitrifying systems, operation of the reactors in flow-through for over 160 days, the use of 16S rRNA as a biomarker, and the significant enrichment of the Firmicutes in reactors regardless of operation suggests an important functional role for these bacteria in cathodic denitrifying biofilms.

In addition to the dominant bacteria, our analysis also demonstrated the enrichment of members of the *Nitrosomonas spp*. exclusively in the loop reactor over time. The maintenance of sequences similar to *Nitrosomonas* over time in the loop system is particularly of interest, as the ability to reduce nitrite to nitrous oxide under anaerobic conditions appears to be a universal trait in these populations (Poth and Focht, 1985; Shaw et al, 2006). Given the rapid turnover rate of 16S rRNA and the significant enrichment of ammonia oxidizing *Nitrosomonas spp*. relative to nitrite oxidizing *Nitrospira spp*. (SI Figure 2), suggests that *Nitrosomonas spp*. may be more than an immigration artifact from the nitrifying reactor and could be functioning in a denitrifying fashion in the loop system. However, this line of evidence does not preclude the activity of other denitrifying bacterial populations, the role of ammonia oxidizing Archaea, or the incomplete dissimilatory reduction of nitrate in the reactor biofilm. Ongoing studies are exploring the ecological role of nitrifying bacteria and Archaea in denitrifying biocathode systems to better understand the populations correlated to the increased nitrous oxide in denitrifying loop-operated biocathodes.

Lack of relationship between dominant members and reactor performance

The performance of the loop reactor in terms of current production and rates of nitrogen removal was notably superior to the three non-loop reactors during this time course. PhyloChip and FISH identified changes in the phylogenetic composition of the ANL reactor that resulted in sample ANL_T2 showing a similar phylogenetic membership of dominant bacteria to the AL samples. Contrary to our initial hypothesis, the phylogenetic affiliation of the most discriminating bacterial members was not associated with the increased performance of the loop reactor, as Firmicutes and Chloroflexi were dominant members of both loop and non-loop reactors.

The lack of correspondence between biological composition and reactor function could be justified using several lines of reasoning. We have considered that perhaps biological change pre-empts operational change and thus at a later time period changes in function would be detectable, with the ANL_T2 producing increased current and more nitrous oxide. We have also considered that it is possible that the discriminating bacteria simply are not functionally relevant to denitrification reactions in these systems and thus changes in the abundance of these populations has no effect on reactor performance. However, since 16S rRNA is considered a proxy for bacterial activity, these bacteria are significantly enriched in the biofilm community, and since this data is consistent with described ecological role of these bacteria as putative denitrifiers, we find these explanations unlikely. Alternatively, we propose that the replacement of Proteobacteria with Firmicutes in the ANL reactor over time without a corresponding change in operation indicates these bacteria are functionally redundant and thus a change in composition does not affect reactor performance (Fernandez et al, 2000; Wohl et al, 2004).

Community structure corresponds to reactor performance

Our study is the first experiment to evaluate differences in community structure (richness and evenness) in either anodic or cathodic microbial fuel cell communities. Although we restricted our analysis to only the most active community members (16S rRNA) and previous studies relied on persistent and active members (16S rRNA gene), the use of the PhyloChip uncovered significantly greater number of OTUs than was reported in prior bioelectrochemical system studies. In comparison to the richness described here (Figure 6, x-axis), only four dominant DGGE bands (Park et al, 2006) or two dominant genera (Gregory et al, 2004) were identified in other studies. Reasons for this discrepancy probably have less to do with vast differences in community richness associated with our reactors, but are more likely attributed to the limitation of the technique (DGGE) or small sampling regimes (<90 clones) that failed to capture a large fraction of the bacterial diversity of the biofilm. As a result of using 16S rRNA as a biomarker, rather than 16S rRNA gene, our results suggest that a much greater diversity of bacteria are not only present but also active members in cathodic denitrifying reactors.

Relative to the non-loop reactors, the loop reactor had a greater number of OTUs, greater evenness, a greater phylogenetic diversity of discriminating taxa and consequently greater overall diversity. These findings support our second hypothesis and demonstrate that changes in the community structure correspond to the functional superiority of the loop reactor. As a corollary, our findings suggest that operation of reactors in non-loop format results in a reduced bacterial diversity of the active communities and may have implications on the functional performance of these reactors.

While 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 macro-organisms (McNaughton 1977; Tilman et al, 2006). Relationships between bacterial diversity and system function are only beginning to be examined for bacterial communities (Yin et al, 2000, Bell et al, 2005, Wittebolle et al, 2009). In our reactors, the active bacterial taxa richness was positively correlated ($p<0.05$) with current production. This finding is consistent with earlier research demonstrating a relationship between increasing bacterial diversity and community respiration rates (Bell et al, 2005). Additionally, it has recently been demonstrated that increased evenness of communities relates to increased ecosystem function and stability in bacterial denitrifying communities with equivalent richness (Wittebolle et al, 2009).

It is possible that the increased overall diversity, in terms of richness and evenness, of the loop reactor was related to the increased performance of this reactor. In the loop reactor the increased bacterial diversity may be a consequence of increased resource diversity as influent from the nitrifier reactor may have expanded the number of niches, allowing this reactor to support greater bacterial diversity. This supposition is supported by the fact that the non-loop reactors converged to similar level of richness regardless of differences in inoculum or reactor design (Figure 6). The increased diversity of the loop reactor could have also resulted in greater functional redundancy within trophic groups, lending to a greater stability and performance of this reactor. Alternatively, it could be argued that immigration from the nitrifying reactor was partly responsible for an artificial elevated diversity of the loop reactor, or a diversity that did not reflect populations active in the biofilm. However, given the rapid turnover of 16S rRNA rather than 16S rRNA gene, consistency in diversity measures over time, and the operation of the reactors in flow-through mode we expect populations that were inactive in the AL reactor to be below detection. These findings demonstrate, for the first time in anodic or cathodic BESs, the potential link between community structure and function and suggest that in order to optimize the bacterial component of these systems, future studies elucidating the relationships between bacterial OTU richness and evenness, phylogenetic diversity, and system performance are necessary.

Conclusions

Our results demonstrate that denitrifying biocathodes can sustain a far greater number of active bacteria OTUs than indicated in previous studies. The performance of the loop reactor in terms of current production and rates of nitrogen removal was notably superior to the three non-loop reactors, and the loop system also contained a greater bacterial OTU richness and evenness. PhyloChip and FISH analyses using 16S rRNA indicated that members of the Proteobacteria and Firmicutes were dominant and active members of the cathodic denitrifying biofilms. However, our analyses suggest that it was the structural aspect of a microbial community, in terms of richness and evenness, rather than the phylogenetic composition, which corresponded best to the elevated performance of the loop reactor. Together our results provide the first characterization of active bacterial communities in denitrifying cathodes. This research also provides a framework for future ecological and physiological microbial research in these systems.

Acknowledgements

We thank Cameron Thrash for discussions pertaining to bacterial physiology as well as Eoin Brodie and Todd DeSantis for informative discussions pertaining to the analysis of PhyloChip data. Funding for KW is through the UC Berkeley Chang Tien Fellowship for Biodiversity research. RAD was supported under a STAR Research Assistance Agreement No. FP-916933 awarded by the U.S. Environmental Protection Agency. BV was supported by the international Ph.D. program in Engineering and Environmental Sciences granted by the University of Cagliari, Italy. BV, SR, and KR thank the Australian Research Council for funding (Grant DP0666927 and DP0879245).

Figure Legends

Figure 1. Schematic of the a) loop operated and b) non-loop operated reactors.

Figure 2a) Current profiles over time for the Australian reactors. Time (days) for the ANL and AL axis is summarized on the bottom and top x-axes respectively. The arrows indicate the biological sampling time points.

Figure 3. Evolution of current (i), nitrate (NO_3^-), nitrite (NO_2^-), and nitrous oxide (N_2O) during denitrification batch experiments performed on the Australian loop-reactor (graph a) and non-loop reactor (graph b). Graph (a) was redrawn after Virdis et al, 2008. Figures (c) and (d) refer to polarization curves and power curves measured at the same period that the batches were performed.

Figure 4. Non-metric Multidimensional Scaling (NMDS) output of PhyloChip hybridization intensity data. NMDS scores for each sample are denoted with filled circles. Statistically different clusters ($p<0.05$) are identified by triangles with the cluster number identified in bold centrally. Cluster I includes samples B1_T1, B1_T2, B2_T1, B2_T2 and ANL_T1 while cluster II includes samples AL_T1, AL_T2, and ANL_T2. Black arrow indicates a shift in the community of the ANL reactor with time.

Figure 5. a) Distribution of the major phyla in the discriminating OTUs in each cluster. b) The class level abundance of two most dominant phyla, Proteobacteria and Firmicutes, in the two clusters.

Figure 6. Rank abundance curves at time point 1 and 2 for the a) Australian loop and non-loop reactors and b) Belgium non-loop reactors. The distribution is graphed with hybridization intensity (relative abundance) on the y-axis and the OTU rank on the x-axis. Rank abundance curves visually represent taxa richness (number of OTUs ranked on the x-axis) and evenness (slope of line) in each of the samples.

Table 1: Phylogenetic identity of the ten most dominant OTUs in each cluster. Each OTU is increased (>1 log) in relative abundance. The accession number corresponds to the 16S rRNA sequence of the probe.

Table 2: Changes in relative abundance of discriminating taxa relative to general Bacteria 16S rRNA probe using FISH in samples ANL_T1 and ANL_T2. Low, medium, and high correspond to a relative abundance of 1-25%, 40-65%, and 75-100% respectively.

Table 1

Phylum	Class	Order	Family	Probe Accession
Cluster I				
Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	X74066
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	X67234
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	X74913
Proteobacteria	Gammaproteobacteria	Unclassified	Unclassified	AJ296549
Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	AJ010297
Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified	
Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	AX554010
Proteobacteria	Gammaproteobacteria	Unclassified	Unclassified	
Proteobacteria	Alphaproteobacteria	Azospirillales	Unclassified	AF524861
Cluster II				
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	AB089032
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	AB088983
Firmicutes	Desulfotomaculum	Unclassified	Unclassified	AB091324
Firmicutes	Mollicutes	Anaeroplasmatales	Erysipelotrichaceae	
Chloroflexi	Thermomicrobia	Unclassified	Unclassified	AY250886
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	AB089035
Firmicutes	Mollicutes	Anaeroplasmatales	Erysipelotrichaceae	AY133091
Chloroflexi	Anaerolineae	Unclassified	Unclassified	AF507690
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	

Table 2

Target	ANL_T1	ANL_T2
Alphaproteobacteria	Low	Low
Betaproteobacteria	Med	Low
Deltaproteobacteria	Low	Low
Gammaproteobacteria	High	Medium
Firmicutes	Low	Medium
Chloroflexi	Low	Medium

References

Amann, R. I., W. Ludwig and K. H. Schleifer (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143-169.

Bell, T., J. A. Newman, B. W. Silverman, S. L. Turner and A. K. Lilley (2005) The contribution of species richness and composition to bacterial services. *Nature* **436**: 1157-1160.

Brodie, E. L., T. Z. DeSantis, D. C. Joyner, S. M. Baek, J. T. Larsen, G. L. Andersen, T. C. Hazen, P. M. Richardson, D. J. Herman, T. K. Tokunaga, J. M. Wan and M. K. Firestone (2006) Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. *Appl Enviro Microbiol* **72**: 6288-6298.

Brodie, E. L., T. Z. DeSantis, J. P. M. Parker, I. X. Zubietta, Y. M. Piceno and G. L. Andersen (2007) Urban aerosols harbor diverse and dynamic bacterial populations. *PNAS* **104**: 299-304.

Blo, M and E.E. Roden (2009) Composition and activity of an autotrophic Fe(II)-Oxidizing, nitrate-reducing encrichment culture. *Appl Enviro Microbiol* **75**: 6937-6940

Clauwaert, P., Rabaey, K., Aelterman, P., DeSchamphelaire, L., Pham, T. H., Boeckx, P., Boon, N., Verstraete, W. (2007) Biological denitrification in microbial fuel cells. *Environ Sci Technol* **41**: 3354-3360.

Clarke KR (1993). Non-parametric multivariate analysis of changes in community structure. *Australian Journal of Ecology* **18:** 117-143.

Colliver, B. B. and T. Stephenson (2000) Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. *Biotechnol Adv* **18**: 219-232.

Demaneche, S., L. Philippot, M. M. David, E. Navarro, T. M. Vogel and P. Simonet (2009) Characterization of denitrification gene clusters of soil bacteria via a metagenomic approach. *Appl Enviro Microbiol* **75**: 534-537.

DeSantis, T., E. Brodie, J. Moberg, I. Zubieta, Y. Piceno and G. Andersen (2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microb Ecol* **53**: 371-383.

Fernandez, A. S., S. A. Hashsham, S. L. Dollhopf, L. Raskin, O. Glagoleva, F. B. Dazzo, R. F. Hickey, C. S. Criddle and J. M. Tiedje (2000) Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. *Appl Enviro Microbiol* **66**: 4058-4067.

Fernández, N., R. Sierra-Alvarez, J. A. Field, R. Amils and J. L. Sanz (2008) Microbial community dynamics in a chemolithotrophic denitrification reactor inoculated with methanogenic granular sludge. *Chemosphere* **70**: 462-474.

Gregory, K. B., D. R. Bond and D. R. Lovley (2004) Graphite electrodes as electron donors for anaerobic respiration. *Environ Microbiol* **6**: 596-604.

Gregory, K. B. and D. R. Lovley (2005) Remediation and recovery of uranium from contaminated subsurface environments with electrodes. *Environ Sci Technol* **39**: 8943-8947.

Kragelund, C., C. Levantesi, A. Borger, K. Thelen, D. Eikelboom, V. Tandoi, Y. Kong, J.V. Waarde, J. Krooneman, S. Rossetti, T.R. Thomsen, P.H. Nielsen (2007) Identity, abundance and ecophysiology of filamentous *Chloroflexi* species present in activated sludge treatment plants. *FEMS Microbiol Ecol* **59**: 671-682

Kohno T, Sei K & Mori K (2002) Characterization of Type 1851 organism isolated from activated sludge samples. *Water Sci Technol* 46: 111–114.

Kowalchuk, G. A. and J. R. Stephen (2001) Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annu Rev Microbiol* **55**: 485-529.

Knowles, R (1982) Denitrification. *Microbiological Reviews* **46**:43-70

Logan, B.E., B. Hamelers,, R. Rozendal,', U. Schröder,, J. Keller,, S. Freguia,, P. Aelterman,, W. Verstraete, and K. Rabaey (2006) Microbial Fuel Cells: Methodology and Technology. *Environ Sci Technol* **40**: 5181-5192

Lueders, T., B. Wagner, P. Claus and M. W. Friedrich (2004) Stable isotope probing of rRNA and DNA reveals a dynamic methylotroph community and trophic interactions with fungi and protozoa in oxic rice field soil. *FEMS Microbiol Lett* **6**: 60-72.

McNaughton, S. J. (1977) Diversity and stability of ecological communities: a comment on the role of empiricism in ecology. *Am Nat* **111**: 515-525.

Park, H. I., J. S. Kim, D. K. Kim, Y.-J. Choi and D. Pak (2006) Nitrate-reducing bacterial community in a biofilm-electrode reactor. *Enzyme Microb Tech* **39**: 453-458.

Park, H.I., Y.J. Choi, and D. Pak. (2005) Autohydrogenotrophic denitrfying microbial community in a glass beads biofilm reactor. *Biotechnol Lett* **13**:949-953.

Poth, M. and D. D. Focht (1985) 15N kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. *Appl Environ Microbiol* **49**: 1134-1141.

R Development Core Team. (2008). R Foundation for Statistical Computing: Vienna, Austria.

Rabaey, K., S.T. Read, P Clauwaert, S. Freguia, P.L. Bond, L.L. Blackall and J. Keller (2008) Cathodic oxygen reduction catalyzed by bacteria in microbial fuel cells. ISME J: 1-9

Schmidt, I., R. J. van Spanning and M. S. Jetten (2004) Denitrification and ammonia oxidation by *Nitrosomonas europaea* wild-type, and NirK- and NorB-deficient mutants. *Microbiology* **150**: 4107-4114.

Shaw, L. J., G. W. Nicol, Z. Smith, J. Fear, J. I. Prosser and E. M. Baggs (2006) *Nitrosospira spp*. can produce nitrous oxide via a nitrifier denitrification pathway. *Environ Microbiol* **8**: 214-222.

Straub, K.L., W.A. Scho, B.E. E. Buchholz-Cleven, and B. Schink (2004) Diversity of ferrous iron-oxidizing, nitrate-reducing bacteria and their involvement in oxygen-independent iron cycling. *Geobiology* **6**:371-378.

Strycharz, S. M., T. L. Woodard, J. P. Johnson, K. P. Nevin, R. A. Sanford, F. E. Loffler and D. R. Lovley (2008) Graphite electrode as a sole electron donor for reductive dechlorination of tetrachlorethene by *Geobacter lovleyi*. *Environ Sci Technol* **74**: 5943-5947.

Thrash, J. C. and J. D. Coates (2008) Review: direct and indirect electrical stimulation of microbial metabolism. *Environ Sci Technol* **42**: 3921-3931.

Thrash, J. C., J. I. VanTrump, K. A. Weber, E. Miller, L. A. Achenbach and J. D. Coates (2007) Electrochemical stimulation of microbial perchlorate reduction. *Environ Sci Technol* **41**: 1740-1746.

Tilman, D., P. B. Reich and J. M. H. Knops (2006) Biodiversity and ecosystem stability in a decade-long grassland experiment. *Nature* **441**: 629-632.

Virdis, B., K. Rabaey, Z. Yuan and J. Keller (2008) Microbial fuel cells for simultaneous carbon and nitrogen removal. *Water Res* **42**: 3013-3024.

Virdis, B., K. Rabaey, Z. Yuan, R.A. Rozendal, and J. Keller (2009) Electron fluxes in a microbial fuel cell performing carbon and nitrogen removal. *Enviro Sci Tech* **42**: 3013-3024.

Wan, J., T. K. Tokunaga, E.L Brodie, Z. Wang, Z. Zheng, D. Herman, T. C. Hazen, M. K. Firestone and S. R. Sutton (2005) Reoxidation of bioreduced uranium under reducing conditions. *Environ Sci Technol* **39**: 6162-6169.

Weber KA, Achenbach LA, & Coates JD (2006) Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nat Rev Micro.* **4:** 752-764

Weber, K. A**.**, F. W. Picardal, E. E. Roden (2001) Microbially Catalyzed Nitrate-Dependent Oxidation of Biogenic Solid-Phase Fe(II) Compounds. *Environ Sci Technol* **35**:1644-1650

Wiegel, J., R. Tanner, F.A. Rainey. The Clostridiaceae. In Prokaryotes volume 4; M. Dworkin, S. Falkow, E. Rosenberg, K.H. Shliefer, E. Stackebrandt Eds.; Springer: New York 2006; pp654-678.

Wittebolle L., M. Marzorati, L. Clement, A. Balloi, D. Daffonchio, K. Heylen, P.D. Vos, W. Verstraete, N. Boon (2009) Initial community evenness favours functionality under selective stress. *Nature* **458**:623-626

Wohl, D. L., S. Arora and J. R. Gladstone (2004) Functional redundancy supports biodiversity and ecosystem function in a closed and constant environment *Ecology* **85**: 1534-1540

Wrighton, K. C., P. Agbo, F. Warnecke, K. A. Weber, E. L. Brodie, T. Z. DeSantis, P. Hugenholtz, G. L. Andersen and J. D. Coates (2008) A novel ecological role of the Firmicutes identified in thermophilic microbial fuel cells. *ISME J* **2**: 1146-1156.

Yin, B., D. Crowley, G. Sparovek, W. J. De Melo and J. Borneman (2000) Bacterial functional redundancy along a soil reclamation gradient. *Appl Enviro Microbiol* **66**: 4361-4365.