A bioelectrical system (BES) for enriching Fe(III)-reducing bacteria

Part II of the final project report for Kate Campbell – July 2010

Introduction

Bioelectrical systems (BES) (also known as bioelectrical reactors, BERs) have recently been explored as a versatile class of techniques to culture organisms, determine biochemical pathways and kinetics, test bioremediation, and generate current through microbial fuel cells (MFCs) [1-3]. Based on the idea of a classical electrochemical cell, there is an inherent flexibility in the range of processes and applications for BESs. The most widely studied BES is a MFC, where bacteria are given an electron donor while the terminal electron acceptor (TEA) is the working electrode surface, resulting in current generation. In this study, the reverse process will be explored; namely, the ability of a working electrode to donate electrons to bacteria by applying a constant voltage across the cell with a potentiostat. The goal is to determine whether the application of a voltage in the presence of iron oxides as the TEA can be used as an enrichment technique for Fe(III) reducing bacteria from an environmental inoculum. Ultimately, the objective of this work is to determine whether different bacterial metabolisms can be targeted by varying the applied voltage during enrichment in a BES.

Methods

Overview of the experiment. The BES system consisted of the anaerobic reaction vessel, the counter vessel, the salt bridge, working, counter and reference electrodes, and a potentiostat (Figure 1). The vessels were 200mL beakers with rubbers stoppers equipped to hold the appropriate electrodes and salt bridge. The potentiostat was used to poise the voltage between the working and counter electrodes at a constant voltage (-600mV), in reference to an Ag/AgCl electrode in the reaction vessel. The reaction vessel contained freshwater medium amended with iron oxide, the inoculum (1mL of fresh sediment from school street marsh (Woods Hole, MA)), one end of the salt bridge, and the working and reference electrodes. All holes in the reaction vessel were plugged to maintain anaerobic conditions to the greatest extent possible without being in an anaerobic chamber. The counter vessel contained the other end of the salt bridge and the counter electrode, and was left open to the atmosphere. Current through the system was measured by the potentiostat. The system was run for about 9.5 days without stirring to allow any biofilms that formed to remain undisturbed.

Analytics. Samples of the supernatant were taken vial a sampling port in the rubber stopper and analyzed for dissolved Fe(II) by the Ferrozine method [4]. At the end of the experiment, the reaction vessel was taken into the anaerobic chamber, and half of the graphite paper was cut from the working electrode to be analyzed by CARD-FISH (δ- and γ-proteobacterial probes, a non-specific binding probe, and Alexa 488 dye). The CARD-FISH probes were chosen because *Geobacter* species are in the δ-proteobacteria, and *Shewanella* species are in the γ-proteobacteria, both of which are known to be successful in microbial fuel cell applications. The HFO/medium in the reaction vessel was homogenized and sampled for total Fe(II) in triplicate by dissolving the HFO in 1% H_2SO_4. The remaining half of the

electrode was re-inserted into the reaction vessel. The microbes were killed with 1% paraformaldehyde, and the cell was reconnected to the potentiostat. Total Fe(II) was measured again after 4 days.

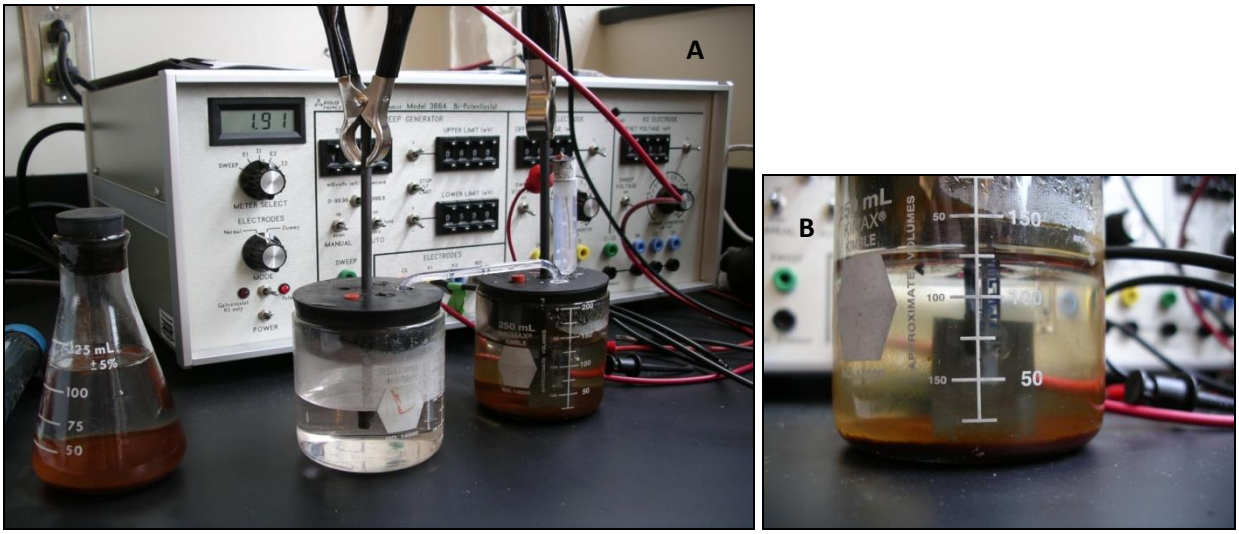
Freshwater medium and Hydrous Ferric Oxide (HFO). 500 mL of sterile freshwater medium without electron donor (organic carbon) was made by adding the appropriate amounts of each stock solution (5mL 100x freshwater base, 5mL 100x NH_4Cl, 0.5mL 1M Na_2SO_4, 0.5mL 1000x Na_2HPO_4, 0.5mL trace elements, 5mL MOPS pH 7.2 buffer, and 50µL 1000x vitamin solution). The electron acceptor added was hydrous ferric oxide (HFO), an amorphous iron oxyhydroxide, because it is considered to be the most bioavailable of the iron oxides due to its high surface area. The HFO was made by titrating a 0.05M ferric chloride solution to pH 8 with 0.1M NaOH, washing three times in 18 MΩ water by centrifugation, and resuspending in water [5]. The HFO was not autoclaved due to substantial mineralogical changes that occur when exposed to elevated temperature and pressure. The HFO was added to the freshwater medium for a final concentration of 0.7g/L HFO. The medium with HFO was then deoxygenated with N_2 in the reaction vessel for >1 hour. 100mL of freshwater medium without HFO was transferred to the counter vessel. This solution was not deoxygenated since the expected half reaction to occur in this vessel was splitting of water into H_2 and O_2.

Reference electrode and salt bridge. The reference electrode was an Ag/AgCl electrode kindly provided by Lars Angenent. The electrode consisted of KCl-saturated agar solution (>4M KCl) filling the inner electrode, connecting an Ag frit on the bottom of the electrode to an Ag wire inserted into the KCl/agar. The Ag wire was connected to the potentiostat. The salt bridge was necessary to maintain charge balance during the reaction. It was made by bending a 1mL glass pipette into a "U" shape and filling it with KCl-saturated agar.

Working and counter electrodes. The working and counter electrodes were kindly provided by Lars Angenent. The counter electrode was simply a graphite rod. The working electrode was a graphite rod with graphite paper attached to the end of the rod with conductive adhesive. Both electrodes were connected directly to the potentiostat.

Experimental controls. One control was established by adding freshwater medium with HFO to a flask, deoxygenating it, and adding a piece of graphite paper. No voltage was applied. Inoculum was added after 5 days so that the control could demonstrate that no Fe(II) was evolved from the abiotic reaction of HFO and graphite, as well as establish a background signal for Fe(II) and microbial community in the presence of an unpoised electrode surface. The control graphite paper was removed at the same time as the working electrode was divided, and analyzed via CARD-FISH.

Figure 1. Picture of the BES system. (A) The reaction vessel is on the right, the counter vessel is in the middle, and the control flask on the left. (B) A detail of the reaction vessel. The graphite paper was allowed to be in direct contact with the HFO on the bottom of the reaction vessel to facilitate electron transfer via a biofilm or electron shuttling compounds.



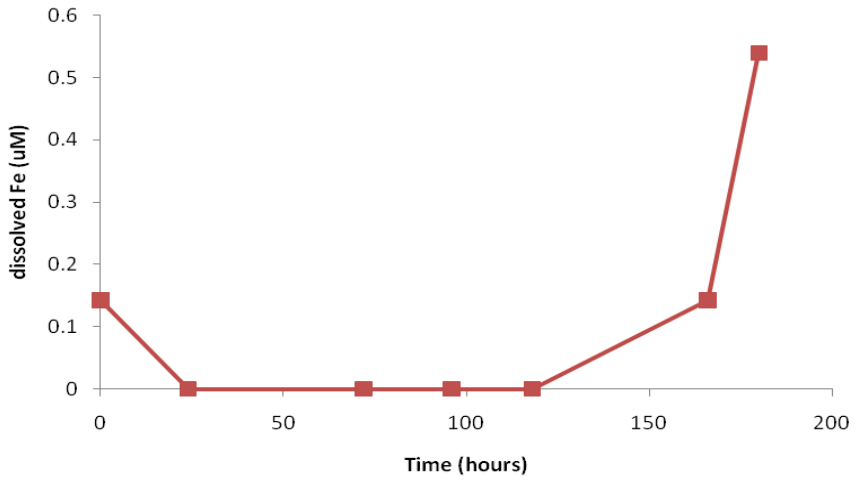
Results and Discussion.

The dissolved Fe(II) was intially detectable immediately after inoculation, but was quickly scavenged by the HFO, which has a high adsorptive capasity for Fe(II) (Figure 2). After 150 hours, the dissolved Fe(II) began to increase, indicating that Fe(III) was being reduced above the background signal. It should be noted that the second to last time point (~0.15 uM Fe(II)) is only slightly above the detection limit of the ferrozine method, and the dissolved Fe(II) signal is quite low, probably because the HFO has adsorbed most of the Fe(II) produced in the cell. Qualitatively, the color of the HFO was darker at the end of the reaction period, consistent with the production and adosrption of Fe(II). The total Fe(II) (dissolved + adsorbed) measured at the end of the incubaton period was 7.3 ± 0.4 µmol/g, compared to 0.5 ± 0.1 µmol/g in the control flask. There was no detectable total Fe(II) signal in the control flask without sediment. Although the control flask was reacted with sediment for a shorter amount of time than the reaction vessel, these results suggest that Fe(II) was produced in the reaction vessel as a result of the applied voltage.

Four days after the paraformaldehyde addition to the cell, the total Fe(II) was 5.2 µmol/g, which is significantly less than the total amount of Fe(II) measured at the end of the live incubation. This indicates that no additional Fe(II) was produced, but also that some Fe(II) was re-oxidized to Fe(III). This strongly supports the idea that the Fe(III) reduction was due to microbial activity rather than an abiotic artifact. Addionally, it suggests that the measured amount of Fe(II) produced above background is probably an underestimate of the actual amount of Fe(II) produced due to reoxidation of Fe(II), possibly by dissolved O_2 leaking into the reaction vessel. Future experiments will improve the experimental design to keep the reaction vessel anaerobic, ideally providing a quantiative measurement of Fe(III)

reduction. In addition, future experiments will run for a longer duration to have a higher Fe(II) signal compared to background.

Figure 2. Dissolved Fe(II) in the reaction vessel over the course of the incubation with an applied voltage of -600mV. At 180 hours, the cell was sampled for total Fe(II) after removing half of the working electrode for CARD-FISH analysis.

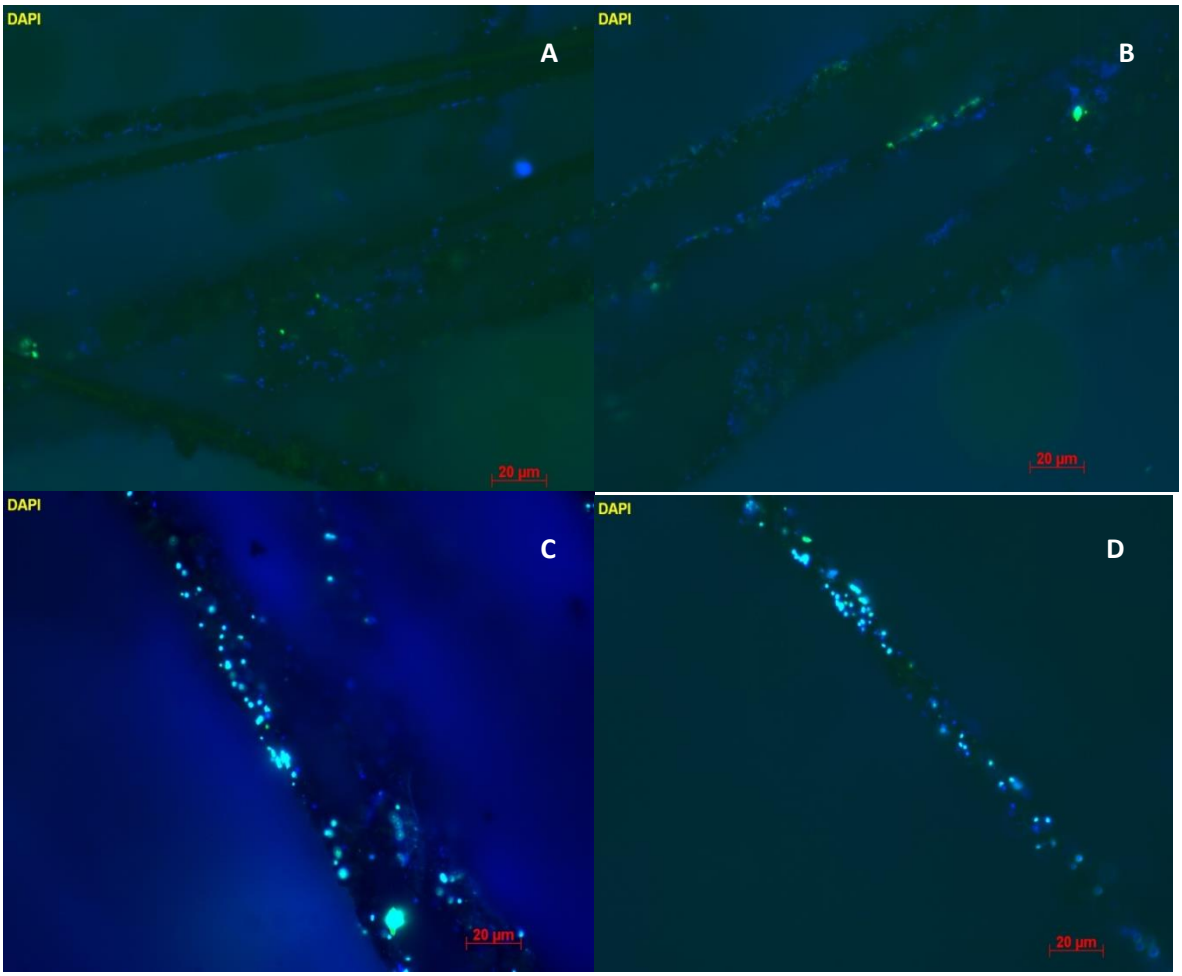


Current was measured periodically on the potentiostat as a way to monitor the progress of the reaction. However, there was substantial variability in current measurements, and even a diurnal cycle possibly related to temperature changes. Before inoculation, the current was 7 µA. Over the course of the incubation, the current rose from 80 µA after inoculation to a maximum of 250 µA. After the paraformaldehyde kill, the current decreased to between 100-150 µA. However, the variability of the current both before and after the kill makes a quantitative interpretation of the current difficult. Future experiments will ensure that the reaction is carried out under temperature-controlled conditions.

The CARD-FISH γ-proteobacterial probe sucessfully hybridized, while the δ-proteobacterial probe did not hybridize, based on the similarity of the signal to the non-specific probe signal. This was possibly due to an error in permeabilization temperature. The γ-probe signal was clear and significantly greater than background non-specific binding signal. The control graphite surface has qualitatively more cells (DAPI signal) attached than the working electrode surface, possibly indicating that the voltage applied to the electrode surface was harmful to the survival of many bacteria or prevented the colonization. The γ-probe signal does not overlap as strongly with the DAPI total cell count on the control graphite surface compared to the working electrode. In fact, there is substantial overlap between the γ-probe signal and the DAPI signal on the working electrode (Figure 3), suggesting an enrichment of γ-proteobacteria on the working electrode.

From this data set, it is not possible to identify community composition. However, the enrichment of γ-proteobacteria is evident from the CARD-FISH data. *Shewanella* species are classified as γ-proteobacteria, and future work will investigate whether this group has been enriched in the BES. Although there was no matches to Shewanella in the metagenome of school street marsh sediment, the signal from this group may be have been obscured in the metagenomic analysis, or the enriched organisms are a different group within the γ-proteobacteria. A comparison of clone libraries of the initial sediment, the control graphite surface, and the biofilm on the working electrode will be the method of choice for future experiments. Once a group of organisms has been identified as the target for the enrichment, specific CARD-FISH probes can be designed to visualize the electrode surface. In addition, a control electrode placed in the reaction vessel without voltage applied (no connection to the potentiostat) will be a more appropriate control sample.

Figure 3. CARD-FISH images of overlaid DAPI (blue) and the γ-proteobacterial FISH probe (green). (A) and (B) are two images of the control graphite paper; (C) and (D) are images of the working electrode after 9.5 days of incubation at -600mV. The strands of graphite paper are visible in the images, with the bacteria attached to the outside of the fibers; they appear to be attached only on the sides due to depth of focus of the microscope.



As a proof-of-concept, these experiments were sucessful in demonstrating that an applied voltage is effective at supplying bacteria with electrons, and that it can be used as an enrichment technique. Future experiments will be conducted to determine whether changing the voltage applied to the working electrode can select for different groups of organisms (e.g., Fe(III) reducers vs. sulfate reducers). This method potentially has many applications for simultaneous enrichments from sediment samples, *in situ* enrichments, and isolation of organisms with novel electron transport ability, such as nanowires.

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References

1. Thrash, J.C. and J.D. Coates, *Review: Direct and indirect electrical stimulation of microbial metabolism.* Environmental Science & Technology, 2008. **42**(11): p. 3921-3931.
2. Thrash, J.C., et al., *Electrochemical stimulation of microbial perchlorate reduction.* Environmental Science & Technology, 2007. **41**(5): p. 1740-1746.
3. Baron, D., et al., *Electrochemical Measurement of Electron Transfer Kinetics by Shewanella oneidensis MR-1.* Journal of Biological Chemistry, 2009. **284**(42): p. 28865-28873.
4. Stookey, L.L., *Ferrozine-A new spectophotometric reagent for iron.* Analytical Chemistry, 1970. **42**(7): p. 779-781.
5. Schwertmann, U. and R.M. Cornell, *Iron Oxides in the Laboratory*. 1991, Weinheim: Wiley-VCH.