

Using cathodic poised potential experiments to investigate extracellular electron transport in the crustal deep biosphere of North Pond, Mid-Atlantic Ridge

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

RJ and BNO formulated the original hypotheses and designed the project. All authors participated in sample collection. RJ performed the experiments, RJ and TD performed DNA extractions and bioinformatic analysis, and all authors participated in data analysis and interpretation. RJ and BNO wrote the paper with contributions from TD.

Keywords

deep biosphere, ocean crust, electromicrobiology, extracellular electron transfer, microbial fuel cells, Iron oxidation, North Pond

Abstract

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The crustal sub-seafloor covers a large portion of the Earth's surface but is poorly understood as a habitat for life. It is unclear what metabolisms support the microscopic cells that have been observed, and how they survive under resource limitation. As the deep crustal subsurface represents a significant portion of the Earth's surface, microbially-mediated reactions may therefore be significant contributors to biogeochemical cycling. In the present study, we used electrochemical techniques to investigate the possibility that crustal subsurface microbial groups can use the solid rock matrix (basalts, etc.) as a source of electrons for redox reactions via extracellular electron transfer (EET). Subsurface crustal fluids and mineral colonization experiments from the cool and oxic basaltic crustal subsurface at the North Pond site on the western flank of the Mid-Atlantic Ridge were used as inocula in cathodic poised potential experiments. Electrodes in oxic microbial fuel cells were poised at -200 mV versus a standard hydrogen electrode to mimic the delivery of electrons in an energy range equivalent to iron oxidation. In this way, microbes that use reduced iron in solid minerals for energy were selected for from the general community onto the electrode surface for interrogation of extracellular electron transfer activity, and potential identification by scanning electron microscopy and DNA sequencing. The results document that there are cathodic EET-capable microbial groups in the low biomass crustal subsurface at this site. The patterns of current generation in the experiments indicate that these microbial groups are active but likely not growing under the low-resource condition of the experiments, consistent with other studies of activity versus growth in the deep biosphere. Lack of growth stymied attempts to determine the phylogeny of EET-capable microbial groups from this habitat, but the results indicate that these microbial groups are a small part of the overall crustal deep biosphere community. This first demonstration of using electromicrobiology techniques to investigate microbial metabolic potential in the crustal deep biosphere reveals the challenges and opportunities for studying EET in the crustal deep biosphere.

Contribution to the field

This work presents original findings on the first use of bioelectrochemical methods to explore the presence of microbes capable of cathodic Extracellular Electron Transport (EET) to gain electrons for energy from solid substrate in the deep crustal biosphere of North Pond on the Mid-Atlantic Ridge. Our results indicate that there are EET-capable microbes present in this environment but that they are not a significant proportion of the microbial community of the deep basalt crust. We highlight which deep marine basalt crust groups show evidence of EET, and demonstrate the use of bioelectrochemistry as a tool to assist in understanding microbial chemolithotrophic energy sources in this ultra-oligotrophic, low biomass system. Considering how this study concerns the novel use of bioelectrochemical techniques to query the presence of EET-capable microbes in the deep marine crustal subsurface and how it relates to the community as a whole, we believe the subject of this study would be relevant to this special issue, and in Frontiers more generally.

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Inclusion of identifiable human data

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In review

Data availability statement

Generated Statement: The datasets generated for this study can be found in the Chronoamperometry, cyclic voltammetry, and scanning electron microscopy data are available from the Biological and Chemical Oceanography Data Management Office (BCO-DMO) at under project 707762: <https://www.bco-dmo.org/dataset/780261> (SEM), <https://www.bco-dmo.org/dataset/780127> (CA profiles), <https://www.bco-dmo.org/dataset/780248> (cyclic voltammetry profiles), and <https://www.bco-dmo.org/dataset/780225> (experimental metadata). 16S rRNA gene sequence data are available at the National Center for Biotechnology's Sequence Read Archive BioProject PRJNA564565 samples 274 SAMN12723345-SAMN12723415..

In review

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25

Abstract

The crustal sub-seafloor covers a large portion of the Earth's surface but is poorly understood as a habitat for life. It is unclear what metabolisms support the microscopic cells that have been observed, and how they survive under resource limitation. As the deep crustal subsurface represents a significant portion of the Earth's surface, microbially-mediated reactions may therefore be significant contributors to biogeochemical cycling. In the present study, we used electrochemical techniques to investigate the possibility that crustal subsurface microbial groups can use the solid rock matrix (basalts, etc.) as a source of electrons for redox reactions via extracellular electron transfer (EET). Subsurface crustal fluids and mineral colonization experiments from the cool and oxic basaltic crustal subsurface at the North Pond site on the western flank of the Mid-Atlantic Ridge were used as inocula in cathodic poised potential experiments. Electrodes in oxic microbial fuel cells were poised at -200 mV versus a standard hydrogen electrode to mimic the delivery of electrons in an energy range equivalent to iron oxidation. In this way, microbes that use reduced iron in solid minerals for energy were selected for from the general community onto the electrode surface for interrogation of extracellular electron transfer activity, and potential identification by scanning electron microscopy and DNA sequencing. The results document that there are cathodic EET-capable microbial groups in the low biomass crustal subsurface at this site. The patterns of current generation in the experiments indicate that these microbial groups are active but likely not growing under the low-resource condition of the experiments, consistent with other studies of activity versus growth in the deep biosphere. Lack of growth stymied attempts to determine the phylogeny of EET-capable microbial groups from this habitat, but the results indicate that these microbial groups are a small part of the overall crustal deep biosphere community. This first demonstration of using electromicrobiology techniques to investigate microbial metabolic potential in the crustal deep biosphere reveals the challenges and opportunities for studying EET in the crustal deep biosphere.

1. Introduction

Oceanic crust and fluids represent a significant portion of the Earth's habitable volume (Edwards et al., 2005; Orcutt et al., 2011b), and may be an important contributor to biogeochemical cycling based on volume alone (Shah Walter et al., 2018). There are microbial cells present in this environment, often in very low cell densities (Früh-Green et al., 2018; Jørgensen and Zhao, 2016; Jungbluth et al., 2013, 2016; Labonté et al., 2017; Meyer et al., 2016; Orcutt et al., 2011b; Smith et al., 2011). Studies documenting the potential for microbial activity in the crustal subsurface (D'Hondt et al., 2019; Jangir et al., 2019; Lever et al., 2013; Meyer et al., 2016; Orcutt et al., 2013a; Robador et al., 2016; Torsvik et al., 1998; Zhang et al., 2016) indicate relatively low rates of activity consistent with the limiting resource and energy conditions in the subsurface (Bradley et al., 2018; Jones et al., 2018; LaRowe and Amend, 2015).

Oxidation of reduced iron bound in cool, oxic crustal basalts may be an important source of chemolithotrophic energy for supporting a crustal biosphere (Barco et al., 2017; Orcutt et al., 2013b). Theoretical calculations suggest that the oxidation of iron and sulfur can support $41 \pm 21 \times 10^{10}$ g C per year of autotrophic biomass carbon formation (Bach and Edwards, 2003), approximating recent estimates of a carbon fixation rate of 10^9 – 10^{12} g C per year based on incubation experiments with globally distributed basalts (Orcutt et al., 2015). Cultivation-based studies indicate the presence of autotrophic and heterotrophic iron oxidation metabolisms in oceanic crust. (Edwards et al., 2003a, 2003b, 2004; Meyer et al., 2016; Russell et al., 2016). If microbial oxidation of iron in the solid crustal matrix is occurring in subseafloor ocean crust, then this could have implications for the redox alteration state of oceanic crust over time, and on in-filling of vein networks with secondary alteration products that might affect fluid flow.

Crustal iron oxidation processes require capturing energy/electrons from a solid substrate and bringing them into the cell for use in an electron transport chain, a process often referred to as Extracellular Electron Transfer (EET) (Hinks et al., 2017; Karbelkar et al., 2016; Lovley, 2011; Rowe et al., 2018). EET metabolisms rely on electron shuttle molecules, external structures such as nanowires, or direct contact of conductive structures in the cell wall (Lovley, 2008b; Nealson and Rowe, 2016; Tremblay and Zhang, 2015). One way to search for these EET microorganisms is by electrochemical techniques (Lovley, 2008a; Martin et al., 2018; Nealson and Rowe, 2016). In these techniques, the sites of reduction and oxidation reactions are physically separated, using metal wires and the movement of ions in fluid to set up a complete circuit of charge flow between the redox reaction. The voltage (i.e., the energy level of the electrons flowing through the circuit) of the system is set to mimic the redox couple energy output. This relies on the principle that EET microorganisms with the cellular machinery to use electrons at that potential can either take electrons from the electrode as if it were the electron donor (referred to as cathodic) or transfer electrons to an electrode as if it were the electron acceptor (referred to as anodic). Most work concerning EET concentrates on a few model microorganisms (Lovley, 2012; Nealson and Rowe, 2016; Rowe et al., 2018) and their use in microbial fuel cells (MFC's) (Nielsen et al., 2008; Reimers et al., 2006; Rittmann, 2017; Santoro et al., 2017). There are a growing number of anodic electrochemical studies in environmental samples to identify electrode-reducing EET-capable microorganisms (e.g. Bond et al., 2002; Lam et al., 2019a; Reimers et al., 2017; Williams et al., 2010). However, there are far fewer cathodic or electrode-oxidizing studies to identify environmental microorganisms capable of solid substrates as electron donors (Gregory et al., 2004; Jangir et al., 2019; Lam et al., 2019b; Rowe et al., 2015, 2018; Strycharz-Glaven et al., 2013). While EET-capable microbes are confirmed in the deep terrestrial subsurface (Badalamenti et al., 2016; Jangir et al., 2016, 2019;

Karbelkar et al., 2016; Summers et al., 2013) and deep marine hydrothermal vents (Pillot et al., 2018; Yamamoto et al., 2017), to our knowledge there have been no studies in oceanic crust.

Here, we used cathodic poised potential MFC experiments to examine the capacity for microbial communities from the cool and oxic crustal subsurface to acquire energy from solid substrates. Source materials for the experiments originated from mineral colonization experiments called FLOCS (Orcutt et al., 2010) that had been deployed at North Pond on the western flank of the Mid-Atlantic Ridge as part of Integrated Ocean Drilling Program (IODP) Expedition 336 (Expedition 336 Scientists, 2012a, 2012b) and on subsequent follow up cruises (Figure 1). The mineral colonization experiments were incubated in subseafloor crustal fluids that are relatively cool and oxic (Meyer et al., 2016; Shah Walter et al., 2018; Tully et al., 2018) for periods of 3-6 years following approaches described elsewhere (Baquiran et al., 2016; Ramírez et al., 2019). In the MFCs, the colonized substrates were incubated under aerobic conditions in sterilized crustal fluid from the same environment, with voltages applied to mimic Fe₂/O₂ redox coupling and encourage EET-capable cells to use electrons from the working electrodes (Figure 2). In brief, our results indicate the potential for EET-capable microbial groups in the crustal subsurface, although the identity of the specific microbial members enriched by this process are unclear due to low biomass limitations of the experiments.

2. Materials and Methods

2.1. Site description and sample collection

North Pond is a sediment-covered depression overlying basalt crust of the western flank of the Mid-Atlantic Ridge at ~4,450 m water depth (Figure 1). Details of the crustal composition may be found elsewhere (Expedition 336 Scientists, 2012a, 2012b). Fluids circulating within the crust at this site are oxic and 2-4°C with very low dissolved organic carbon and dissolved metal concentrations (Meyer et al., 2016).

Fluid and biofilm-containing mineral samples for this study come from CORK observatories installed at IODP Holes U1382A and U1383C as described elsewhere (Edwards et al., 2012). In brief, instrument strings containing OsmoSampler systems (Wheat et al., 2011) were deployed at different depths within the holes from 2011-2017. Additional OsmoSampler systems were deployed at the wellhead of the CORKs in 2014 (Table 1, Figure 1) and were inoculated with bottom seawater, making them useful for identifying differences between crustal subsurface and bottom seawater inoculated microbial communities. OsmoSampler systems included Flow-through Osmo Colonization Systems (FLOCS) for mineral colonization experiments, as described elsewhere (Edwards et al., 2012; Orcutt et al., 2010; Orcutt and Edwards, 2014; Ramírez et al., 2019; Wheat et al., 2011). Each FLOCS contained sterile (autoclaved and ethanol-rinsed) substrates including crushed basalts, pyrite, pyrrhotite, or inert glass beads housed within polycarbonate cassettes and sleeves. Fluids were introduced into the FLOCS via the OsmoSampler pumps, allowing fluid microbial communities to colonize the substrates. Effluent of the FLOCS experiments were captured in Teflon coils in such a way to allow a time series of dissolved ion chemical composition to be constructed, following methods described in detail elsewhere (Edwards et al., 2012; Orcutt et al., 2011a; Wheat et al., 2011).

All FLOCS were recovered in October 2017 during cruise AT39-01 of the RV *Atlantis* with the ROV *Jason* (Woods Hole Oceanographic Institution) following methods described elsewhere (Ramírez et al., 2019). In brief, the instrument strings with the downhole FLOCS were

pulled up to the ship on a wire, then immediately disassembled to store the FLOCS in a cold room. Wellhead FLOCS incubated in milkcrates attached to crustal fluid umbilicals at the seafloor were also collected on this cruise with the ROV. FLOCS contents were distributed inside an ethanol-rinsed and UV-irradiated HEPA-filtered hood using sterile instruments. Substrates for the experiment were stored cold (2-4°C) and in the dark in sterile centrifuge tubes with ultrafiltered (0.22 µm-mesh Millipore Sterivex and 0.02 µm-mesh Whatman Anotop 25 filters) crustal fluid until incubation at the shore-based laboratory. Parallel samples for initial characterization of the substrate biofilms were transferred to sterile cyrovials, flash frozen with liquid nitrogen, and stored at -80°C until analysis. In addition to the FLOCS samples, 4-10L samples of raw and unfiltered crustal fluids were collected into ethanol-rinsed cubitainers after collection into gamma-irradiated bags on the seafloor using a Mobile Pumping System as described in detail elsewhere (Cowen et al., 2012; Jungbluth et al., 2016; Meyer et al., 2016). Cubitainers were stored cold (2-4°C) and in the dark for approximately one year before beginning the experiments.

The Teflon coils with fluid samples from the FLOCS were sub-sectioned at sea and analyzed for total silica and manganese following established protocols (Wheat et al., 2011). Prior work at North Pond has documented crustal fluid concentrations of 157 µM silica and 0.1 µM manganese and bottom seawater concentrations of 48 µM silica and <0.05 µM manganese (Meyer et al., 2016). The similarity of the FLOCS silica and manganese concentrations to those of crustal fluids (Table 1) is indicator that the FLOCS chambers were flushed with crustal fluid.

2.2. Cathodic poised potential experiments

A detailed description of the protocol used is available elsewhere (Jones and Orcutt, 2019). In brief, glass two-cell, three-electrode MFC systems (Adams and Chittenden, CA, USA) were used as incubations chambers, run in parallel with a multichannel potentiostat (model CHI1030C, CHI Instruments, TX, USA). Nafion 117 proton exchange membrane (Fuel Cell Store, TX) separated the half-cells. Cells were filled with distilled water and autoclaved prior to filling with media and sample inocula.

Ag/AgCl reference electrodes (Gamry Instruments, PA, USA part 932-00018 and/or Analytical Instrument Systems, NY, USA) were calibrated before each run by immersion in 3 M NaCl and compared against a known electrode kept only for that purpose (max +/- 20 mV drift from the value of lab master electrode). Working electrodes (WEs) were 2 × 4 cm² Indium Tin Oxide (ITO) coated glass slides (Delta Technologies Ltd, CO, USA part CB-50IN-1111), constructed for MFC as described elsewhere (Rowe et al., 2015). Counter electrodes (CEs) were carbon cloth with a 4 × 4 cm² surface area. Electrodes were sterilized by rinsing with 80% ethanol, air-drying, then exposing to UV radiation for 15 minutes per side.

Each experiment consisted of five treatments (Table 2): Three MFCs filled with buffered and double autoclaved crustal fluid (cool and oxic) and inoculated with colonized mineral samples. Colonized minerals were not pretreated to loosen cells from the mineral surfaces, as preserving biomass was considered more important. Chronoamperometric (CA) technique with a WE continuously poised at -200 mV versus a standard hydrogen electrode (SHE) was used as electron donor to incubate these cells. These are referred to as the “Echem” treatments. One MFC filled with the same fluid and WE but without any sample inoculum – referred to as the “Fluid” treatment. And finally, one glass bottle with a mixed sample inoculum and ITO electrode without any voltage applied – referred to as the “Offline” treatment. Samples were transferred

194 into the MFCs in HEPA-filtered, UV-irradiated biosafety cabinet or hood using sterile tools. An
195 aliquot of each sample was also collected into a sterile plastic tube for microbial community
196 analysis representing a “time zero” (T0) condition that may have changed from the Shipboard
197 (SB) initial condition. For the Echem and Fluid treatments, the counter cell redox couple was
198 H₂O/O₂. Testing (data not shown) determined that a strong kill control method was necessary to
199 achieve sterility of the crustal fluid, consisting of 1 h autoclaving at 121°C, incubation at room
200 temperature in the dark for ~24 h to allow for spore germination, and then another 1 h
201 autoclaving at 121°C before cooling down to 4°C for the addition of substrate. Sodium
202 bicarbonate was added to the media (0.1 M) as a buffer. MFCs were incubated at 4°C in the dark.

203 Once the MFCs were constructed, a cyclic voltammetry (CV) sweep was performed for
204 each cell with the potentiostat before each poised potential experiment at a scan rate of 0.1 V s⁻¹
205 and sample interval of 0.001 V, across -1 to 1 V range. Then the chronoamperometry
206 experiments began by applying a constant voltage (-200 mV versus SHE) to the Echem and
207 Fluid MFCs. Current generation was monitored and recorded until current changes began to
208 decline after a period of approximately 15 days. At the end of each CA experiment, two further
209 CV sweeps were performed: one with the incubated ITO electrode (Tend i) and one with a fresh
210 ITO electrode dipped into the incubated media (Tend ii).

211 212 *2.3. Post-processing cathodic poised potential samples*

213
214 After CV scanning, the ITO electrodes were carefully removed from the MFCs and split
215 into two sections using a diamond scribe and tweezers for microscopy and DNA analysis. One
216 piece was serially dehydrated in ethanol solutions (10 min steps each at 50:50, 60:40, 70:30,
217 80:20, 90:10 and 100:0 ratios of ethanol:distilled water) and hexamethyldisilazane (10 min) and
218 air dried for 3 days in a HEPA-filtered biosafety cabinet in preparation for scanning electron
219 microscopy (SEM), following methods available elsewhere (Braet et al., 1997; D’Angelo and
220 Orcutt, 2019b; Hazrin-Chong and Manefield, 2012). Dehydrated slide pieces were then mounted
221 onto 12-mm-diameter SEM stubs with double-sided carbon tape, sputter coated with gold with a
222 Denton Vacuum Desk IV cold sputter instrument (Denton Vacuum LLC, Moorestown, NJ), and
223 imaged with a Zeiss Supra25 SEM at between 5-10 kV and a working distance between 3-9 mm.
224 The second ITO piece was vigorously rubbed with a distilled water-soaked sterile cotton swab
225 (sterile phosphate buffered saline solution-soaked swab for NP14) to remove cells for DNA
226 extraction.

227 In addition, aliquots of the residue FLOCS sample material in the MFCs were collected at
228 the end of the experiment for “time final” (Tend) microbial community composition analysis. As
229 with T0 samples, the aliquots were transferred into sterile plastic tubes and stored frozen at -80°C
230 until analysis.

231 232 *2.4. DNA extraction, quantification, sequencing, and bioinformatic processing*

233
234 The FastDNA kit from MP Biomedicals (Irvine, CA, USA) was used for all DNA
235 extractions following manufacturer’s instructions with the exception of using a Retsch MM 400
236 shaker (frequency 20 r/s for 5 min) instead of vortexing as described in detail elsewhere
237 (D’Angelo and Orcutt, 2019a). From the Echem and Fluid samples (Tables 2 and 3), the ITO
238 swab end was cut into a sterile plastic tube and subjected to a freeze-thaw cycle (8 min at -80°C
239 then thawing at room temperature for 10 min or until ice barely melted into water) before

transferring into a FastDNA kit tube for DNA extraction. For the Shipboard and NP12 samples, 0.232 µg/µl polyadenine (polyA, final concentration) was added to the sample at the first step to increase DNA yield from low biomass samples. For each batch of samples extracted, a no template control (NTC) was included as a check for contamination. DNA concentrations were measured by Q-bit Fluorometer 3.0 (Thermo Fisher Scientific, Waltham, MA USA) using the Qubit HS dsDNA kit (Thermo Fisher Scientific, Waltham, MA USA) according to manufacturer instructions. Note that inclusion of polyA interferes with DNA quantification following this approach (Table 3).

Aliquots of DNA extracts were sent to the Integrated Microbiome Resource facility at Dalhousie University (Halifax, Canada) for sequencing of the V4-V5 hypervariable region of the 16S rRNA gene via Illumina Mi-Seq technology using the 515F/926R primer pair (Parada et al., 2016; Walters et al., 2015). Sequencing was performed on an Illumina MiSeq using 300 × 300 bp paired-end V3 chemistry.

Raw sequenced reads were processed at the same time as sequenced reads from mineral incubation experiments taken from the same environments, as well as additional no-template-control samples (data not shown, but full data available at the NCBI Sequence Read Archive BioProject PRJNA564565 samples SAMN12723399-489), using modifications of the standard DADA2 pipeline Version 1.12 (Callahan et al., 2016) to construct unique amplicon sequence variants (ASVs). This was done to directly link ASVs between the two datasets and to give the DADA2 algorithm the most possible data to use for sequence inference. Thirty base-pairs were trimmed off of the start of each read, forward reads were truncated at 250 base-pairs, and reverse reads were truncated at 200 base-pairs. This allowed for 50 BP of overlap for the forward and reverse reads to be merged after sequence inference. Approximately 100 million bases from ~500K reads were used by DADA2 to infer the base-call error rates in the data. Inference of ASVs, merging of forward and reverse reads, and chimera removal resulted in 2,011 ASVs ranging from 345 – 360 BP in length. These sequences were assigned taxonomy by the naïve Bayesian classifier built into the DADA2 package using the Silva v132 database. Scripts used to process the data are available via <https://github.com/orcuttlab/northpond>.

2.5. Data availability

Chronoamperometry, cyclic voltammetry, and scanning electron microscopy data are available from the Biological and Chemical Oceanography Data Management Office (BCO-DMO) at under project 707762: <https://www.bco-dmo.org/dataset/780261> (SEM), <https://www.bco-dmo.org/dataset/780127> (CA profiles), <https://www.bco-dmo.org/dataset/780248> (cyclic voltammetry profiles), and <https://www.bco-dmo.org/dataset/780225> (experimental metadata). 16S rRNA gene sequence data are available at the National Center for Biotechnology's Sequence Read Archive BioProject PRJNA564565 samples 274 SAMN12723345-SAMN12723415.

3. Results and Discussion

3.1. Evidence for Fe₂/O₂ cathodic EET in North Pond crustal microbial communities

Only a few of the cathodic continuously poised potential experiments with colonized substrate samples from North Pond incubated in MFCs set at -200 mV vs. SHE (Table 2) resulted in an increase in current density relative to the double-autoclaved crustal fluid only

treatment (Figure 3). Current responses with values greater than the fluid only treatments were in basalt or glass wool treatments in the NP11, NP12, and NP14 experiments. While the current responses between the samples and the fluid only treatments only varied by a few $\mu\text{A cm}^{-2}$, we note that other environmental cathodic EET experiments have observed similar patterns between biotic and abiotic treatments (Jangir et al., 2016; Karbelkar et al., 2016; Lam et al., 2019b; Li and Nealson, 2015; Rowe et al., 2015). In NP11, which was three basalt replicates from the Hole U1382A downhole incubation (Table 2), there was considerable variation between the signals. This may be because of founder effects or from variation in viable cell biomass between sample inoculum. The basalt signal for NP12, which was the same substrate inoculum, is most similar to NP11 replicate iii, suggesting EET-capable biomass is the driver of the observed signals, as these samples came from a similar section of substrate. In some experiments (i.e., NP13 and NP15), the fluid treatment had an increase in current density compared to the samples (Figure 3), suggesting that some hardy microbial groups that survived the extreme pressure/temperature changes of double autoclaving are also capable of EET. However, NP13 and NP15 curves indicated possible electrode interference, so this interpretation should be viewed with caution. Specifically, the NP13 Fluid reference electrode drifted out of calibration during the course of the incubation (data not shown), and the NP15 Echem basalt treatment exhibited a very noisy profile until day 5. The MFCs incubated with metal sulfide substrates (i.e., pyrite in NP12 and pyrrhotite in NP13-15) barely displayed any current response. Cumulatively, these results indicate that: (1) microbial groups capable of cathodic EET at -200 mV versus SHE (roughly the Fe_2/O_2 redox coupling) are present in the crustal subsurface, but at heterogenous density; and (2) that cathodic EET-capable microbial groups may still exist in cold and oxic crustal fluids that are subjected to intense heating (i.e., double autoclaving).

The strongest current response of $\sim 10 \mu\text{A cm}^{-2}$ increase compared to the control was observed in the glass wool treatment from the U1382A downhole samples (i.e., experiments NP11 and NP12; Figure 3). This result was unexpected, given the inert nature of the colonized substrate as compared to basalt or metal sulfides. However, the glass wool samples in the FLOCS were located downstream of the cassettes containing basalts and metal sulfides, possibly functioning as a sieve that concentrated biomass exiting those samples. Supporting this assessment, the lower starting current for this sample may be an indicator of higher starting biomass for this treatment.

It is notable that the current response from the two experiments with downhole-incubated FLOCS samples (i.e., experiments NP11 and NP12) had similar or increased current density responses to those observed in the wellhead bottom-seawater-incubated FLOCS (Figure 3); despite the wellhead samples having roughly one order of magnitude more starting material in the MFCs as compared to the downhole samples (Table 2). This indicates a lower amount of EET-capable microbial groups in the bottom seawater inoculated samples versus the downhole samples. We attribute this difference to a presumed selection for EET-capable microbial groups in the crustal subsurface compared to bottom seawater. There also may be an effect from the different incubation times on the colonizing biofilms in the FLOCS (i.e., four years or bottom water FLOCS versus six years for downhole FLOCS; Table 2), which allowed more EET-capable groups to colonize in the subsurface samples.

Considering the probable low starting biomass on crustal subsurface FLOCS (i.e., crustal fluid at this site has cell densities of 10^3 - 10^4 cells ml^{-1} (Meyer et al., 2016; Tully et al., 2018)) and rock core samples are estimated at 10^4 cells per gram rock (Jørgensen and Zhao, 2016)), it is remarkable that any current response was detected from these crustal subsurface samples, and at

current densities comparable to much shallower and higher cell density marine environments (Lam et al., 2019b). This is particularly so given the presumed low-energy, low-activity nature of the deep marine crustal subsurface microbial community. Current response was most rapid in the first 5 days of the experiment, leveling off for the remainder of the experiment (Figure 3). However, previous work (Meyer et al., 2016) also noted a quick response to incubation conditions by the North Pond crustal fluid microbial community, proposing this as evidence that the microbes were poised to quickly take advantage of transient resources as an adaptive advantage in their resource limiting environment. This suggests that metabolic activity, rather than growth of cells, explains this current response, similar to prior experiments (Deng and Okamoto, 2018). Activity rather than growth in these experiments makes sense given that no additional resources (i.e., vitamins, minerals, carbon) were provided in the MFC media. EET-capable microorganisms are known to need additional organic carbon (Koch and Harnisch, 2016).

In each experiment, metal sulfides had the weakest current increase, often almost indistinguishable from the control (Figure 3). This is contrary to expectation, as pyrite and pyrrhotite are more reactive than basalt, providing a more favorable substrate for iron oxidation. For comparison, in an anoxic crustal subsurface environment, metal sulfide samples had higher cell densities than basalt samples (Orcutt et al., 2011a), and sulfides have also been observed to be preferentially colonized at the seafloor (Edwards et al., 2003b; Orcutt et al., 2011a). The result of our experiment suggests that: 1) replicating the Fe_2/O_2 redox couple with poised potential at -200 mV may be impacted by sulfide from the metal sulfides, 2) microbial communities colonizing the metal sulfides preferred other redox reactions than those involving Fe_2/O_2 , 3) *in situ* EET-capable microbial communities preferentially colonize the dominant substrate versus minor components of the crust at this site (i.e., basalt is more prevalent than metal sulfides at North Pond: Expedition 336 Scientists, 2012a, 2012b), or 4) that EET-capable microbes on pyrrhotite may have remained attached to the mineral and did not migrate to the electrode. Additionally, when pyrrhotite oxidizes, it preferentially loses iron in dissolved form (Belzile et al., 2004), making it less attractive to iron oxidizers that require surface-bound iron.

3.2. Evidence of redox reactions in cathodic EET-capable microbial communities from cyclic voltammetry

CV scans of attached biomass were performed immediately after CA scans to gain insight into conductive pathways of attached biomass and/or in shuttle molecules released into the fluid (Figure 4). Scan configurations were characteristic of voltammetry in the absence of substrate (LaBelle and Bond, 2009), adding to evidence that these communities were resource limited in this experiment. In this configuration, peaks in a scan correspond to an EET mechanism. Peaks in these curves represent the mid-point potential of a given mechanism, essentially the optimum voltage at which an EET mechanism functions (LaBelle and Bond, 2009).

CV scans show similar patterns across the experiments, with current density peaks at approximately -0.8, -0.25 and +0.1 V vs Ag/AgCl except for NP12 and NP13 fluid, which had peaks at -0.65 and +0.35 V in the Tend i and -0.8 and +0.1 in the Tend ii with fresh electrode (Figure 4). This indicates that there is some EET activity in the fluid control despite double-autoclaving and taking measures to ensure sterility. It is less likely that abiotic reactions are responsible for these peaks, as these would be more similar to each other if there were no biology involved. The CV-observed EET-activity in the controls is particularly strong in the NP12, NP13

and NP15 fluid scans, which correlates with higher CA current density in those same samples (Figure 3).

In some instances – the NP11, NP2 and NP15 curves particularly – initial experiment scans have a peak at +0.1V that disappears or is overreached in Tend curves, indicating that there was an abiotic reaction present in the initial seawater that changes by the end of the experiments. These changes are likely due to changes in chemistry in response to the microbial communities present.

NP11, NP12 basalt, NP13 pyrrhotite and NP15 curves, with the exception of the fluid control, show a Tend i CV curve with the old electrode that has higher peaks than that of the Tend ii CV curves with fresh electrode. This indicates that biofilm organisms with direct contact are responsible for the EET in these cells. All other samples showed a higher current response at the end of an experiment with a fresh electrode. This indicates that planktonic cells and extracellular shuttle mechanisms were more EET-capable in these experiments. These differences in capability between fractions of a single environment are noted elsewhere, but remain an understudied part of microbe-EET understanding (LaBelle and Bond, 2009; Lam et al., 2019b). This result is perhaps surprising given that releasing electron shuttles would seem an expensive strategy in the low-resource deep marine crustal subsurface (Jones et al., 2018).

The CV peak occurring at approximately -0.25 V vs. Ag/AgCl is likely indicating the mechanism that is giving the electrode community energy, as the CA experiment voltage is set to -0.4 V vs. Ag/AgCl (-0.2 V vs SHE). From the CV scans, it appears that the set voltage is not optimum for this community, however. Overpotential may play a role in this and having a peak close to but not a match for a CA voltage is also seen with isolation of EET capable microbes from other environmental samples (Jangir et al., 2016; Lam et al., 2019b; Rowe et al., 2015). This peak, in combination with that of the +0.1 V vs Ag/AgCl, may also be indicative of cytochrome-based EET (Bewley et al., 2013).

There is a diffuse peak between -0.9 to -0.7 V vs. Ag/AgCl. This peak covers the range at which hydrogen is abiotically generated but is in addition to the abiotic signal (Deng and Okamoto, 2018). This may indicate a capacity for using hydrogen as a source of electrons. The capacity to use hydrogen as an electron donor is widespread among acidophilic iron-oxidizers (Hedrich and Johnson, 2013) and there is also some evidence this metabolism is present in neutrophilic iron-oxidizers (Mori et al., 2017), indicating a possible alternative explanation. This peak is also associated with some environmental iron-oxidizers (Lam et al., 2019b), though these appear to have a wide range of potential redox values they can use (Summers et al., 2013).

In many of our experiments, there was a prominent peak at approximately +0.1 V vs. Ag/AgCl, indicating that this community has the capacity to directly use electrodes as a terminal electron acceptor. Anaerobic metabolisms should be unfavorable in the bulk oxic conditions of North Pond, but this result may indicate anaerobic microniche in this habitat that support this type of metabolism. It may be that microbial groups with anaerobic metabolic potential are more well adapted to take advantage of anaerobic micro-niches within the cracks and crevices of the fluid flow system, or that the conditions within the FLOCS facilitated micro-anaerobic pockets within the sample chambers. This evidence corroborates other work on the microbial community at this site, which observed taxa and metabolisms connected to anaerobic processes (Tully et al., 2018). As the ability to perform both anodic and cathodic electron transfer is identified in as many as 20 EET species to date (Koch and Harnisch, 2016; Rowe et al., 2018), our results suggest that the co-occurrence of solid substrate oxidation and reaction metabolisms in the same

microbial communities, as we observe in our experiments, is not out of the ordinary. Future anodic experiments could explore these metabolisms in the crustal subsurface in more detail.

3.3. Morphology of cathode-attached microbial groups

Viewed under SEM, the morphology of particles on the Echem sample ITO electrodes are consistently ultra-small coccoids of 100-300 nm diameter (example provided in Figure 5, all data available in BCO-DMO dataset). These ultrasmall coccoids are not seen in the fluid-only treatments, though spheres of less than 100 nm are occasionally present on those. 100-300 nm coccoids without observable attachment structures are also present on polished chips of basalt and pyrrhotite/pyrite from the downhole experiments at this same site (data available at <https://www.bco-dmo.org/dataset/756152>). These polished mineral coupons are not directly comparable to the poised potential experiments described here, as the bioelectrochemical samples were crushed mineral sands and not polished chips. They are from the same locations and are comparable minerals, however, so give some idea of morphological diversity.

We presume that these small coccoids are cells that have colonized the electrodes. If correct, the size of these cells would classify them as ultra-small bacteria (Ghuneim et al., 2018). If these are cells, this very small size may indicate adaption to resource limited conditions of the deep crustal environment, as suggested for other resource limited environments (Kuhn et al., 2014; Schut et al., 1993), or it may indicate cells in a starvation configuration (Álvarez et al., 2008). A starvation response is consistent with the chronoamperometry profiles showing limited current response after the first few days, which seem to indicate activity rather than growth.

No pilli or attachment structures were detected in any SEM image, indicating that electron transport does not happen through conductive structures. Electron transport is therefore likely through direct contact of some mechanism embedded in the cell wall or via electron shuttle mechanisms. There is currently too little information to speculate on what these cathode-oxidizing mechanisms might be, beyond the CV peaks showing little similarity to flavin-mediated mechanisms (Marsili et al., 2008).

3.4. Microbial groups in North Pond crustal subsurface sample capable of cathodic EET

The low biomass expected from the FLOCS samples (Ramírez et al., 2019), as well as on the ITO electrodes, necessitated a stringent protocol of including numerous no template extraction controls (NTCs) for bulk DNA extraction and sequencing (Labonté et al., 2017; Ramírez et al., 2019; Sheik et al., 2018). Instead of simply removing any sequence groups that appeared in any NTC from sample sequence libraries, we present the full results of all taxonomic groups in all samples including NTCs (Figures 6 & 7), with samples grouped by treatment type rather than by experiment, to show trends more clearly.

Overall, the Echem ITO electrode swabs revealed that the dominant taxonomic groups in the sequence data overlapped with the dominant groups in the NTC's sequence libraries (Figures 6, 7). This suggests overamplification of DNA contaminants in these extremely low biomass samples, as is known to occur (Chandler et al., 1997; Ramírez et al., 2019; Sheik et al., 2018), swamping out any signal of any cells that may have attached to the ITO electrodes. It may also indicate cross-contamination from the samples into the NTCs during sample handling, as some of the taxonomic groups that appear in the NTC samples are not "typical" sequence contaminant groups, such as Thioalkalispiraceae (Figure 7). Because of these uncertainties in provenance, taxonomic groups that appear in the NTCs are not discussed further.

Of the remaining taxonomic groups not observed in the NTCs, the majority of these groups observed in the Echem samples were also observed on the ITO electrodes incubated in the Offline controls without voltage applied (Figures 6 & 7). This indicates cathodic EET did not enrich these taxa.

Only a small fraction of the total sequences in this study are found on the Echem samples but not in the NTCs or Offline treatments (Figure 6 & 7), which may indicate that they are involved in the cathodic EET observed in the experiments. One of the basalt treatments from the U1382A downhole NP11 experiment (RJ_NP11i from Table 3) had a few sequences grouping within the Spirochaetes, Synergistetes, and Tenericutes phyla (Figure 6). The Spirochaetes phyla was also observed in the sequence libraries from the bottom seawater NP15 experiment Echem samples from basalt and glass wool (RJ_15NP15 and RJ_13NP15), and it was also enriched in a NP15 Tend sample (RJ_18NP15) compared to Shipboard or T0 samples where it is absent or very rare. The NP15 experiment had current response that was inconclusive for indicating EET activity, so it is not clear if this phylum is responding to stimulating conditions for cathodic EET or to some other factor. Spirochaetes members have been identified elsewhere in crust-associated subsurface sediment (Liao et al., 2011) and can perform acetogenesis (Leadbetter et al., 1999; Miura et al., 2016), which is a known EET-associated metabolism (Igarashi and Kato, 2017; Yang et al., 2012). They were also detected in a shallow subsurface sediments EET study (Lam et al., 2019b). Flavins are noted as potentially metabolically important in some Spirochaetes (Gutiérrez-Preciado et al., 2015; Radolf et al., 2016) which are a known EET mechanism in other phyla (Xu et al., 2016; Yang et al., 2012; Zhao et al., 2019). However, characteristic flavin-based peaks, as described elsewhere (Fuller et al., 2014; Light et al., 2018; Marsili et al., 2008), are absent from in our data. There are also marine Synergistetes that can metabolize hydrogen (Jumas-Bilak and Marchandin, 2014; Miura et al., 2016), but these are described as strict anaerobes and are not described as acetogens specifically (Jumas-Bilak and Marchandin, 2014). Tenericutes members occur in a bioelectrochemical study, though little information is available on their specific role (Liu et al., 2019). These groups have yet to be investigated for EET capability directly.

The Fluid treatment from the NP13 experiment (RJ-NP13-11), which had the strongest current response of all samples in that experiment (Figure 3) despite going through double autoclaving, had sequences falling into this non-NTC/non-Offline category that grouped within the Chloroflexi and Nanoarchaeaeota phyla (Figure 6). Chloroflexi strain *Ardentica maritima* 110 is EET capable (Kawaichi et al., 2018). Nanoarchaeaeota-associated sequences were also detected in the NP14 Echem sample from basalt (RJ_NP14_43), which also had the strongest current response in that experiment (Figure 3). There are no specific EET studies involving Nanoarchaeaeota, though they have a gene for cytochrome bd-I ubiquinol oxidase subunit I, a protein that shuttles electrons from the host's membrane coupled to O₂ reduction (Jarett et al., 2018). A syntrophic quinone electron transfer mechanism has been shown to support Nanoarchaeaeota in co-culture (Smith et al., 2015) and interspecies electron transfer is a known mechanism (Lovley, 2017) as are using cytochromes as an EET mechanisms (Rosenbaum et al., 2011; Santos et al., 2015). CV peaks that may indicate a cytochrome mechanism (Bewley et al., 2013) are present in in our data. Other archaea show some evidence of EET capability (Uria et al., 2017). Particle size and coccoid morphology observed by SEM are also consistent with Nanoarchaeaeota members (Huber et al., 2003). This may indicate that Nanoarchaeaeota are involved in the cathodic EET activity observed in those experiments.

The NP13 glass wool sample (RJ_NP13-21), which showed the second strongest current response in that sample (Figure 3), had a small fraction of Shewanellaceae-associated sequences which were not observed in the NTCs or Offline samples (Figure 7). This taxon was also observed in a few shipboard, T0 and Tend samples, indicating that this group is a crustal generalist. Shewanellaceae are well known for their ability for anodic and cathodic EET across multiple environments (Lovley, 2012; Rowe et al., 2018; Zou et al., 2019). Our results suggest that Shewanellaceae can also perform similar electron transfer reactions in the crustal subsurface.

Several Echem samples had Beggiatoaceae-associated sequences that did not appear in any other samples (Figure 7). This taxon is commonly detected on sediment surfaces and at shallow depths rather than the subsurface (Amend and Teske, 2005; Jørgensen et al., 2010; Teske et al., 2016), so it is a rare member of the crustal deep biosphere that may have responded to the cathodic EET stimulating conditions of this experiment. No EET study has specifically looked at Beggiatoaceae, and it is very rarely mentioned in a bioelectrochemical context. Cultured members of this Proteobacteria family are sulfide oxidizers that can use oxygen or nitrate as a terminal electron acceptor. Beggiatoaceae also metabolize acetate (Burton et al., 1966; Güde et al., 1981; Strohl et al., 1981), which may be being generated on the cathode as a product of acetogenesis (Min et al., 2013) or possibly as a waste product from EET-capable microbes.

Finally, the NP14 Echem sample with pyrite (RJ_NP1416), which did not show any current response (Figure 3), had non-NTC/non-Offline sequences grouping within the Elusimicrobia phylum (Figure 6) and the Micavibrionaceae family of Proteobacteria (Figure 7). As this experiment did not indicate EET current response, it is not likely that these taxa are involved in EET.

4. Summary

These results indicate that solid-substrate-utilizing EET-capable microbes are present in the oxic crustal subsurface and the deep sea, but those that potentially oxidize iron with this mechanism are likely minor overall contributors to the general community despite the energetic advantages to do so. It may also be that this mechanism is one of many these microbes are capable of, reflective of a strategy of taking advantage of resources as they become available in North Pond, as suggested elsewhere (Tully et al., 2018). This may also be an example of an available niche that is not well filled (Cockell et al., 2016). Moreover, if the cathodic EET-capable microbial groups require organic carbon, then the extreme carbon-limitation of this oligotrophic basalt environment (Mason et al., 2009) is a limiting factor to this subset of the North Pond community, as suggested for other oligotrophic environments (Morono et al., 2011; Ziebis et al., 2012). Despite the evidence for cathodic EET reactions, this incubation approach was not successful for conclusively determining which members of the low biomass crustal subsurface are responsible for these reactions, although there are indications that Nanoarchaeota and Shewanellaceae taxa may be involved. Overall, this study shows that cathodic EET-capable microbial groups may be more widespread in the general environment than previously considered, and that this enrichment approach to searching for them can be useful to quantify their activity and metabolic potential in challenging environments such as the cool, oxic basalt of North Pond.

Author Contributions

RJ and BNO formulated the original hypotheses and designed the project. All authors participated in sample collection. RJ performed the experiments, RJ and TD performed DNA extractions and bioinformatic analysis, and all authors participated in data analysis and interpretation. RJ and BNO wrote the paper with contributions from TD.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Tables

Table 1. Summary of mineral colonization experiments (i.e. FLOCS) and inoculating fluids used in the poised potential experiments, including deployment and recovery information about the FLOCS experiments and the source fluids used as inoculum and/or media, and the concentrations of dissolved silica and manganese within the FLOCS at the time of recovery. The latitude, longitude, and water depth of the North Pond CORKs at Holes U1382A and U1383C are 22° 45.385 N, 46 ° 4.899W, 4483 m and 22° 48.135N, 46° 3.179W, and 4414 m, respectively. Abbreviations: BW, bottom water; mbsf, meters below seafloor; NA, not applicable; X336, IODP Expedition 336. Dates in YYYY-MM-DD format

Experiment ID	NP11	NP12	NP13	NP14	NP15
FLOCS ID	U1382A downhole	U1382A downhole	U1383C wellhead BW	U1383C wellhead BW	U1382A wellhead BW
FLOCS Name	MBIO upper	MBIO upper	JP FLOCS Red	JP FLOCS Blue	JP FLOCS green
FLOCS deployed depth (mbsf)	151	151	0	0	0
FLOCS source fluid depth (mbsf)	151+	151+	0	0	0
Fluid source depth (mbsf)	151+	151+	151+	250+	151+
FLOCS substrates	Basalt	Basalt, Pyrite, Glass wool	Basalt, Pyrrhotite, Glass Wool	Basalt, Pyrrhotite, Glass Wool	Basalt, Pyrrhotite, Glass wool
FLOCS deployment date	2011-10-12	2011-10-12	2014-04-07	2014-04-08	2014-04-06
FLOCS deployment dive #	X336	X336	J2-766	J2-771	J2-769
FLOCS recovery date	2017-10-19	2017-10-19	2017-10-20	2017-10-20	2017-10-22
FLOCS recovery dive #	J2-1031	J2-1031	J2-1032	J2-1032	J2-1034
Fluid collection date	2017-10-12	2017-10-12	2017-10-13	2017-10-11	2017-10-12
Fluid collective dive #	J2-1027	J2-1027	J2-1028	J2-1026	J2-1027
FLOCS Si (μM)	65-139	65-139	38-245	78-197	55-93
FLOCS Mn (μM)	0.1-0.2	0.1-0.2	0.9-3.8	0.1-20.5	1.7-5.8

Table 2. Summary of poised potential experimental set-ups, indicating contents of the working electrode (WE) cell, the media used in the cells (ultra-filtered and double autoclaved fluid from either CORKs U1382A or U1383C supplemented with 10 mM sodium bicarbonate as a buffer). In all microbial fuel cells, the working electrode consisted of an 8 cm² indium tin oxide coated WE poised at -0.2 V versus a standard hydrogen electrode; the offline experiments had the same electrode with no voltage applied.

Exp. #	Channel	WE sample (g material added to microbial fuel cell)
NP11 U1382A downhole FLOCS + U1382A crustal fluid media		
1		Media-only abiotic control (0)
2		B516 Basalt i (0.56)
3		B516 Basalt ii (0.18)
4		B516 Basalt iii (0.08)
Offline		B516 Basalt (0.30)
NP12 U1382A downhole FLOCS + U1382A crustal fluid media		
1		Media-only abiotic control (0)
2		B516 Basalt (0.66)
3		B518 Glass Wool (0.44)
4		B517 Pyrite sand (1.47)
Offline		Mixture of B516, B517 + B518 (1.53)
NP13 U1383C wellhead BW FLOCS + U1383C shallow crustal fluid media		
1		Media-only abiotic control (0)
2		Glass Wool (1.92)
3		Pyrrhotite (4.26)
4		Basalt (5.51)
Offline		Mixture of glass wool, pyrrhotite + basalt (12.66)
NP14 U1383C wellhead BW FLOCS + U1383C deep crustal fluid media		
1		Media-only abiotic control (0)
2		Glass Wool (2.7)
3		Pyrrhotite (6.84)
4		Basalt (7.59)
Offline		Mixture of glass wool, pyrrhotite + basalt (14.93)
NP15 U1383C wellhead BW FLOCS + U1382A crustal fluid media		
1		Media-only abiotic control (0)
2		Glass Wool (24.82)
3		Pyrrhotite (3.67)
4		Basalt (5.03)
Offline		Mixture of glass wool, pyrrhotite + basalt (13.85)

Table 3. Summary of DNA extractions and 16S rRNA gene amplicon sequence analysis (after quality control filtering) for each experiment and treatment, including in the original samples after collection and direct preservation on the ship (Shipboard or SB), at the start of the experiment after 1+ year of sample storage (T0), on the ITO electrode at the end of the experiment (EChem), on the sample material in the WE cell at the end of the experiment (Tend), in an electrochemical cell with only sterile fluid (Fluid), in an electrochemical cell without voltage applied (Offline), and in a no template negative DNA extraction controls run with the same batch of samples (NTC). Sample IDs match those available in Sequence Read Archive (SRA). DNA concentration reported as ng DNA per microliter of extract. Asterisk indicates samples that were extracted with a modification to the protocol that included polyadenine to increase DNA yield from low biomass samples, which interferes with the Qubit DNA quantification method. Abbreviations: ASV, amplicon sequence variant; BDL, below detection limit; NA, not applicable; PE, Paired end.

Sample ID	Treatment	Substrates	ng DNA μL ⁻¹	Total PE Sequences After Filtering	Total ASV
NP11					
TD_B516*	Shipboard	Basalt	0.01	53970	125
RJ_NP1112	T0	Basalt	0.01	89	9
RJ_NP11i	EChem	Basalt	0.01	45017	429
RJ_NP11ii	EChem	Basalt	1.76	1465	33
RJ_NP11iii	Fluid	NA	BDL	89	14
RJ_NP101	Fluid	NA	0.02	113	14
RJ_NP1113	Offline	Basalt	0.01	13843	83
RJ_NP1117	NTC	NA	0.01	13567	35
NP12					
TD_B516*	Shipboard	Basalt	0.01	53970	125
TD_B517*	Shipboard	Pyrite	1.44	34401	55
TD_B518*	Shipboard	Glass wool	1.04	15534	57
RJ_NP1226*	T0	Glass Wool	0.01	34406	38
RJ_NP1228*	T0	Basalt	3.12	667	84
RJ_NP1227*	T0	Pyrite	0.01	50710	68
RJ_NP1221*	Fluid	NA	1.23	19	6
RJ_NP1222*	EChem	Glass Wool	0.01	0	0
RJ_NP1224*	EChem	Basalt	0.01	3	1
RJ_NP1223*	EChem	Pyrite	3.6	0	0
RJ_NP1225*	Offline	Mix	0.01	594	27
TD_181022NTC*	NTC	NA	0.55	91	14
NP13					
TD_181214-1*	Shipboard	Basalt	1.47	84949	90
TD_181214-2*	Shipboard	Pyrrhotite	0.79	29112	118
RJ_NP13-71	T0	Glass Wool	BDL	7277	52
RJ_NP13-61	T0	Basalt	BDL	51879	112

RJ_NP13-81	T0	Pyrrhotite	BDL	1752	30
RJ_NP13-11	Fluid	NA	BDL	2527	46
RJ_NP13-21	EChem	Glass Wool	0.03	30698	56
RJ_NP13-41	EChem	Basalt	0.04	50924	29
RJ_NP13-31	EChem	Pyrrhotite	0.10	41572	21
RJ_NP13-51	Offline	Mix	0.09	32814	64
RJ_NP13-12	Tend	Glass Wool	BDL	22746	43
RJ_NP13-32	Tend	Basalt	BDL	12124	46
RJ_NP13-22	Tend	Pyrrhotite	0.05	79736	83
RJ_NP13-42	NTC	NA	BDL	794	11
TD_181214_NTC*	NTC	NA	0.57		10
NP14					
TD_181213-2*	Shipboard	Basalt	0.98	41627	92
TD_181213-3*	Shipboard	Pyrrhotite	0.76	27753	51
RJ_NP14-73	T0	Glass wool	0.01	31656	71
RJ_NP14-63	T0	Basalt	0.01	10112	46
RJ_NP14-83	T0	Pyrrhotite	BDL	55635	73
RJ_NP1414	Fluid	NA	0.01	12158	56
RJ_NP1415	Echem	Glass wool	0.01	17767	53
RJ_NP14-43	Echem	Basalt	BDL	8092	72
RJ_NP1416	Echem	Pyrrhotite	0.01	17381	66
RJ_NP14-53	Offline	Mix	0.04	42493	78
RJ_NP14-52	Tend	Glass wool	BDL	19912	52
RJ_NP14-62	Tend	Basalt	BDL	20405	71
RJ_NP14-72	Tend	Pyrrhotite	BDL	223	12
RJ_NP14-82	NTC	NA	BDL	6	1
TD_181213_NTC*	NTC	NA	0.64	1204	17
NP15					
TD_181214-4*	Shipboard	Basalt	0.77	49317	129
TD_181214-5*	Shipboard	Pyrrhotite	1.1	39062	109
RJ_17NP15	T0	Glass wool	0.02	5460	23
RJ_25NP15	T0	Basalt	0.02	5101	75
RJ_16NP15	T0	Pyrrhotite	BDL	404	17
RJ_12NP15	Fluid	NA	0.0	26	2
RJ_13NP15	Echem	Glass Wool	BDL	51	5
RJ_15NP15	Echem	Basalt	BDL	44	4
RJ_14NP15	Echem	Pyrrhotite	BDL	27	3
RJ_11NP15	Offline	All	BDL	21	3
RJ_18NP15	Tend	Glass Wool	BDL	67716	674
RJ_22NP15	Tend	Basalt	0.01	3723	59
RJ_21NP15	Tend	Pyrrhotite	BDL	447	21
RJ_24NP15	NTC	NA	BDL	1603	47

Figure Captions

Figure 1. Overview of sampling from the North Pond crustal subsurface for this study. Top: Schematic of FLOCS colonization experiments and fluid sampling from CORK observatories installed during IODP Expedition 336. The primary locations targeted in this study – Holes U1382A and U1383C – are highlighted in yellow. FLOCS colonization experiments were deployed both downhole (green) and at the wellheads of the CORKs and accessing crustal fluid sourced from depth via stainless steel umbilical tubing (pink, with Ws representing a FLOCS sourcing the shallow sealed horizon at W_D sourcing the deeper sealed horizon). Figure modified from (Edwards et al., 2012a) with permission. Lower left: Photograph of downhole FLOCS colonization experiment. Reprinted with permission from Edwards et al. 2012. Lower middle: Photograph of wellhead FLOCS experiment. Lower right: Deployment of wellhead FLOCS experiments in a milkcrate with OsmoSamplers connected to CORK wellhead umbilical. Photo courtesy RV *Atlantis* cruise AT39-01 ROV *Jason* dive J2-1032, 2017, chief scientist Geoff Wheat, University of Alaska Fairbanks, US National Science Foundation, © Woods Hole Oceanographic Institution.

Figure 2. Overview of cathodic poised potential experiments for enriching for microorganisms from North Pond subsurface crustal samples capable of extracellular electron transport (EET) when supplied with -200 mV (versus standard hydrogen electrode) and oxygen. Top: Conceptual cartoons of experiment principles. Middle: Photograph of a complete two-cell unit, with Reference Electrode (RE) and ITO-coated glass Working Electrode (WE) in the cell on the left and the Counter Electrode (CE) in the cell on the right separated by a Nafion Proton Exchange Membrane (PEM). Bottom: Schematic of a complete two-cell unit.

Figure 3. Chronoamperometry profiles of current (normalized to electrode surface area) versus incubation time in cathodic poised potential experiments at -200 mV (versus standard hydrogen electrode), with channels and conditions as listed as in Tables 1 and 2. A: NP11. B: NP12. C: NP13. D: NP14. E: NP15. Breaks in data records indicate periods when data were not recorded due to glitches in software. Note the breaks in the y-axes in the NP11, NP14, and NP15 experiments, and that ‘-’ here denotes current direction and not mathematical negative so increasingly negative values represent increasing current density of the electron flow direction out of the electrode.

Figure 4. Cyclic voltammetry (CV) scans of indium tin oxide (ITO) electrodes from cathodic poised potential experiments. In each plot, red lines are scans of electrodes immediately before incubation, blue lines are scans of electrodes immediately after incubation, and purple scans are from fresh ITO electrodes inserted into incubated media. Overall, electrodes have current peaks at -0.8, -0.35 and 0.2 V vs Ag/AgCl on both the incubated ITO electrode at end of experiment (blue) and on a fresh ITO electrode inserted into incubated media (purple), indicating redox couples likely equivalent to H₂/O, Fe₂/O₂ and an as yet unknown mechanism, respectively. Note that ‘-’ here indicates current and voltage direction and not mathematical negative so increasingly negative values represent increasing current density in the opposite electron flow direction. Plots are average of triplicate CV scans at 0.1 V/s (duplicate for NP11 Tend i, NP11 Tend ii, NP12 Tend i and NP13 Tend i).

Figure 5. Representative scanning electron microscopy (SEM) images of particle morphology observed on indium tin oxide (ITO) electrodes in NP14 Glass Wool (top), NP14 Basalt (middle), and NP14 fluid control (bottom) on termination of incubations indicates very small coccoid-shaped particles, possibly cells, adhering to the electrodes in the live samples. Scale bars 1 μ m.

Figure 6. Relative abundance of taxonomic groups at the Phyla level in poised potential experiment samples, based on 16S rRNA gene (V4-V5 hypervariable region) Illumina sequence data, with sample names matching those provided in Table 2. Sample order grouped by Treatment type, and taxonomic groups sorted as taxa that appear at least once in a no-template-control blank DNA extraction versus those that do not.

Figure 7. Relative abundance of Proteobacteria taxonomic families as a subset of data shown in Figure 6.

In review

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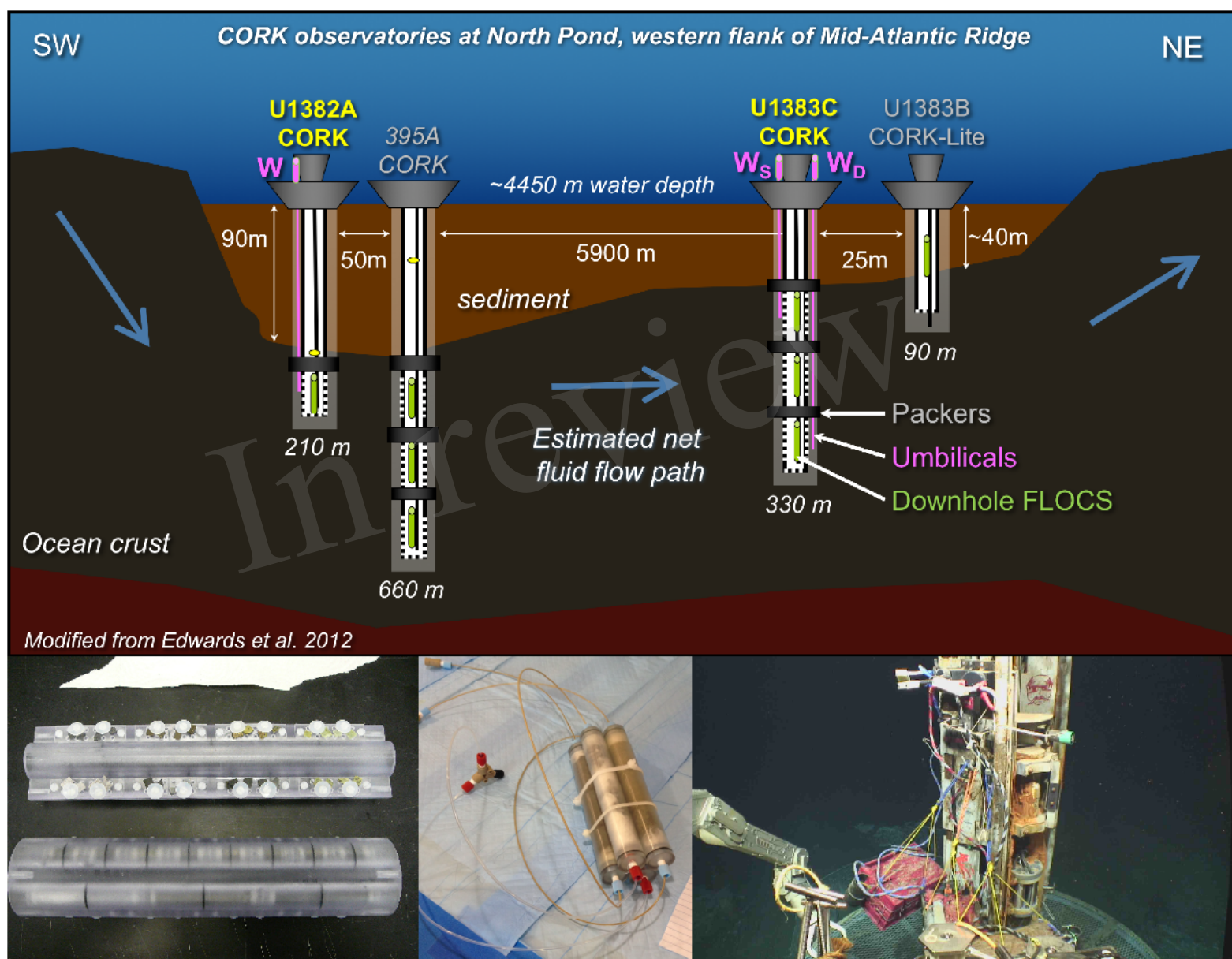
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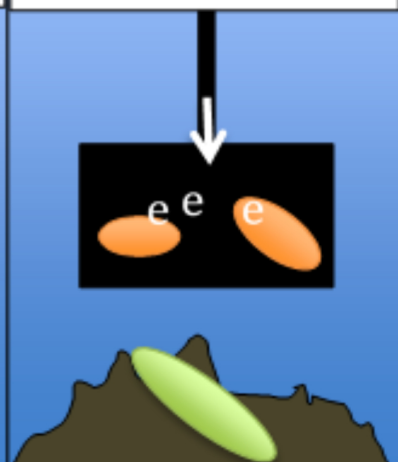
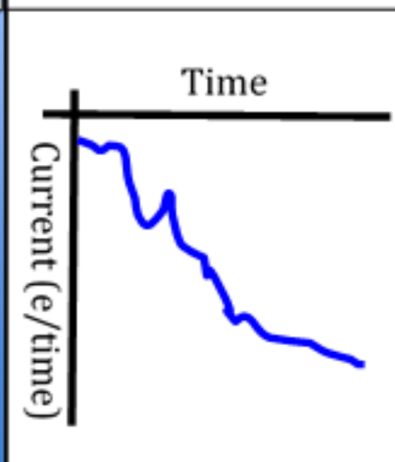
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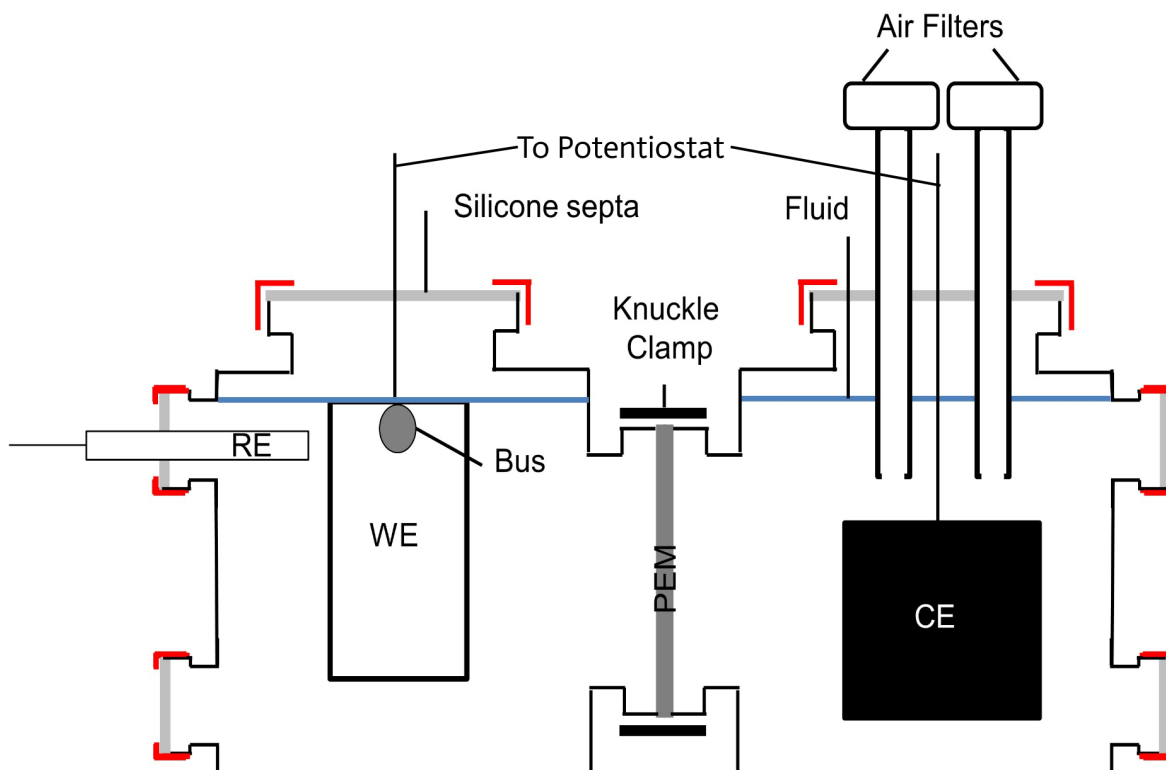
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In review

Figure 1.TIF



Rock community is a mix microbes of capable of EET and not	Selecting EET microbes by giving electrons at specific energy	Microbe activity changes electron demand and so rate of electrons/time
		



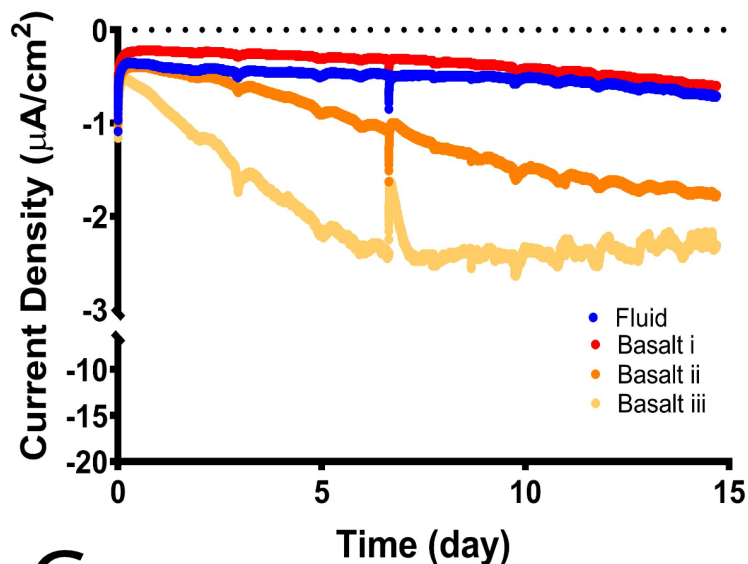
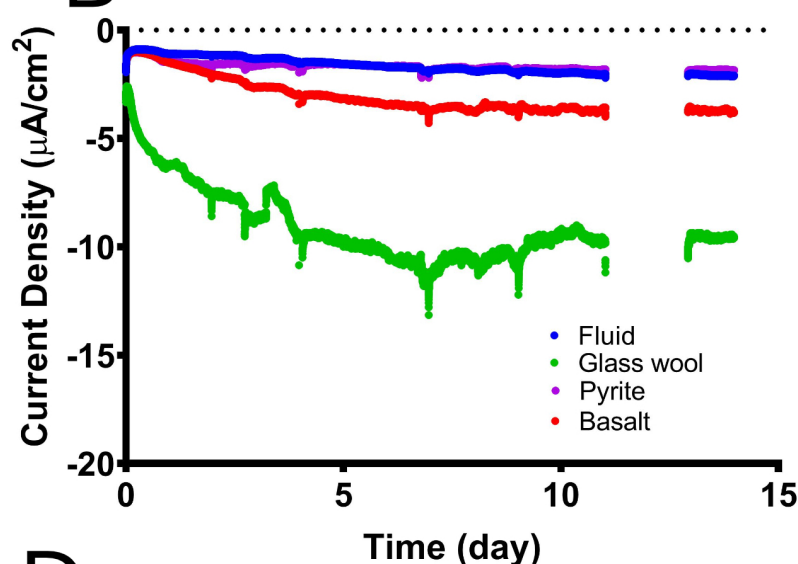
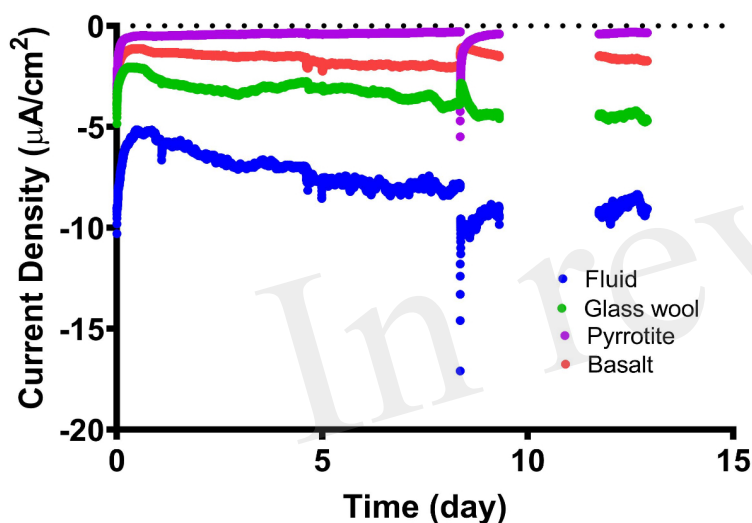
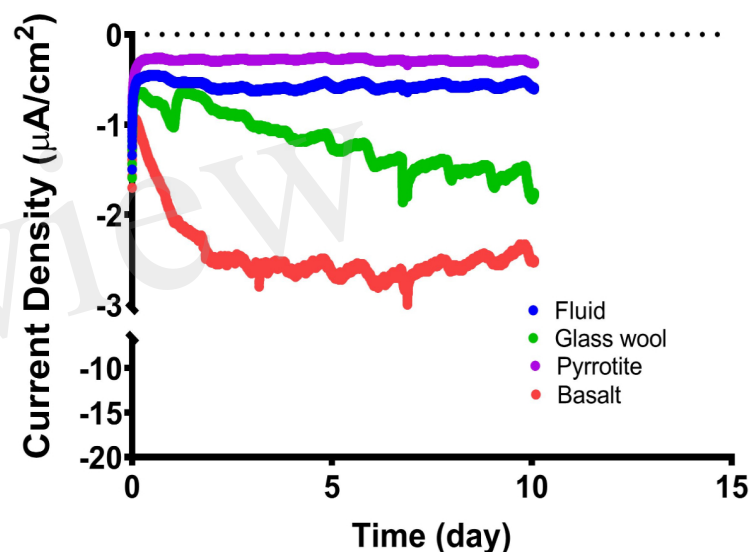
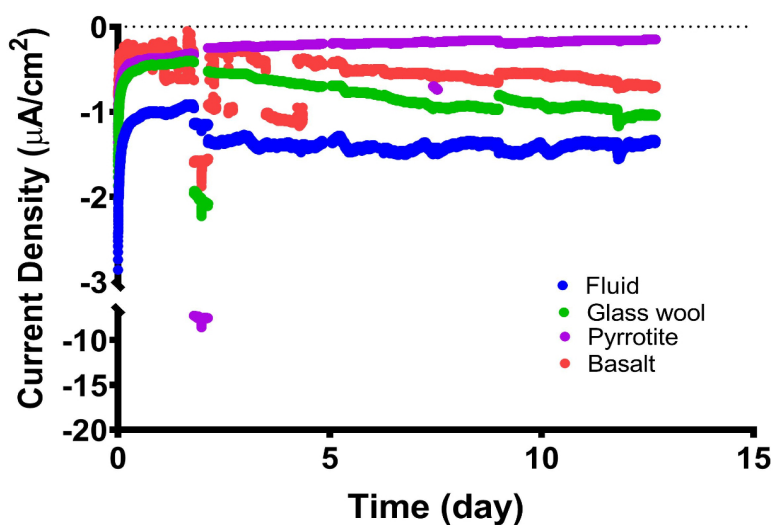
A NP11 - U1382A downhole**B** NP12 - U1382A downhole**C** NP13 - U1383C wellhead BW**D** NP14 - U1383C wellhead BW**E** NP15 - U1382A wellhead BW

Figure 4.JPEG

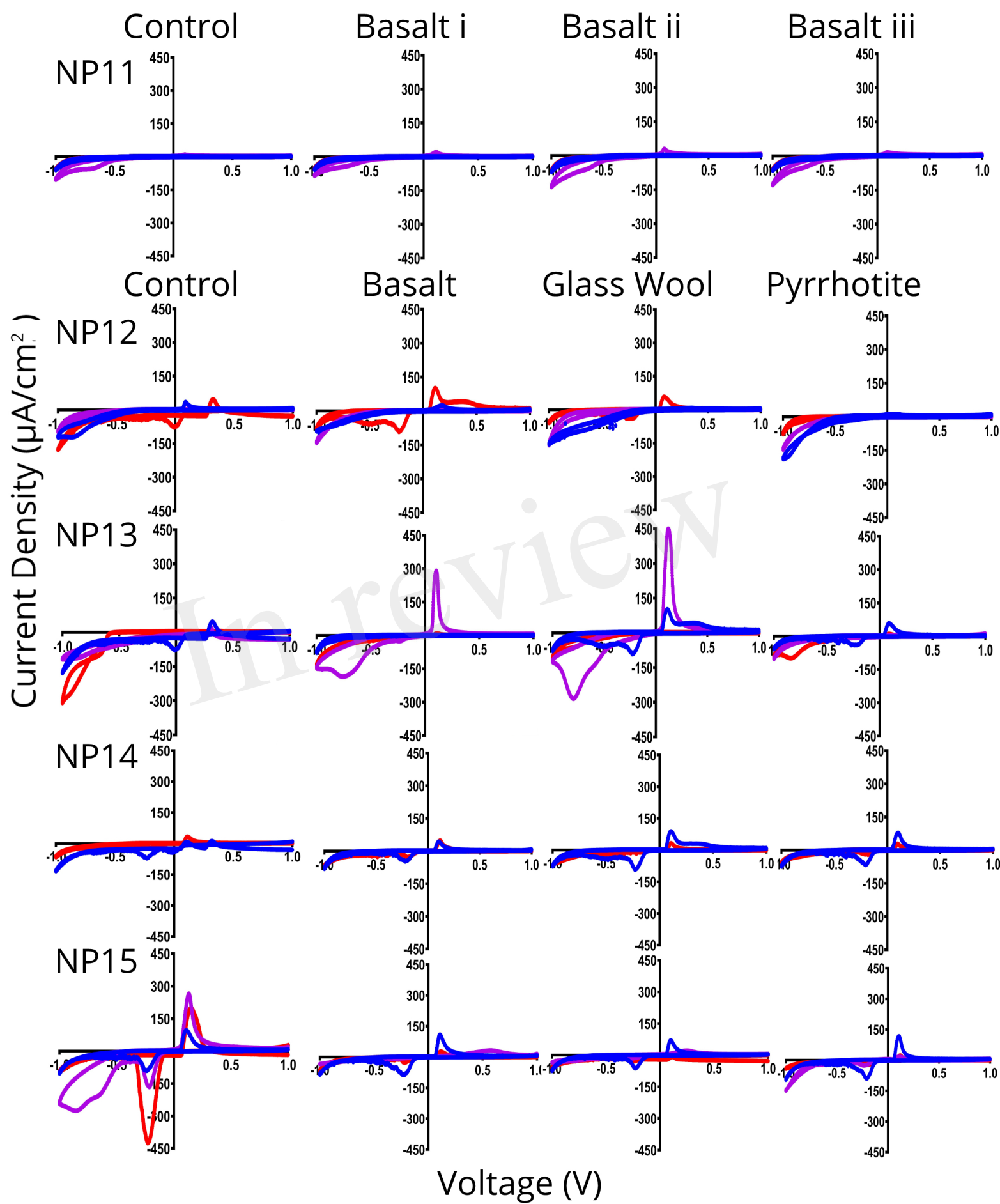
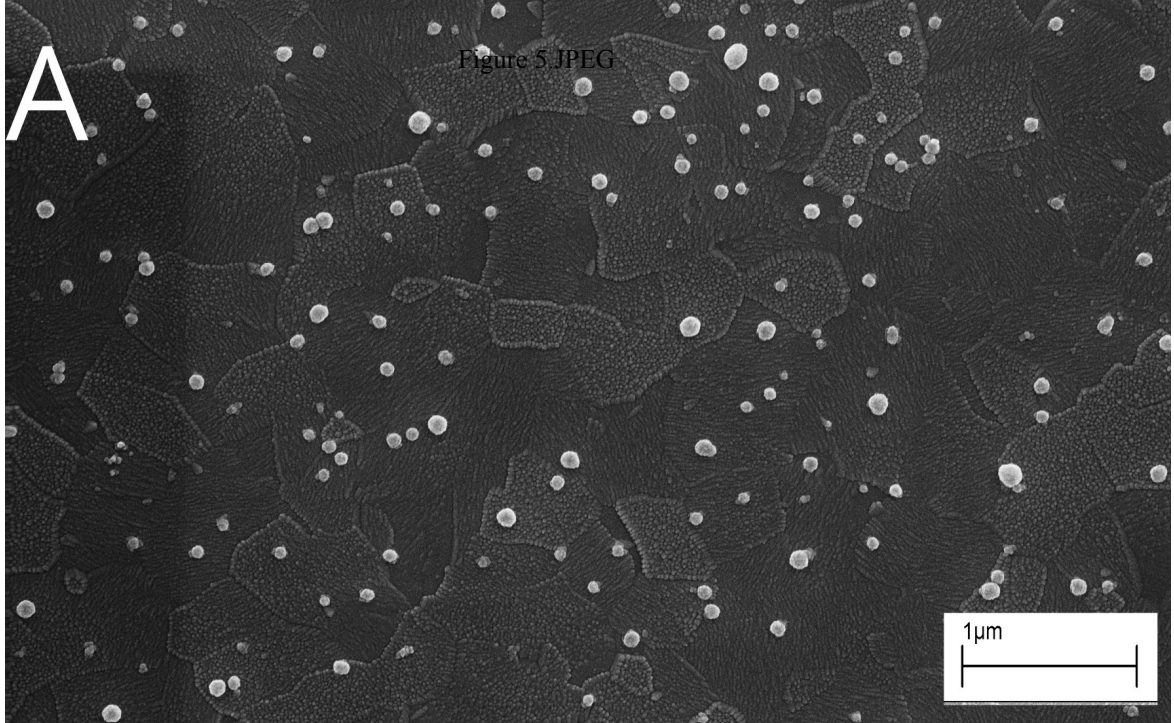
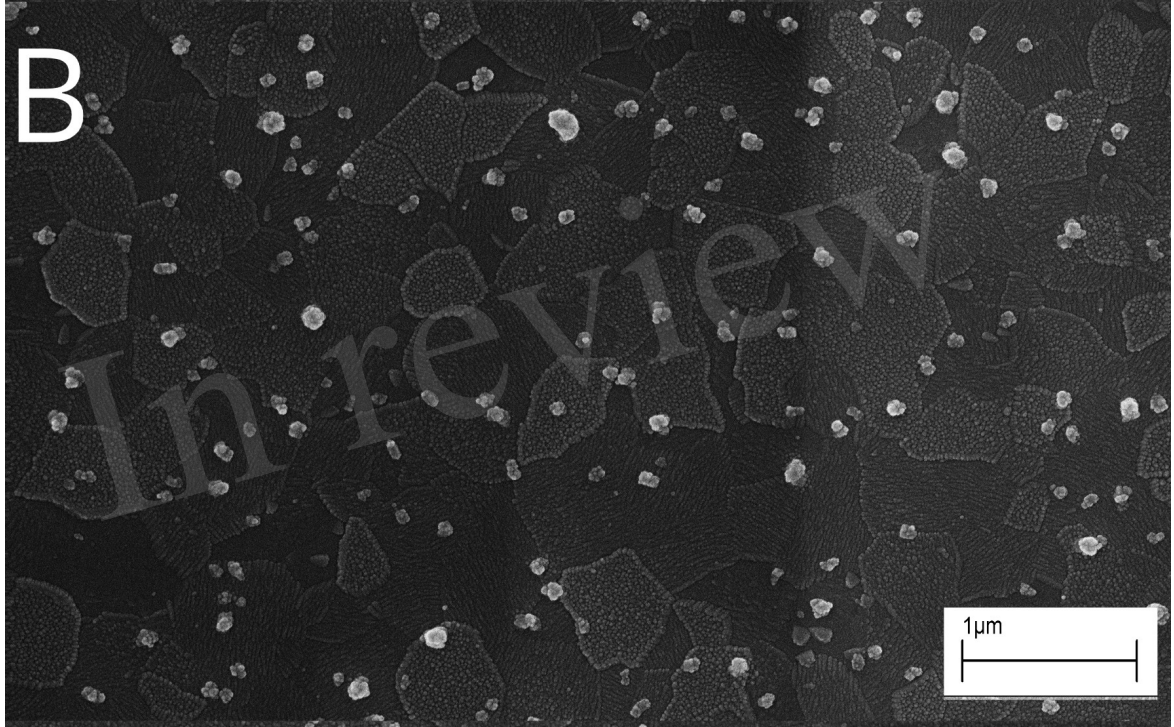


Figure 5.JPEG

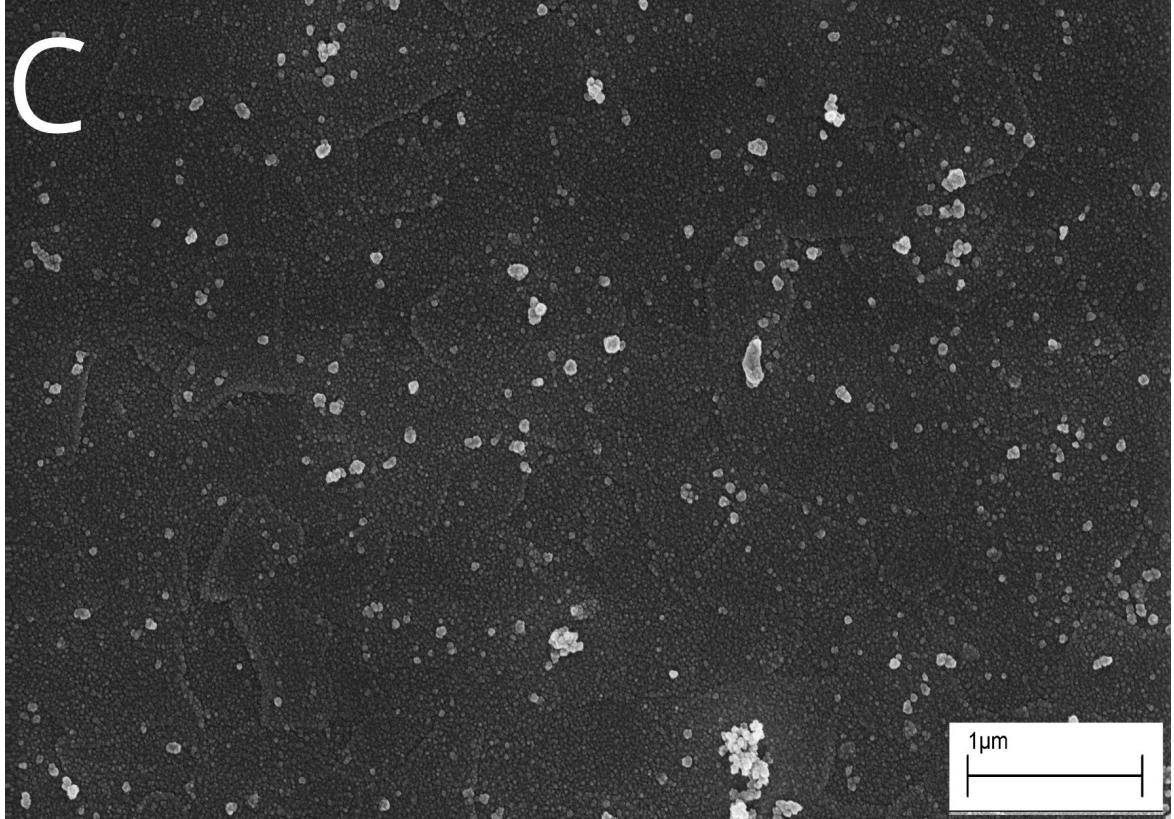
A



B



C



Phylum

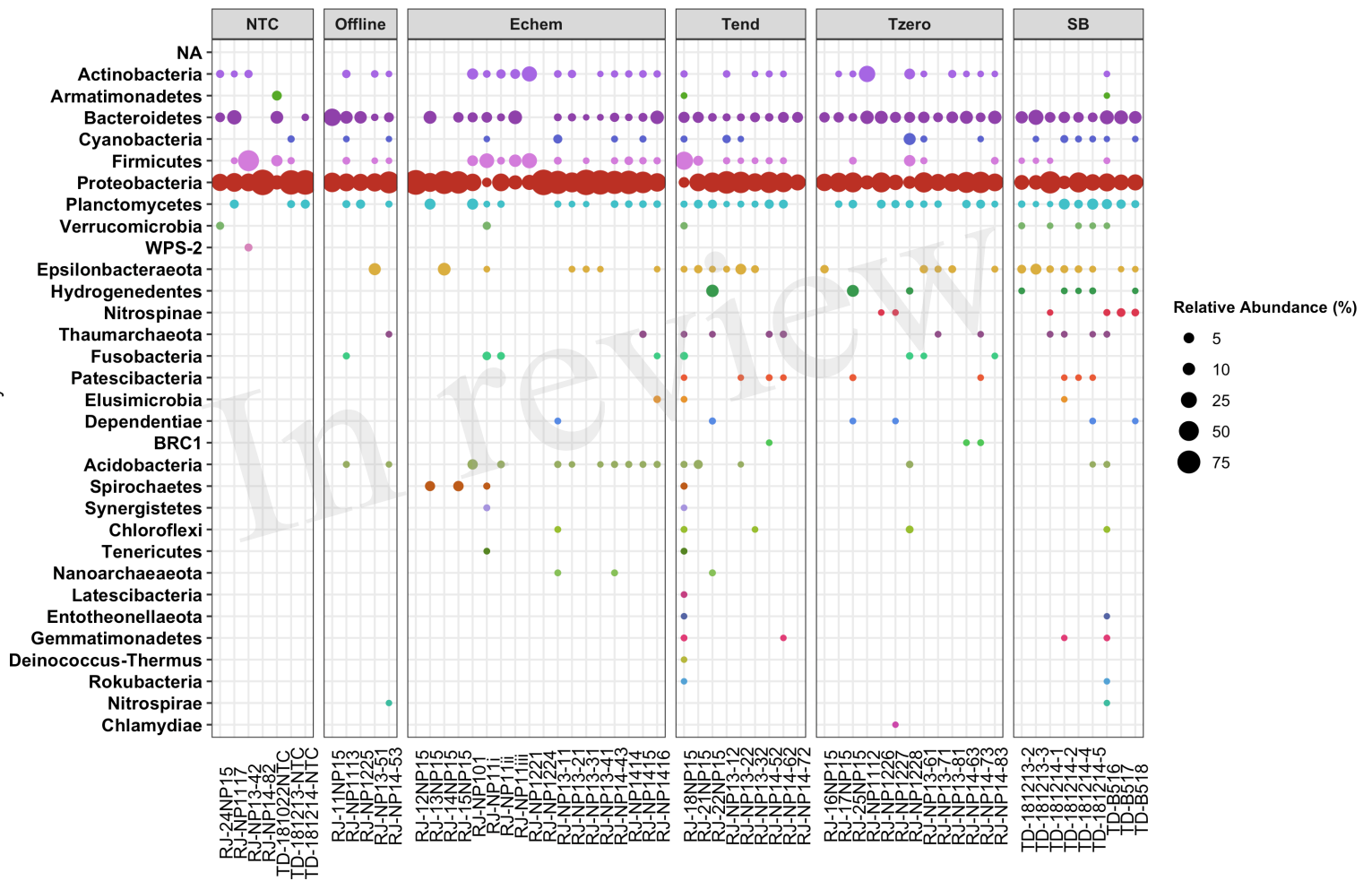


Figure 7.JPEG

