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Protozoan grazing reduces the current output of microbial fuel cells

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HIGHLIGHTS

- Inhibition of eukaryotic grazing increased power output of sediment MFC 2–5-fold.
- Geobacteraceae and *Euplotes* were enriched on current-harvesting anodes.
- Anaerobic protozoa prey on *G. sulfurreducens* in pure culture studies.
- Protozoan grazing can reduce current up to 91% in *G. sulfurreducens* fuel cells.
- Anode biofilms are 4-fold thinner in fuel cells with protozoa.

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ABSTRACT

Several experiments were conducted to determine whether protozoan grazing can reduce current output from sediment microbial fuel cells. When marine sediments were amended with eukaryotic inhibitors, the power output from the fuel cells increased 2–5-fold. Quantitative PCR showed that Geobacteraceae sequences were 120 times more abundant on anodes from treated fuel cells compared to untreated fuel cells, and that Spirotrichea sequences in untreated fuel cells were 200 times more abundant on anode surfaces than in the surrounding sediments. Defined studies with current-producing biofilms of *Geobacter sulfurreducens* and pure cultures of protozoa demonstrated that protozoa that were effective in consuming *G. sulfurreducens* reduced current production up to 91% when added to *G. sulfurreducens* fuel cells. These results suggest that anode biofilms are an attractive food source for protozoa and that protozoan grazing can be an important factor limiting the current output of sediment microbial fuel cells.
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1. Introduction

Although many applications have been proposed for microbial fuel cells, powering electronic monitoring devices with energy harvested from marine sediments is one of the few applications that appear to be currently practical (Franks and Nevin, 2010; Logan, 2009). However, the output of sediment fuel cells is relatively low (4–55 mW/m^2) (Girguis et al., 2010), which has led to many investigations designed to identify factors limiting current production in order to develop strategies to increase current output and expand the types of devices that can be powered with sediment fuel cells.

One strategy to enhance power output is to increase the anode surface area available for microbial colonization. Unfortunately, studies have shown that as the surface area is increased the power output may not scale linearly (Ewing et al., 2014) and large surface anodes are unwieldy and difficult to deploy. Another approach is to increase the supply of electron donor to anode microbes. Current output can be directly related to the rate that fermentable organic matter is degraded within sediments (Wardman et al., 2014; Williams et al., 2010), which can be increased with the addition of particulate organic matter (i.e. cellulose, chitin, or algal biomass) that can be fermented to electron donors that will support current production (Cui et al., 2014; Rashid et al., 2013; Rezaei et al., 2009). However, the technical difficulty of amending sediments with organic material, especially in deep environments, as well as the need for repeated additions of the organics for long-term deployments, limits the applicability of this approach. Another approach is to provide electron donors for current production within the anode itself (Nevin et al., 2011), but this strategy is not applicable

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for the multi-year operation desired for many applications of sediment fuel cells.

A proven strategy for increasing current densities in laboratory studies is to colonize anodes with those microorganisms that have the most effective current production capabilities. Characteristics that confer high current densities include the ability to directly transfer electrons to electrodes and the formation of electrically conductive biofilms that permit cells that are part of the anode biofilm but not in direct contact with anodes to contribute to current production (Lovley, 2012). To date, the combination of these characteristics has only been documented in members of the Geobacteraceae (Malvankar and Lovley, 2014). The common predominance of Geobacteraceae on anodes harvesting current from sediments and other environments suggests that microorganisms with these favorable characteristics already have a competitive advantage in colonizing anodes in many instances (Lovley, 2012). In laboratory studies it is possible to genetically engineer microorganisms with improved current capabilities (Leang et al., 2013), but the feasibility of preemptively colonizing anodes of sediment fuel cells with engineered microbes has not been intensively investigated.

In open environments, such as sediments, there may be factors other than substrate availability limiting the growth of Geobacteraceae in anode biofilms. For example, when the growth of *Geobacter* species in the subsurface was stimulated with the addition of high concentrations of electron donor in the form of acetate, a bloom of protozoa accompanied increases in *Geobacter* growth and substantially lowered the accumulation of *Geobacter* biomass below that expected in the absence of protozoan grazing (Holmes et al., 2013). An anode biofilm also represents a substantial enrichment of Geobacteraceae likely to enhance grazing opportunities for protozoa. Amoeboid protozoa have been shown to consume bacteria within a biofilm at rates of 1465 bacteria h^{-1} (Rogerson et al., 1996) and mixed cultures of amoeba and flagellates can consume 55–75% of the cells comprising a biofilm (Zubkov and Sleigh, 1999). Here we report on sediment and defined culture studies that suggest that protozoan grazing on anode biofilms may be an important factor limiting the current output of sediment microbial fuel cells.

2. Methods

2.1. Sediment microbial fuel cells

Marine sediments and overlying water were separately collected from The Great Sippewissett Marsh (West Falmouth, MA) as previously described Holmes et al. (2004) at a depth of 0.5 m in canning jars that were filled to the top and then sealed. Water from the sampling site was also collected in plastic containers as previously described Holmes et al. (2004). Six 1-L glass beakers were filled 1/4 full with anoxic sediment and then the beakers were completely filled with water from the site. Prior to fuel cell construction, three of the sediments were amended with 200 mg/L each of the eukaryotic inhibitors cycloheximide and colchicine. This concentration of inhibitors was selected because it has previously been shown to inhibit protozoan growth in sediment microcosms (Holmes et al., 2014; Schwarz and Frenzel, 2005). In order to ensure that the eukaryotic inhibitors could not act as electron shuttles, current generated by *Geobacter sulfurreducens* in two-chambered 'H-type' fuel cells with acetate (10 mM) provided as an electron donor in the presence of both inhibitors was monitored (Fig. 1). These pure culture studies showed that similar current outputs were obtained by *G. sulfurreducens* grown in the presence or absence of inhibitors, indicating that they could not act as shuttles. Power generated by sediment

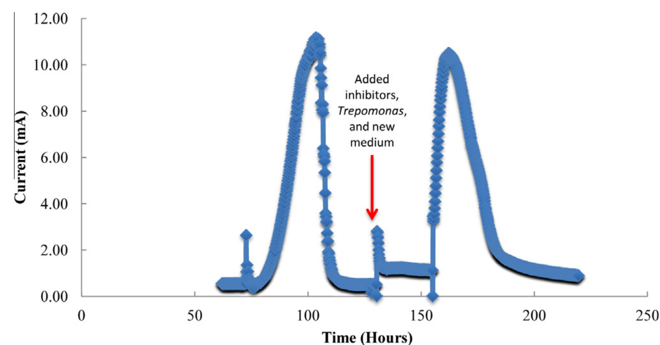

Fig. 1. Current generated by H cell inoculated with *G. sulfurreducens* strain PCA grown with acetate (10 mM) provided as the electron donor in the presence and absence of *Trepomonas* and eukaryotic inhibitors cycloheximide and colchicine.

fuel cells constructed with autoclaved sediments in the presence and absence of inhibitors was also monitored to ensure that the inhibitors did not have an abiotic impact on current produced by sediment fuel cells (Supplementary Fig. 1).

As previously described by Holmes et al. (2004), graphite (grade G10, Graphite Engineering and Sales, Greenville, MI) anodes (3.9 cm × 3.9 cm × 0.3 cm) were placed 2–5 cm below the sediment surface, and electrically connected to cathodes, constructed of the same size and material, that were suspended in overlying seawater, which was continuously bubbled with air. A resistor of 560 Ω was included in the circuit between the anode and cathode. Current and voltage measurements were collected with a Keithley model 2000 multimeter (Keithley Instruments, Cleveland, OH). Current produced by all 6 fuel cells was monitored for 43 days at an incubation temperature of 18 °C.

2.2. Defined culture studies

Trepomonas agilis strain RCP-1 (ATCC 50286), *Breviata anathema* (ATCC 50338), and *Hexamita inflata* strain AZ-4 (ATCC 50268) were purchased from the American Type Culture Collection (ATCC). *Heteromita* strain DH-1 was isolated from sediments collected from a uranium-contaminated aquifer located in Rifle, Colorado and has 18S rRNA and β-tubulin gene sequences that are 97% and 92% identical to the flagellate, *Heteromita globosa* (D. Holmes et al., manuscript in preparation). *G. sulfurreducens* was obtained from our laboratory culture collection.

Strict anaerobic culturing and sampling techniques were used throughout (Balch et al., 1979). All protozoan cultures were grown anaerobically with *G. sulfurreducens* provided as the food source. *B. anathema* was maintained on medium containing a 1:3 mixture of simplified ATCC medium 1171 (mucin, Tween-80, and rice starch were omitted) and standard ATCC medium 802 (Sonneborn's Paramecium medium); *T. agilis* was maintained on ATCC 1171 TYGM-9 medium; *H. inflata* was maintained on ATCC 1773 *Hexamita* medium; and *Heteromita* strain DH-1 was maintained on ciliate mineral medium consisting of (per liter distilled water): 0.125 g K_2HPO_4, 0.025 g NH_4Cl, 0.4 g NaCl, 0.2 g $MgCl_2{\cdot}6H_2O$, 0.15 g KCl and 0.25 g $CaCl_2{\cdot}2H_2O$, and 1% wheat starch (Sigma–Aldrich).

For batch culture grazing studies, *G. sulfurreducens* strain PCA^T (ATCC51573) was grown in a bicarbonate-buffered freshwater medium (Lovley and Phillips, 1988) with fumarate (40 mM) provided as the electron acceptor and H_2 as the electron donor at 22 °C in the dark under N_2–CO_2 (80:20). Once cells reached stationary phase (OD_{600nm} of ~0.8), protozoa were added to the medium. *G. sulfurreducens* cells were counted with acridine orange staining and epifluorescence microscopy as previously described (Hobbie et al., 1977). Cells were diluted 100-fold, fixed with a 10%

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