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HIGH-GRADIENT MAGNETIC SEPARATION METHODS FOR ANAEROBIC EXTRACTION OF MAGNETIC MINERALS FROM MARINE SEDIMENTS

Kyle S. Metcalfe and Victoria J. Orphan

Division of Geological and Planetary Sciences,
California Institute of Technology, Pasadena, California, 91125

ABSTRACT

Evidence suggests microorganisms inhabiting marine sediments may use magnetic minerals as conduits for extracellular electron transfer (EET). However, existing techniques to extract magnetic minerals do not maintain anoxic conditions necessary for downstream culturing of obligately anaerobic microorganisms. Here, I present anaerobic methods for the extraction of magnetic minerals from marine sediments, with the aim of using these extracted minerals for culturing of the attached microorganisms.

INTRODUCTION

Conductive magnetic minerals such a magnetite (Fe_3O_4) present in marine sediments may serve as a conduit for electron transfer between microorganisms living in these sediments. Experimental work with anaerobic enrichment cultures has shown that magnetite amendments can stimulate the metabolic activity of syntrophic microorganisms [1–6]. This suggests microorganisms employing EET may associate *in situ* with conductive minerals as a way to

facilitate electron transfer between syntrophic partners. Indeed, microorganisms known to employ EET as a core part of their metabolism such as *Geobacter* sp. have been shown to precipitate fine-grained magnetite [7, 8] although the degree of physical association between these authigenic phases and the cell is unclear. To examine microbial communities attached to magnetic minerals *in situ*, extraction of such minerals from the bulk sediment matrix must be performed.

Previous methods for extraction of magnetic minerals from marine sediment have primarily been performed under aerobic conditions [9]. These techniques employed high-gradient magnetic separation (HGMS) to extract magnetic minerals from marine sediment, as HGMS is effective for extracting fine-grained (< 100 μm) magnetic minerals [10–12] with crystal sizes similar to those of magnetite often observed in marine sediments [13]. These techniques have been primarily developed for paleomagnetic study of sediments, and thus have typically been unconcerned with maintaining anaerobic conditions. Also, methods developed for compatibility with downstream molecular biology work (e.g. [9]) also were not primarily concerned with maintaining anaerobic conditions, as a priority was placed on molecular study. However, this precludes the culturing of obligate anaerobes from these separation techniques. Syntrophic microorganisms found to be stimulated by magnetite enrichments (e.g. *Geobacter* sp. and *Methanosarcina* sp.) are sensitive to O_2, and thus using these techniques to extract such microorganisms for culturing would be ineffective.

Here, we present methods for the extraction of fine-grained magnetic minerals from marine sediments, while maintaining anaerobic conditions and other considerations necessary for downstream culturing work.

METHODS

Briefly, HGMS was performed on marine sediments by use of an apparatus in which a peristaltic pump circulated a diluted marine sediment sample through a magnetic column of nickel foam placed inside a magnetic field generated by a Frantz L-1 Isodynamic Separator (S.G. Frantz Co., Trenton, NJ). The methods described here were developed further from those detailed by Harrison and Orphan, particularily their use of a magnetic column (in their case using steel wool as the magnetic matrix) inside a Frantz separator. The methods presented here differ from previously-published methods in several respects. This method focuses on highly magnetic minerals (cheifly, magnetite), given the strength of the evidence for the capability for this mineral to stimulate syntrophic EET. This method also maintains anaerobic conditions in the separation apparatus, useful for downstream culturing.

HGMS Apparatus Assembly

Before assembly of the main apparatus, 2 butyl stoppers penetrated each by 4 sharp 17G 3.5” nickel-plated cannulas with Luer hubs (Cadence Science, Cranston, RI) were prepared for subsequent sterilization by autoclave. The apparatus consisted of 11 lengths of Cole-Parmer Masterflex® silicone tubing, 95802-05, ID = 0.125”, OD = 0.25” (Vernon Hills, IL) and were autoclave-sterilzed along with the butyl stoppers, Cole-Parmer polypropylene Luer fittings, 1x 250 mL and 1x 1 L Pyrex bottles with a magnetic stir bar placed in the 250 mL bottle, 1x 125 mL serum vial, and nickel foam for the magnetic column (see “HGMS Column” below). Tubing was connected with fittings (fittings were wrapped in Parafilm to aid in preventing O_2 leaking into the

line) to create 3 parallel circuits (Figs. 1, 2) for use in circulating in sequence 1) the diluted sediment sample through the magnetic column to collect magnetic minerals in the column while the Frantz was active , 2) 3X PBS for removal of residual non-magnetic minerals from the line, and for 3) elution of magnetic minerals into a 125 mL serum vial after the Frantz was turned off. Circulation was controlled by a Cole-Parmer Masterflex L/S peristaltic pump.

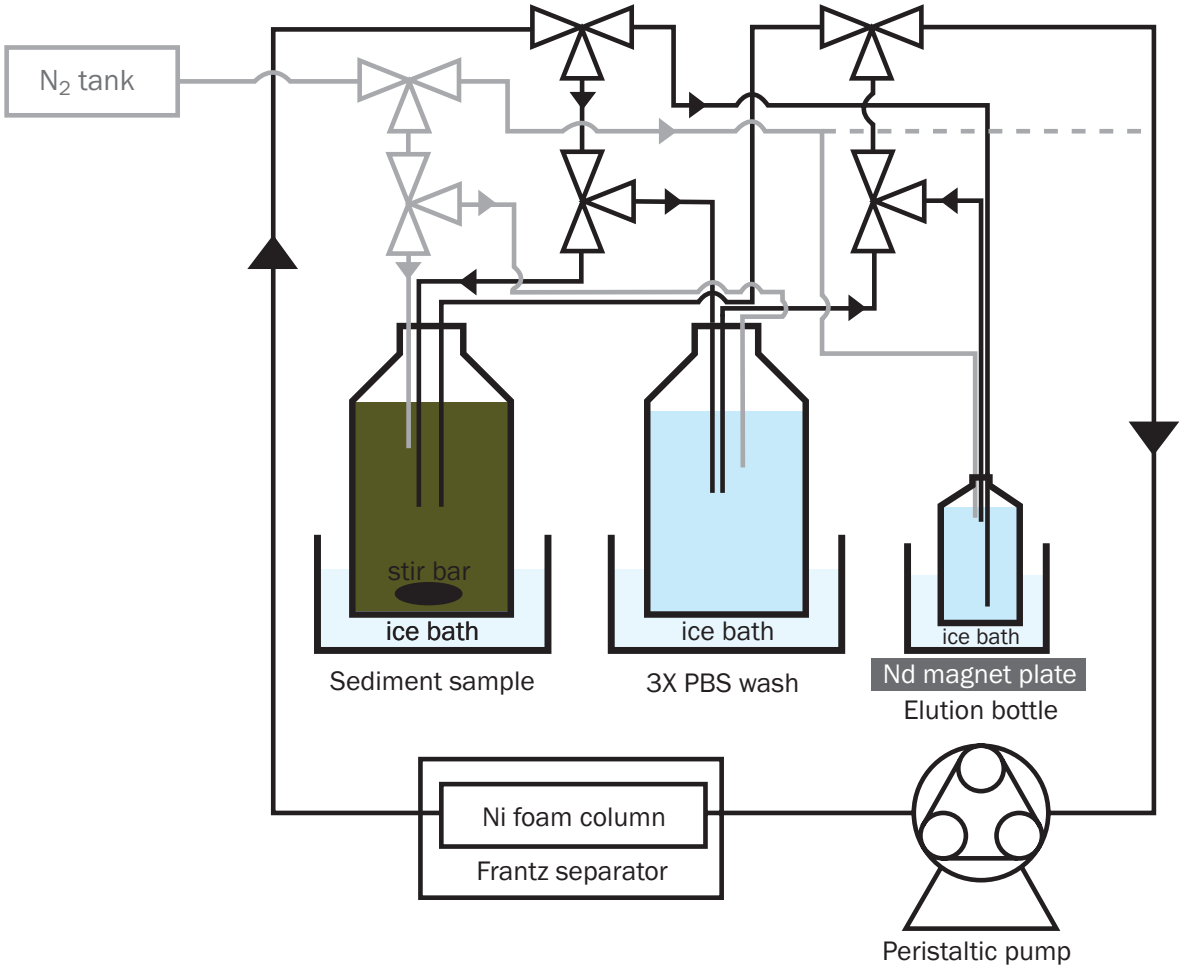

Figure 1. Schematic of anaerobic HGMS apparatus. N_2 vents for bottle sparging are omitted for clarity. Dashed line indicates temporary connection to sparge line with N_2.

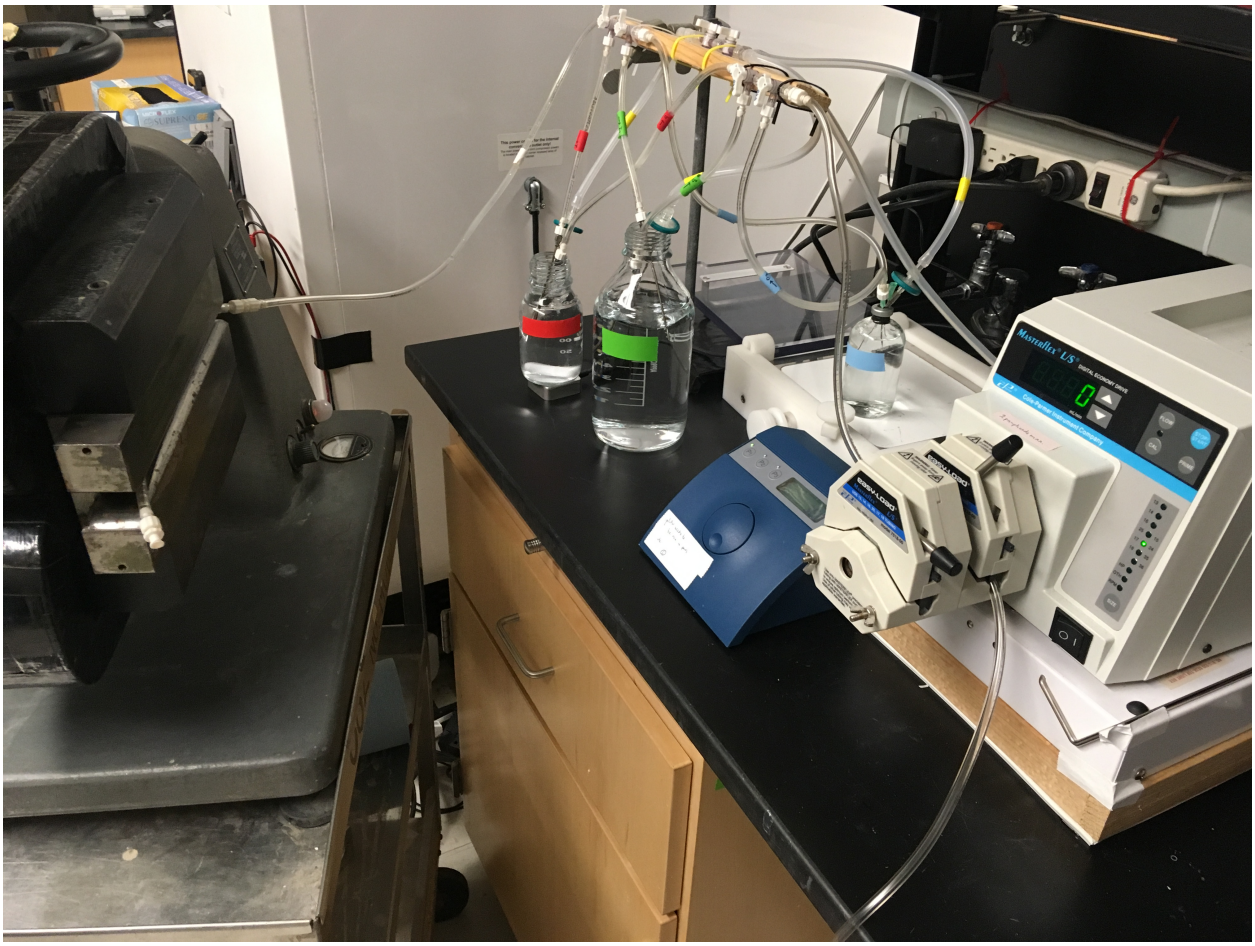

Figure 2. Picture of the apparatus during an early stage of development. Omitted in this image are the prepared butyl stoppers with sharp cannulas and the ice baths. The connection between the peristaltic pump and the column was removed to position the Frantz separator at an angle as to easily image the full apparatus. Lines are labeled in accordance to a correponding bottle color label. Lines labeled with yellow are N_2 sparging lines.

HGMS Column

One autoclaved segment of tubing, cut to the length of the Frantz separator chute, was prepared for use as the magnetic column. Nickel foam (Item no. EQ-bcnf-16m-2, 80-110 pores per inch, pore diameter = 0.25 mm, MTI Corporation, Richmond, CA) was used as the magnetic matrix for the column, selected for its similar filament size but superior porosity to steel wool or thin wire used in previous studies to extract fine-grained magnetic minerals [9]. Ni foam was prepared for use in the column using a 0.125" diameter hole punch (Mayhew Steel Products, Turners Falls, MA) to create 50 Ni foam discs. These discs were placed in a small beaker and autoclaved. NiO produced on Ni foam surfaces from autoclaving (which reduces recovery of magnetic minerals by ~50%) was removed by cleaning Ni discs within the beaker in a sonication bath of 37% HCl for 5 min. The hazardous $NiCl_4^{2-}$ complex produced from this sonication was disposed in an appropriate waste container, and Ni foam was then cleaned by three 5 min washes of 100% ethanol in a sonication bath. Waste ethanol was placed in a separate hazardous waste container. Ni foam was dried under UV-C light and added to the autoclaved tubing segment by use of a sterile cannula. The cannula was magnetized by a Nd magnet through the wall of a sterile glass test tube and used to pick up Ni foam discs for placement in the column under a sterile flame. Fittings were placed on the ends of the column and the column was then connected with the rest of the apparatus.

HGMS Operation

After the apparatus was constructed, 1x 250 mL, 1x 1 L, and 1x 125 mL 3X PBS were filter-sterilized (0.2 μm) into the sterile Pyrex bottles and serum vial and N_2 sparged for 15 min in an ice bath. These vessels were sealed with butyl stoppers and 400 mM Na_2S was added to each vessel to obtain 4 mM Na_2S to remove residual O_2. These bottles were placed in a 10˚C cooler until use. Three ice baths were placed on the lab bench adjacent to the Frantz separator for placement of the Pyrex bottles and serum vial. A submersible stirrer with external control was placed in the water bath.

The Pyrex bottles were then prepared and attached to the apparatus, taking care to quickly perform these next steps as to maintain anaerobic conditions in the bottles. First, the two autoclaved butyl stoppers prepared with sharp cannulas were placed in a Coy anaerobic chamber along with the 250 mL and 1 L bottles retrieved from the 10˚C cooler. The butyl stoppers sealing the Pyrex bottles were quickly exchanged for the stoppers prepared with sharp cannulas, and the top of the Pyrex bottles were wrapped in Parafilm to provide a temporary seal. Bottles were then removed from the chamber, placed in the ice baths, and connected to the apparatus via Luer fittings (Fig. 1). These bottles were then continuously sparged with N_2 during the operation of the apparatus. Additionally, the N_2 line for sparging the 125 mL bottle during elution was temporarily reconnected to the segment of the apparatus including the column to remove as much O_2 as possible from the line (Fig. 1). The immersed stirrer was set to 750 RPM.

10 mL of sediment sample (~2:1 water:sediment) was then injected into the 250 mL Pyrex bottle containing the stir bar. N_2 sparging of the column and connected lines was stopped and

circulation of the sediment sample through the apparatus was initiated by switching three-way valves to only circulate through the sample bottle. The peristaltic pump was then set to a flow rate of 40 mL/min and the Frantz separator was switched on and set to 0.3 A, targeting magnetite [14]. Sample was circulated through the apparatus for 13 min, equivalent to >2 complete circulations of the sample bottle volume.

After 13 min, the 'wash' step was performed to dilute contaminating minerals in the line from the first magnetic capture step. The peristaltic pump was paused and the three-way valves swiched to only circulate through the 1 L 'wash' bottle of 3X PBS with 4 mM Na_2S. Circulation of the wash bottle then commenced at the same flow and magnetic parameters as used in the initial magnetic capture step.

After 13 min of circulation of the wash bottle, the elution step was performed. The 125 mL serum vial for capturing eluted magnetic minerals was retrieved from the 10˚C cooler, placed in an ice bath, and attached to the apparatus using 1x 7 cm 22G needle (inflow to vial) and 1x 2.5 cm 22G needle (outflow from vial). Sparging with N_2 was initiated using 2x 2.5 cm 22G needles. The peristaltic pump was again paused, three-way valves switched appropriately, and the Frantz separator was switched off. Elution was initiated by setting the peristaltic pump to a flow rate of 40 mL/min. Circulation proceeded for 7 min, after which the peristaltic pump was shut off and the bottle was detached from the apparatus by needle removal. The bottle was then placed in a 4˚C cooler for downstream culturing efforts.

Results from Control Experiments

To test the efficacy of the methods presented here, a control experiment was performed in which recovery of fine-grained magnetite synthesized following published protocols [15] mixed with nonmagnetic clay (ISCz-1, Clay Minerals Society, Chantilly, VA) was measured. A mixture of 34.7 mg synthetic fine-grained magnetite and 500 mg ISCz-1 was suspended in 250 mL DI and circulated through the apparatus following the protocol detailed above. The mass of magnetic particles recovered in the elution bottle was measured by filtration of the elution bottle through a 0.4 μm polycarbonate filter. After drying and weighing, 26.3 mg material was recovered on the filter, corresponding to >70% recovery; no more than ~0.5 mg of the material remaining on the filter was estimated to be ISCz-1, as this is approximate mass of ISCz-1 per mL after dilution from the volume of the wash bottle. Negligible magnetite was retained on the magnetic stir bar, as observed with natural sediment samples.

Preliminary Results

Recovery of magnetic minerals and attached microbial communities using the HGMS apparatus detailed here was performed on a methane seep sediment sample from bottle PC-KD (see Chapter 2 of this thesis for sampling details). The elution bottle was prepared using artificial seawater media from published work [16] and was pressurized to 2 bar with CH_4. Epifluorescence microscopy was performed on PFA-fixed samples from the elution bottle, revealing the presence of microbial aggregates with morphology similar to that of ANME-SRB consortia (Fig. 3). Reflected light microscopy revealed the presence of reflective domains ~2-3 μm in diameter,

potentially representing magnetite attached to ANME-SRB exteriors. 16S rRNA amplicon sequencing of these incubations documented abundant reads assigned to the Heimdallarchaeota. These incubations were maintained at 4˚C for future experimental work.

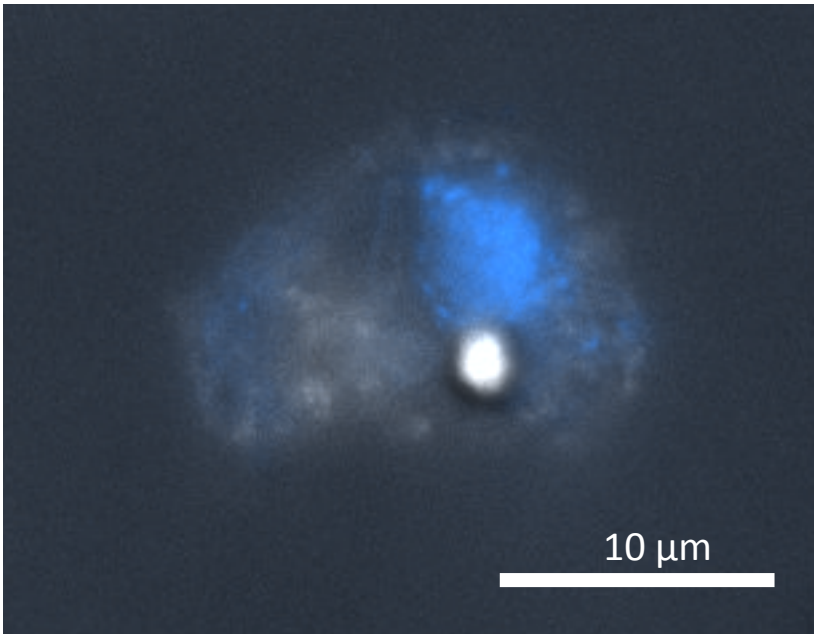

Figure 3. Epifluorescence microscopy of microorganisms recovered from HGMS separation of methane seep sediment. Putative ANME-SRB consortia were stained by DAPI (*blue*) and were observed attached to reflective particles (*white*).

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