

Attracting Resident Magnetotactic Bacteria in the Little Sippewissett Salt Marsh

Christa Heyward

Introduction

Magnetotactic bacteria are anaerobic, sulfate-reducing bacteria characterized by an ability to mineralize magnetite (Fe_3O_4) or greigite (Fe_3S_4) (Lefevre 2011). Chains of magnetite or greigite produce a magnetic moment or torque that allows this organism to orient itself and swim along the earth's magnetic field (Lins, 2003, Simmons 2007, Lefevre 2011). Magnetite crystals are primarily bullet- or arrowhead- shaped, while greigite crystals have irregular shapes (Lefevre 2011). Most magnetotactic bacteria produce either magnetite or greigite; however, in some cases the production of magnetite or greigite can be influenced by the concentration of hydrogen sulfide available during growth (Lefevre 2011). In this case the production of magnetite or greigite is regulated by expression of separate clusters of *mam* genes, which are required for magnetosome synthesis (Lefevre 2013a).

One type of magnetotactic bacteria, called Many-celled Magnetotactic bacteria or MMBs, only exists as aggregates of 10-60 genetically identical cells with a central core where cells are absent (Simmons 2007, Lefevre 2011). The MMB structure is surrounded by a polysaccharide layer (Keim 2004). Individual cells contain chains of greigite magnetosomes with an average diameter of 60-90nm (Simmons 2007 (Pósfai 1998)). MMBs are broadly distributed in microaerophilic and anaerobic environments and in both fresh and salt water environments (Simmons 2007). MMBs are highly motile, with individual cells all possessing flagella; however, the

mechanism by which the movement of the aggregate is coordinated has yet to be determined (Simmons, 2007). Observation of cell motility reveals a 'ping pong' motion as they reach the edge of a water droplet, which varies according to the strength of the magnetic field applied (Simmons 2007 (Greenberg, 2005)).

The MMB prefer low-sulfide environments ($<100\mu\text{M}$) and the greatest concentrations are found at the interface between oxic and anoxic zones. MMBs can be found in close proximity to photo and chemo-synthetic sulfide oxidizers such as the purple sulfur bacteria that form the berries in Little Sippewissett salt marsh. MMBs have been isolated from the slightly sulfidic sediments of Little Sippewissett salt marsh previously (Simmons 2007, Shapiro 2011). The goal of this project was to compare the composition of MMBs among four berry ponds found in the Little Sippewissett salt marsh (Figure 1A).

Materials & Methods

Sample Collection.

All magnetotactic bacteria were collected from the Little Sippewissett salt marsh, Falmouth, MA. MMBs were obtained from four ponds, all with visible aggregates of purple sulfur bacteria known as berries. For this reason, each collection pond is identified as a berry pond followed by a corresponding number based on the work of previous investigators at the site (See Figure 1A and work by E. Wilbanks from a previous Microbial Diversity course.) Sediment cores were also collected at berry ponds 1 and 2 and the number of MMBs present was counted from the sediment

surface to a depth of up to 50mm below the sediment surface at increments of 3mm. Cores were obtained using a modified syringe (Figure 1E) and 3mm of sample was



Figure 1: MMB collection

A. Map of the Little Sippewissett sampling site. B. Berry pond 2 with fluffy Thiothrix indicative of a high sulfur environment. C. High Sulfur berry pond with milky white water containing elemental sulfur. D. The same pond as C when the water is clear. Elemental sulfur is still visible at the sediment layer. E. Sample sediment core. F. Example of 3mm core samples enriching for MMBs.

placed in cups with magnets attached (Figure 1F). Samples were gently shaken for 15-30 seconds and then incubated for at least 30 minutes to allow for the sediment to settle and MMBs to migrate to one of the magnets. Samples visualized by microscope were always taken from the north-facing magnet for consistency. To prepare

the hanging-droplet slide 5 μ L of bacteria were collected using a pipette and placed on the coverslip.

Wolfes's Mineral Solution (1L)

500mL of Milli-Q H₂O, 1.5g Nitritotriacetic Acid, pH solution to 6.5, 3.g of MgSO₄ * 7 H₂O, 0.5gMnSO₄ * H₂O, 1g NaCl, 0.1g FeSO₄ *7H₂O, 0.1g CoCl₂ *6H₂O, 0.1g CaCl₂, 0.1g

ZnSO₄ * 7H₂O, 0.01g CuSO₄ * 5H₂O, 0.01g AlK(SO₄)₂ * 12 H₂O, 0.01g Na₂ MoO₄ * 2H₂O, 0.01g H₃BO₃. Bring volume up to 1000mL. Store in the dark at 4°.

Basylnski's Complex anaerobic liquid growth medium (1L)

Basal Media: 20g NaCl, 6g MgCl₂, 3.24g NaSO₄, 1g CaCl₂, 0.55g KCl, 2.4 g HEPES.

To 1L of Basal Medium add: 0.2mL of 1% resazurin, 0.3g NH₄Cl, 0.2g yeast extract and 1g Casamino acids. The medium was bubbled with N₂ for 30 minutes and sealed using the Hungate Method and the Super Special German Anaerobic caps. Medium was then autoclaved. After autoclaving and cooling the medium was placed inside the anaerobic chamber. Once in the chamber 5mL of anaerobic Wolfe's mineral solution, 0.5mL of anaerobic vitamin solution, 2.8mL of 0.5M KHPO₄, 2.8mL of filter-sterilized, anaerobic 0.8M NaHCO₃ 28mL of XM NaSO₄, 5 drops of 1M H₂S (where each drop is approximately 10uL) and 11.3mL of filter-sterilized XM cysteine-HCl.

Sheri Simmons' MMB isolation medium (20mL/vial)

Filter-sterilize marsh seawater sample. Add 20mL of filtered seawater and 2µL of resazurin to Hungate vials and bubble with N₂ while heating. Seal using the Hungate method. Add 100µM Na₂S (It took three extra drops from the 0.1M Na₂S bottle to completely eliminate any pink from the resazurin).

Alternative Salt Water Medium (1L)

970mL of Salt Water Base (lab manual recipe), 2.4g HEPES, 1mL of 1M K₂HPO₄, 0.3g of NH₄Cl, 7g of Sodium Acetate, and 2µL of 1% resazurin. Medium was bubbled with N₂ for 30 minutes and sealed using the Hungate method and the Super Special German Anaerobic caps. Autoclave medium. After medium has cooled, place the medium in the anaerobic chamber and add 5mL of anaerobic filter-sterilized Wolfe's

Mineral solution, 1mL of filter-sterilized, anaerobic 100X vitamin solution, 2.8mL of 0.8M NaHCO₃, 2.0mL of 0.2M Cysteine-HCl, 1.6mL of 1M Na₂S, and 28mL of 1M Na₂SO₄.

MMB Enrichment

Sheri Simmons' MMB isolation medium was used during the enrichment of MMBs for their use in downstream applications such as electron microscopy, community analysis and confocal microscopy. Samples of marsh sediment were collected from 4 different berry ponds at Little Sippewissett Salt marsh and brought back to lab. In lab, the samples were mixed gently and then magnets were affixed to the sides of the collection with the following orientation. S→N:bucket: S→N. Magnetotactic bacteria were allowed to aggregate at the magnets for at least an hour but not more than 2 or 3 as this retains the MMBs in the oxic portion of the sample, an environment which is not favorable for their survival. After an hour, MMBs were removed using a needle and syringe and placed in one of the 3 media described above. MMBs from multiple enrichments were pooled and the process was repeated multiple times moving sample from the first anaerobic vial to the next. After 3 consecutive enrichments samples were processed for scanning electron microscopy.

Microscopy



Migration of MMBs along a magnetic field was visualized on the Zeiss Axio **XXX** microscope

Figure 2: Sample hanging droplet slide

5uL of sample is placed on a coverslip and inverted over a washer.

using the hanging droplet method (Figure 2). The hanging droplet method is essential for permitting migration of MMBs across the slide. Samples were also processed for scanning electron microscopy. MMBs harvested using magnets were enriched in Sheri Simmon's Filtered Sea Water medium and then isolated again using a 1mL needle. ~100uL was removed from the vial by removing the cap in a hood and using the syringe to remove the volume nearest the North-facing magnet. ~100mL was placed in 10mL of 2% glutaraldehyde diluted in PBS. Samples were fixed overnight at 4°C and filtered onto 2µm filter paper. The filter was subsequently washed three times with PBS and the allowed to air dry. The filter was then passed through serial dehydration treatments using 50%, 70%, 85%, and three times 100% ethanol washes. Samples were then taken to the microscopy facility for critical point drying, sputter coating and visualization on a Zeiss Supra 40VP scanning electron microscope.

Clone Library Production

Droplets obtained using the enrichment scheme described above were viewed. After confirming the presence of MMBs, the sample was collected from the coverslip and used for community analysis. Each sample was boiled for 15 minutes at 95°C for 10 minutes and then 2µL of sample was added to PCR tubes to amplify the 16S sequence. The presence of 16S was confirmed by gel electrophoresis. Afterwards bands from each berry pond were cut out of the gel and purified using the Omega Bio-Tek Gel extraction kit according to the manufacturer's instructions. The fragments were subsequently cloned into the TOPO-TA vector and electroporated into TOP 10 One Shot electrochemical cells according to the manufacturer's

instructions. Samples were plated on LB ampicillin plates at either a 1:10 or 1:25 dilution and incubated at 37°C overnight followed by monitoring for colony formation. Ninety-six colonies were then picked for sequencing in order to determine the composition of MMBs at each berry pond location.

The resultant sequences were then compared to those in the NCBI database using the online version of BLAST and analyzing 20 sequences at a time. The BLAST results were then reviewed for any matches with previously identified magnetotactic bacteria. Putative magnetotactic sequences were then used to create a phylogenetic tree with their closest neighbors using ARB SINE alignment service. To create the tree, a fasta file and an ARB database with near full-length bacteria sequences were downloaded to a working directory (using the term `wget` and pasting the link into the terminal). Open these databases by typing `tar xvzf` and then the file name into the terminal. Then type `arb` to open the tree analysis program. Import the sequences you identified (must be `.txt` not `.doc`) and then click `fasta`. With `gap`. Click `use found names` and then close the resultant window. Now store imported sequences before they have been altered by clicking the “# marked” tab. Once you can view your sequences, change all - - - dashes to ... so that ARB knows that these are missing sequences and not insertions or deletions. Click the dashes before and after your sequences to and then push the period button to change the dashes to dots. Go to the alignment editor and change the view to “hide gaps.” Click “tree,” “add species to existing tree,” “ARB Parsimony –Quick add marked” and then pick the appropriate reference bacteria. The tree was pruned by removing all but the most closely related sequences to the sequences. Mark a subset of bacteria to

use for the tree using the sequences identified as closely related to the magnetotactic bacterial sequences using the BLAST results or from broader resources such as publications. Build your reference set by highlighting sequences of interest. This will cause the number of marked sequences to increase. Left click to select, right click to deselect. Sequences can be searched by accession number by clicking species, search and query, change search fields to accession and type in the accession number.

Results & Discussion

Previous work done by course alumni Orr Shapiro and Roland Hatzenpichler and subsequently published used magnetotactic bacteria obtained from berry pond 1, which they identified as having the highest concentration of magnetotactic bacteria. All ponds contain elevated concentrations of sulfur and it was hypothesized that higher levels of sulfur thought to be favorable for MMB sulfate reduction. In contrast to berry pond 1, berry pond 2 had large fluffy growths of *Thiothrix*, an organism associated with high levels of sulfur (Figure 1B). Berry pond 4 was predicted to have the highest levels of sulfur because the water was a milky white color at the time of sampling, although water was not collected at that time (Figure 1C). Taking a sample of surface water at the time of collection and subsequent analysis using ion and liquid chromatography is recommended in the future. This information would be using in helping to explain potential differences in the concentration of MMBs in different berry ponds in the future. At a later visit to berry pond 4, the water was not a milky color but did show signs of elemental sulfur deposits on the sediment floor

(Figure 1D). This change is indicative of the dynamic environment that is characteristic of the Sippewissett salt marsh. In addition, longitudinal analyses of ion concentrations as well as tracking of MMB numbers (or other microbes of interest, i.e. berries, acromatium, etc) will provide valuable insight into habitat preferences. The number of magnetotactic bacteria from each berry pond was counted using the hanging droplet method and using a magnetic field to collect the bacteria at the North-facing or South-facing edge of the water droplet on the microscope stage. The number of north-seeking bacteria and south-seeking bacteria were quantified separately. Previous literature notes the predominance of North-seeking bacteria in the northern hemisphere and South-seeking MMBs in the southern hemisphere; however, in some samples, roughly equivalent numbers were recovered for each and variation correlated more with sample sight than with seeking direction. Berry Pond 2 possessed a greater density of MMBs compared to

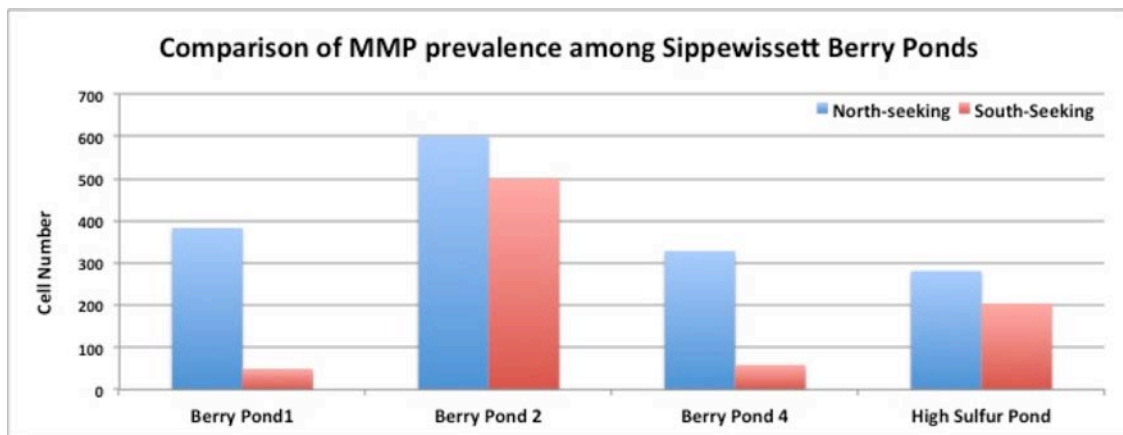


Figure 3: MMB Density among Little Sippewissett Berry Ponds

Samples from four locations in the Little Sippewissett salt marsh were assessed for the density of MMBs. Data were collected for North-seeking MMBs and South-seeking MMBs.

the other three ponds analyzed (Figure 3). Exact numbers were difficult to quantitate from berry pond 2 due to the density of cells at the droplet edge. The star in Figure 3 indicates that the number shown is probably a drastic under-estimate of the actual numbers. The identification of berry pond 2 as having the highest concentration of berries is in contrast to the findings of previous projects. It is possible that this finding reflects the variability in the environment or collection methods. In the future, it is recommended that samples be collected at several times of day, at various tides, and repeated on multiple days. The numbers here reflect densities obtained from a single visit to the marsh. In addition, aliquots of MMBs from the same sample should be counted multiple times and subsequently averaged.

In addition to comparing the quantity of MMBs between ponds, the distribution of MMBs at various depths below the surface of the sediment was also assessed

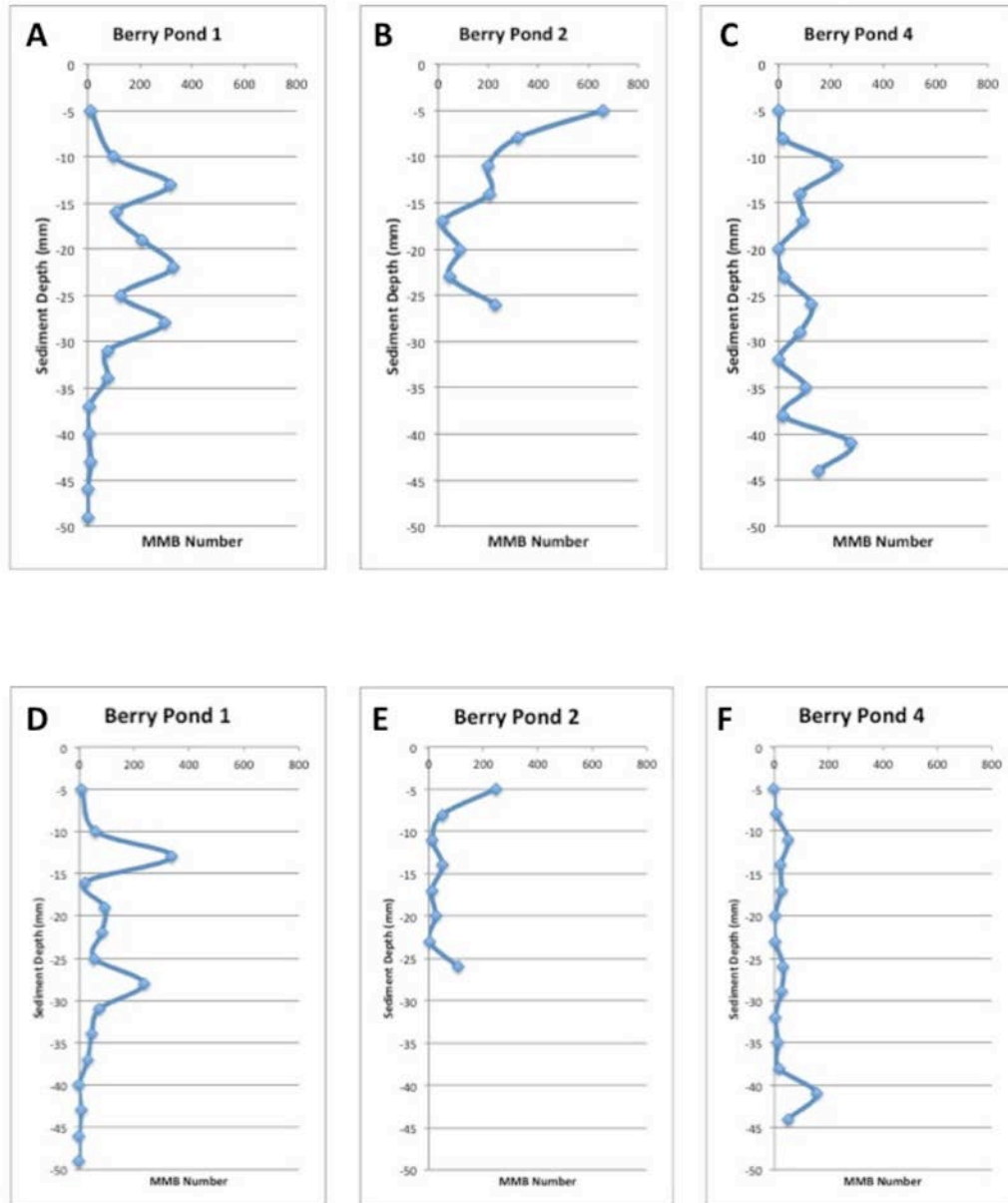


Figure 4: Daytime density of MMBs with increasing sediment depth
MMBs were collected during the day and counted at 3mm increments for North-seeking (A-C) and South-seeking (D-F) aggregates from berry ponds 1, 2, and 4.

(Figure 4). No specific depth was determined to be optimal for MMB growth. MMBs were found at most depths studied but the numbers appear to decrease about 30-

40mm depth. Due to the fact that the greatest concentrations of MMBs are found at the interface between oxic and anoxic zones and this interface changes with the time

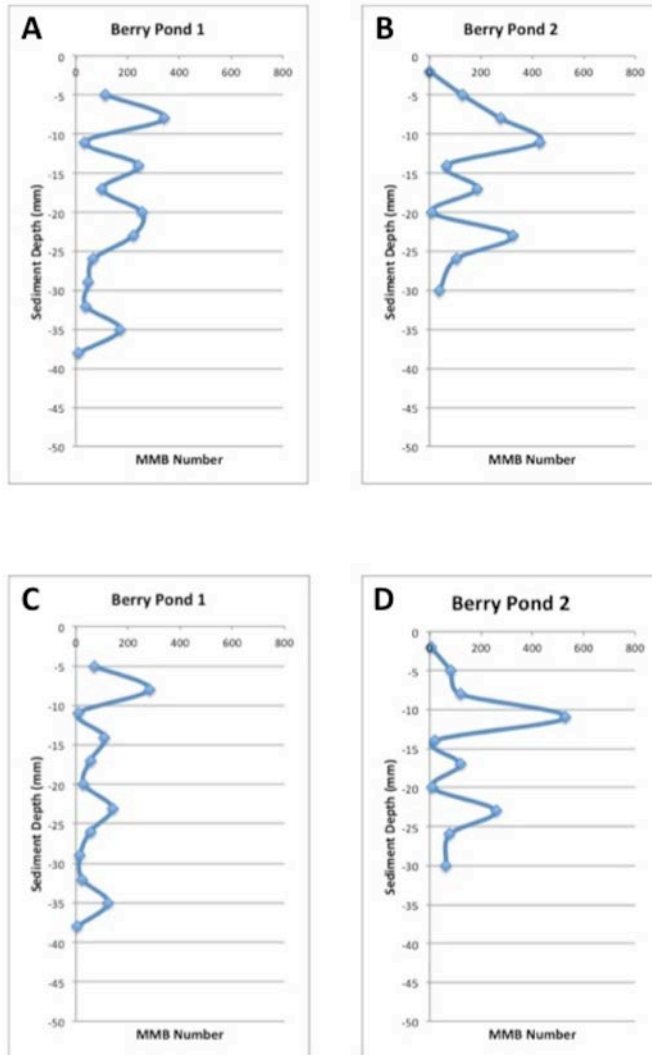


Figure 5: Nighttime density of MMBs with increasing sediment depth

MMBs were collected at night and counted at 3mm increments for North-seeking (A&B) and South-seeking (C&D) aggregates from berry ponds 1 and 2.

of day and that previous miniprojects suggested that the depth at which you find MMBs may change in a diurnal fashion (Shapiro et al.), the depth at which the most MMBs can be found was

compared between day and night samples (Figure 5). Comparison of Figure 4A and 5A, 4B and 5B, 4D and 5C, and 4E and 5D

reveals opposite trends in MMB numbers indicating that this aspect requires

further investigation

before a thorough

conclusion can be reached.

Similar to the findings of

Shapiro and Hatzenpichler, the number of MMBs retrieved from MMB 1 was higher

at night than during the day; however, the opposite was true for berry pond 2. The aggregate structure of MMBs found in the Little Sippewissett salt marsh was

confirmed by visualization using scanning electron microscopy (Figure 6A). Figure 6 shows several images of MMB associations containing 20 or more cells. In several

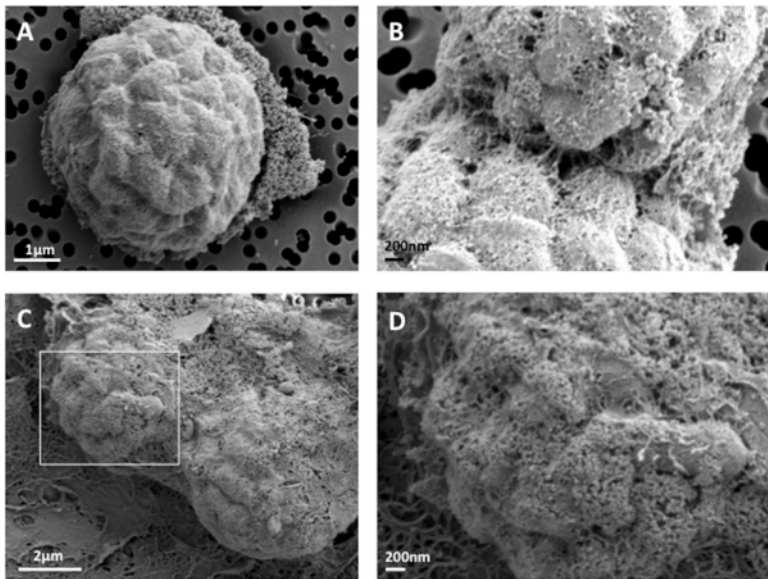


Figure 6: Scanning Electron Micrographs of MMBs

A. An MMB aggregate. B. Region connecting two MMB aggregates. C. Two MMB aggregates where the polysaccharide covering has been damaged. D. A magnification of the box in C.

cases, two aggregates were seen in very close association with one another and these associations may represent occurrences where an aggregate is dividing into two (Figure 6 B & C). While MMBs were not found as

individual cells, in some cases the outer polysaccharide covering was disrupted during processing and individual rod-shaped cells could be seen in the aggregate (Figure 6D). In the future, developing a protocol for disruption of the aggregates before SEM in order to visualize the internal structure would be interesting. Flagella were not present on the surface of the MMB aggregates. These structures may not

be maintained throughout the sample preparation procedure. The association of these cells during the fixation and SEM preparation process cannot be ruled out and therefore no concrete conclusions can be reached concerning the nature of this particular interaction.

Finally, the diversity of MMBs in the Sippewissett salt marsh samples was

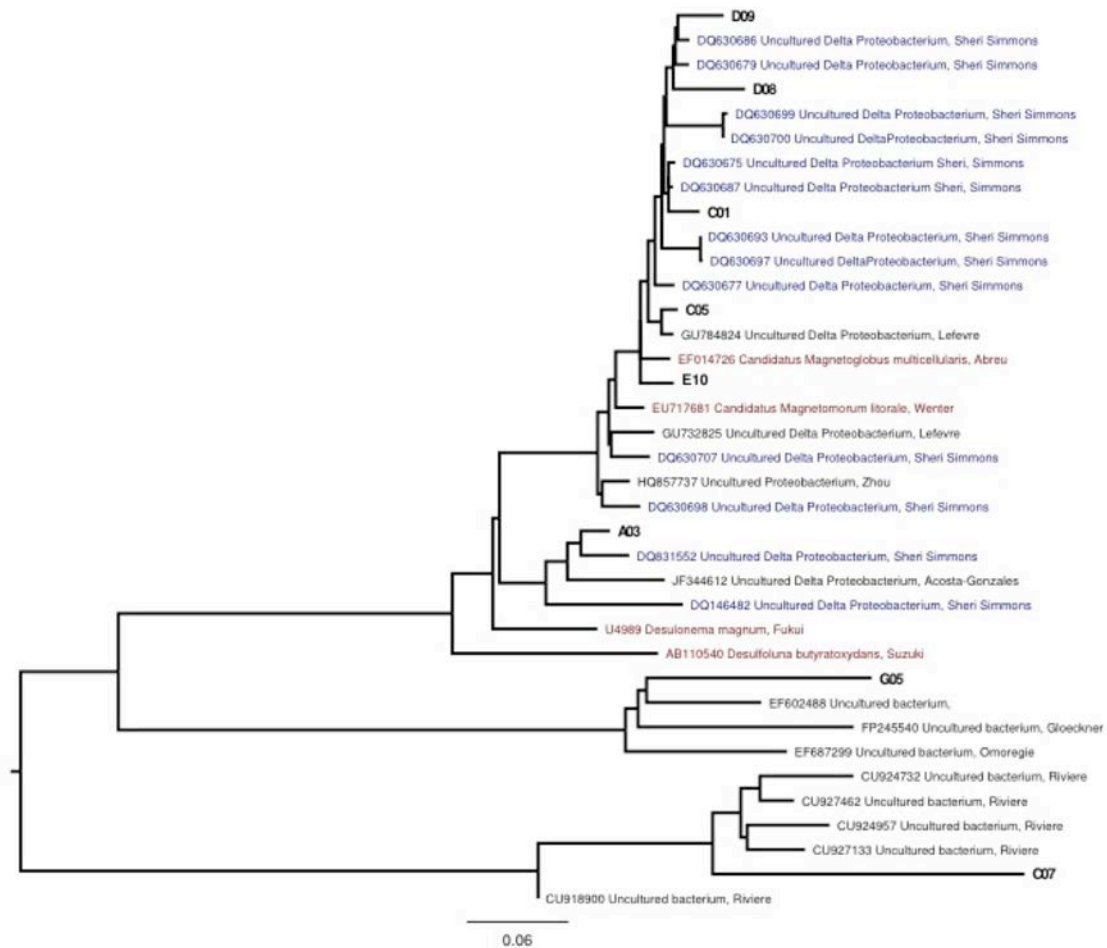


Figure 7: Phylogenetic analysis of Little Sippewissett MMB enrichments
 The 16S sequence for D09, D08, C05, C01, D10, A03, G05, and C07 was compared to the NCBI BLAST database to identify closest relatives. Sequences identified as belonging to MMBs are highlighted in Blue. Sequences from candidate or confirmed MMB species are highlighted in red.

investigated by colony PCR of the 16S sequences. The majority of 16S sequences turned out to be from bacteria that are not magnetotactic such as Thiomicrospira, Oceanospirillaceae and other common marine bacteria. The results of 16S sequencing identified eight sequences potentially from MMBs based on comparison to the NCBI BLAST sequence database. Of these eight sequences, four, C01, C05, D08 and D09 were closely related to sequences for MMBs from the Sippewissett salt marsh submitted by Sheri Simmons. Each of these isolates was obtained from berry pond 2. Sequence E10 was very closely related to the candidate MMB species isolated by Abreu, *Candidatus Magnoglobus multicellularis*. A03 was somewhat related to *Desulfonema magnum* a magnetotactic bacterial species isolated by Suzuki et al.

Much remains to be learned from the magnetotactic bacteria of Little Sippewissett salt marsh. Suggestions for the future include isolation of magnetotactic bacteria early on in the course followed by the subsequent genome sequencing by 454. Although this was undertaken by Esther Singer in the past, Esther notes that her results contained considerable contamination from other organisms, especially Eukaryotes. As magnetotactic bacteria have yet to be isolated, this will most likely continue to be an issue. The media suggestions in the methods and materials were included as a starting place for individuals looking for protocols for the potential isolation of MMBs. Although this was an original goal of mine, time constraints did not permit the completion of this goal. I used Sheri Simmons' media protocol which does require heating in addition to the bubbling in order to get completely anoxic

media. Questions still to be answered include, how do these organisms communicate with each other and how motility is coordinated, both of which are more complex than can be addressed in a six-week course. Good luck to the next student who takes the torch up on this project. The magnetos will not disappoint.

References

- Greenberg, M., Canter, K., Mahler, I., and Tornheim, A. Observation of magnetoreceptive behavior in a multicellular magnetotactic prokaryote in higher than geomagnetic fields. (2005) *Biophysics Journal* **88**: 1496-1499.
- Keim, C.N., Abreu, F., Lins, U., Lins de Barros, H., and Farina, M. Cell organization and ultrastructure of a magnetotactic multicellular organism. (2004) *Journal of Structural Biology* **145**: 254-262.
- Lefèvre, C.T., Menguy, N., Abreu, F., Lins, U., Pósfai M., Prozorov, T., Pignol, D., Frankel, R.B., and Bazylinski, D. A Cultured Greigite-producing magnetotactic bacterium in a novel group of sulfate-reducing bacteria. (2011) *Science* **334**: 1720-1723.
- Lefèvre, C.T., Trubitsyn, D. Abreu, F., Kolinko, S., Jogler, C., Paula de Almeida, L.G., de Vasconcelos, A.T.R., Kube, M., Reinhardt, R., Lins, U., Pignol, D., Schüler, D., Bazylinski, D. and Ginet, N. Monophyletic origin of magnetotaxis and the first magnetosomes. (2013a) *Environmental Microbiology* **epub**
- Lefèvre, C.T., Trubitsyn, D. Abreu, F., Kolinko, S., Paula de Almeida, L.G., de Vasconcelos, A.T.R., Lins, U., Schüler, D., Ginet, N., Pignol, D., Bazylinski, D. Monophyletic origin of magnetotaxis and the first magnetosomes. (2013b) *Environmental Microbiology* **epub**
- Lins, U., Freitas, F., Keim, C.N., Lins de Barros, H., Esquivel, D.M.S., and Farina, M. Simple homemade apparatus for harvesting uncultured magnetotactic microorganisms. (2003) *Brazilian Journal of Microbiology* **34**: 111-116.
- Shapiro, O.H., Hatzenpichler, R., Buckley, D. H., Zinder, S.H., and Orphan, V.J. Multicellular photo-magnetotactic bacteria. (2011) *Environmental Microbiology Reports* **3**: 233-238.
- Pósfai M., Buseck, P.R., Bazylinski, D., and Frankel, R.B. Iron sulfides from magnetotactic bacteria: structure, composition, and phase transitions. (1998) *American Mineral* **83**: 1469-1481.
- Simmons, S.L., and Edwards, K.J. Unexpected diversity in populations of the many-celled magnetotactic prokaryote. (2007) *Environmental Microbiology* **9**: 206-215.