# Enrichments for phototrophic iron and manganese oxidizers from Little Sippewissett and School Street Marsh

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## *Summary*

The phototrophic oxidation of manganese was proposed to predate oxygenic photosynthesis based on biochemical and geochemical reasoning, however no modern-day organism with this capacity is known. In this project inoculum from Little Sippewissett and School Street Marsh was used to enrich for novel Fe(II) oxidizers and potential phototrophic Mn(II) or Mn(III) oxidizers. Sulfate reducers defined initial enrichments, in part likely because the previously sourced iron rich mats from School Street marsh had not sufficiently well developed after a flooding. In subsequent enrichment attempts these contaminations were minimized and the enrichments characterized by pigment analysis and microscopy. Several phototrophs growing at high manganese concentrations were found.

## *Electron donors for photosynthesis*

Photolithoautotrophs use radiant energy and inorganic electron donors to catalyze the reduction of $CO_2$ into organic molecules [1]. Oxygenic phototrophs use $H_2O$ as electron donor, while anoxygenic phototrophs can utilize reductants such as reduced carbon compounds, $H_2$, $H_2S$, Fe(II), $NO_2^-$ and As(III). [2-4]

Oxygenic photosynthesis, which first evolved in cyanobacteria and later via chloroplasts spread to algae and plants, is the predominant form of primary production in the biosphere. It is able to catalyse the four-electron transfer required for the oxidation of water to molecular oxygen by utilizing two photosystems acting in concert. This architecture increases the redox potential of electrons via the absorption of two photons and thereby creates sufficient oxidizing power for the photolysis of water. In contrast, anoxygenic photosynthetic bacteria have a single photosystem that catalyzes one-electron transfer reactions and ultimately depends on electron donors that have redox potentials significantly more negative than the water/oxygen pair ($E_0$'=+0.82V; *c.f.* Figure 1). Over the last two decades several species of purple bacteria have been described that use Fe(II), As(III) and $NO_2^-$ to donate electrons to reaction centers, whose midpoint potential at physiological pH typically is about +0.45V. The ecological niches for metal oxidizing forms of photosynthesis arguably have been much more widespread before the biological invention of oxygenic photosynthesis.

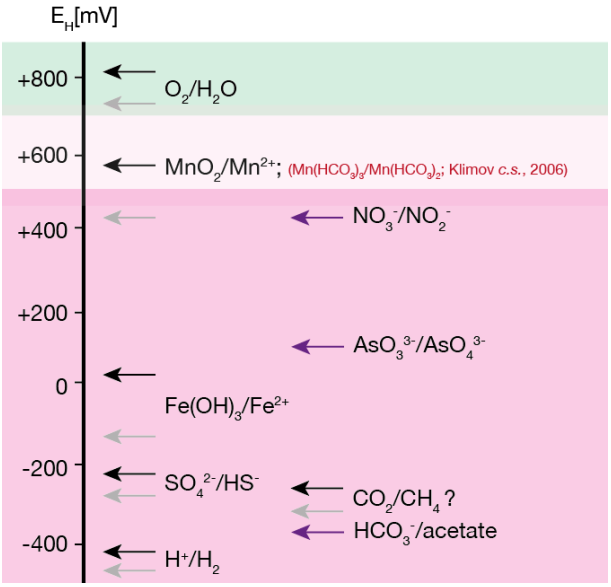


Figure 1. Redox potential of some important redox couples in water at pH 7 (black arrows) and pH 8 (grey arrows) taken from Sigg *et al.* [5] Values indicated by purple arrows are standard redox potentials taken from other literature [1,3,6]. Pink and green areas indicate redox potentials below the midpoint potential of a typical bacterial reaction center at +450mV and PSII, respectively.

Manganese is the second most abundant transition metal after iron in the Earth crust (approx. 950ppm) and it is an important inorganic cofactor of many enzymes including superoxide dismutase, ribonucleotide reductase and the water-oxidizing complex of photosystem II. Furthermore, the ionic radius of hydrated $Mn^{2+}$ and $Mg^{2+}$ are similar so that the two divalent ions compete for binding to nucleic acids.

In the environment manganese occurs in three different oxidation states, +II, +III, and +IV [7]. In general, Mn(II) is thermodynamically favored in the absence of oxygen and at low pH, whereas Mn(III) and Mn(IV) become more favorable in the presence of oxygen and at high pH. Mn(II) is the principal soluble form, and can exist at up to millimolar concentrations in waters, even in the presence of oxygen. Various bacteria, including cyanobacteria, are known to catalyze the oxidation of manganese, usually outside of the cell.

The Mn(II)/Mn(IV) redox couple would typically be too high to allow donation of electrons to a photosystem, however in complex with bicarbonate the redox potential decreases. This may allow Mn(II) to act as reductant of PSII or potentially even a bacterial reaction center [6,8]. Further support for this hypothesis comes from biochemical studies on bacterial reaction center mutants which had some activity to oxidize Mn(II) [9,10].

The oxidation of Mn(II) with oxygen is thermodynamically favourable but kinetically hindered and is accelerated by five orders of magnitude by biological or a combination of biological and abiotic processes [7,11]. Recent geochemical evidence for manganese oxidation before the rise of atmospheric oxygen points towards the ancient existence of a phototrophic live-style based on Mn(II) oxidation [12]. The discovery of these

deposits can be viewed in analogy to banded iron formations, which provide evidence for widespread photoferrotrophy before oxygenic photosynthesis [13].

Together these observations hint towards the existence of phototrophic oxidation of manganese before the evolution of oxygenic photosynthesis. However, there is no support to date for the existence of this metabolic capacity in the modern biosphere and it is not at all clear if, due to the high redox potential of the Mn(II)/Mn(IV) redox couple, this metabolism could persist and colonize a biological niche.

During several previous projects at the Microbial Diversity course related to this topic, several students in the course have obtained enrichments for ferro-phototrophs. In 2009 the student Benjamin Tully has followed his enrichments with sequencing of the *pioB* gene and found a sequences cluster specific for an enrichment culture with manganese.

## Goals of the mini project

The goal was to employ traditional enrichment techniques in this project for the isolation of Fe(II) and potential manganese phototrophs. The Fe(II) enrichments subsequently could be used in a second step to select for organisms that show activity to oxidize Manganese(II) in addition of Fe(II). Mn(III) was tested as an alternative electron donor to allow the one-electron oxidation to Mn(IV), as was elevated pH.

## Methods

### *Medium and inoculum*

The enrichment media were based on the medium used by Widdel *et al*.[4] For fresh water medium 0.3 g $NH_4Cl$, 0.5 g $KH_2PO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$ and 0.1 g $CaCl_2 \cdot 2H_2O$ were dissolved in one liter of MilliQ purified water. For salt water medium 22g NaCl, 3.5 g $MgCl_2 \cdot 6H_2O$, 0.5 g KCl and 0.05 g $CaCl_2$ were added in addition. The solutions were then autoclaved at 15 psi and 121°C for 30 minutes and subsequently cooled under bubbling with 20 % $CO_2$/80 % $N_2$. Once cooled, 22ml of $NaHCO_3$ (sterile, autoclaved under $CO_2$ headspace) and 1 ml each of the Microbial Diversity course 2013 Trace metal and Vitamin mix as well as 1 ml of Vitamin B12 solution (5 mg in 50 ml $H_2O$) were added. The pH was adjusted to 7.0 with HCl, if required. Fe(II) and Mn(II) were added from 1 M stock solutions prepared anaerobically. Any iron precipitate in the stock solution was removed by filtration. Upon addition of metal ions to the medium a precipitate formed, in the case of Fe(II) likely $Fe[PO_4]_2 \cdot 8H_2O$ or $FeCO_3$ [14] and also for manganese likely phosphate and carbonate minerals. Typically 20 ml medium were inoculated in 50 ml serum bottles , sealed with blue butyl rubber stopper and kept under pressure in 20% $CO_2$/80%$N_2$.

DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) was added to some of the samples to inhibit the growth of cyanobacteria and photosynthetic eukaryotes. A 0.1 M stock (1000x) was prepared in acetone, using a glass vial.

Cycloheximide was added to some enrichment cultures to inhibit the inadvertent growth of eukaryotes, mainly diatoms and other algae. A 50 mg/ml stock solution was prepared in water and diluted 1000-fold in samples.

Enrichments incubated in the light were placed ~30 cm from a 40 W incandescent bulb and kept at 30 °C. Dark controls were kept in a sealed container.

Inoculum for the enrichment cultures was sampled from Little Sippewissett (marine) and School Street Marsh (brackish), collecting approximately the first 1-2 cm of the sediments, mixed with bottom water. The sampling sites in School Street Marsh were primarily chosen because a presence of a $MnO_2$ sheen at the water surface. Only later in the middle of July appeared an iron rich mats reminiscent to the ones used in previous studies (see student report by N. Caiazza, 2006). The presence of $MnO_2$ was confirmed with leucoberbelin blue. The inoculum was kept anaerobic, was thoroughly vortexed before use and only supernatant of the slurry was used to inoculate the enrichment cultures in order to avoid extra input of organic carbon.

Salt water medium was used for samples from Little Sippewissett, whereas a 1:1 mixture of fresh water and salt water medium was used for samples from School Street Marsh. Salinity at School Street Marsh was typically 15 ‰ (brackish) and samples from Little Sippewissett had a salinity of 32-36 ‰ as determined by a refractometer.

*Mn(III)-pyrophosphate synthesis*

2 mL of 100 mM sodium pyrophosphate-HCl (pH 7.5) were mixed with 4 mL of 1 M HEPES-KOH pH 7.5 and then 320 uL of 0.1 M $MnCl_2$ were added and mixed well. 400 uL of 20 mM K-permanganate Mn(VII) were then added and the solution incubated overnight on a rotating shaker at 4 °C. The next day, the solution was sterilize filtered through at 0.2 µM filter. The filtered solution was treated as a 5 mM Mn(III) stock.

*Agar shake tubes*

Agar shake tubes were prepared by dissolving 3 %(w/v) washed Noble agar in water and autoclaving it at 15 psi and 121 °C for 30 min. The molten agar was then transferred to a water bath at 55 ºC and bubbled with 20% $CO_2$/80 % $N_2$. 3 ml agar were transferred into sterile Balch tubes and kept anaerobic by bubbling with a long, sterilized needle. 6 ml anaerobic culture (containing 0.9 ml inoculum) was added, avoiding air. The tubes were immediately closed using a blue rubber stopper, mixed and placed on ice until the agar had solidified. For higher dilutions (*e.g.* 1:100) the culture was first diluted in anaerobic medium, which was then used for preparing the agar shake tube.

*Leucoberbelin blue test*

$MnO_2$ can be readily detected by smearing a small amount of metal sheen from the environment or an isolated colony on Whatman paper and adding a drop of

leucoberbelin blue reagent (LBB; refrigerated, filter-sterilized 0.04% leucoberbelin blue plus 1 % glacial acetic acid). Formation of a bright blue color indicates presence of $MnO_2$.

*Ferrozine assay*

Dissolved ferrous iron was measured using the calorimetric ferrozine assay. Fe(II) reacts with ferrozine molecules to form an intensely purple complex, which can be quantified at 562nm.

0.1 %(w/v) ferrozine in 50 %(w/v) ammonium acetate was prepared and stored in the dark at 4 °C. 10 %(w/v) hydroxylamine hydrochloride (HAHC) was prepared in 1 M HCl.

Calibration curves were prepared with $(NH4)_2Fe(SO4)_2 \bullet 6H_2O$ in 1 M HCl, measuring appropriate blanks.

Dissolved Fe(II) was quantified by mixing 0.9 ml 1 M HCl with 0.1 ml filtered sample under anoxic conditions and transferring 0.1 ml of this solution to 0.9 ml 1 M HCl in a plastic cuvette. 1 ml ferrozine solution was added and absorbance at 562 nm measured after 10 min.

Total iron (Fe(II) plus Fe(III)) was measured by mixing 0.1 ml sample with 0.9 ml 6 M HCl under anoxic conditions. After incubation for 5-24 h the sample was centrifuged and 0.1 ml of the supernatant were mixed with 0.9 ml 1 M HAHC in a cuvette. 1 ml ferrozine solution was added and absorbance measured at 562 nm after 10 min incubation.

The assay was used to determine the Fe(II) concentration of the stock solution and to estimate iron content of inoculum used to start enrichments.

*Ion chromatography*

Ion chromatography was performed on a Dionex ICS-2100 by injecting 1 ml filtered and desalted solution. Desalting was performed using a Dionex OnGuard II Ag/H 2.5cc cartridge. Quantification was performed by calibration with a mix of standards. Sulfate concentration in sediment collected were typically around 24 mM for Little Sippewissett and 16 mM for School Street Marsh.

*Pigment analysis*

Pigment analysis was commonly performed *in extracto*. For this a suitable amount of cell material was pelleted and resuspended in 1 mL 7:2 acetone/methanol mix. After incubation for 5 min in the dark, the sample was centrifuges at maximum speed for 2 min. Acetone methanol mix was used as blank and UV/VIS spectra recorded from 350 to 1000 nm.

## Results

The initial enrichment cultures were prepared in original Widdel medium and 1:10 dilution of slurry supernatant. Agar shake tubes were prepared in 1:10 and 1:100 dilutions. 1 mM and 10 mM Fe(II), as well as 0.1-100 mM Mn(II) were used for a broad screening of growth conditions.

After four days Fe(II) containing agar shake tubes showed the formation of black colonies. Exchange of the headspace revealed $H_2S$ smell and it was concluded that $H_2S$ had been formed in the cultures, causing the precipitation of FeS. Mn(II) cultures did not show precipitation, likely because of the greater solubility product of manganese (II) sulphide compared to FeS. Over time also 1:100 dilution agar tubes showed strong FeS precipitation, but also pigmented cells occurred. Despite $H_2S$ production the predominant coloration was green and the acetone extracted pigment spectrum had a prominent peak at 662 nm, corresponding to Chl*a*. A minor peak was detected at 766 nm, which is in agreement with the absorption of bacteriochlorophyll. Analysis of 16S sequences from these samples by 454 sequencing indicated a diverse composition of the microbial communities with predominantly members of proteobacteria, cyanobacteria, bacteroides, fermicutes and chlorobi. 1mM Mn(II) cultures here appeared to stimulate the growth of algae, as indicated by a high abundance of chloroplast 16S sequences in this sample. Microscopy of these initial enrichments revealed autofluorescence in the red channel for cells with a diameter of 2 μm, which is in agreement with the growth of cyanobacteria. Brownish colonies appeared but were negative in the LBB test and showed the presence of diatoms under the microscope.

After more than two weeks, growth was also observed in 100mM Mn(II) cultures. A green culture in agar shake tube from School Street marsh revealed a Mn(II)-resistant algal colony (Figure 2 A +B). The LBB test was negative.

Liquid cultures from Little Sippewissett showed growth of disk-shaped greenish aggregates which were embedded at the bottom of the tube in precipitated manganese carbonate (Figure 2 C-F; 0.1M Mn(II)). These aggregates were also LBB negative and showed growth of thin filamentous cells, perhaps a Prosthecochloris-like green sulfur bacterium.

Furthermore, spherical aggregates of cyanobacteria grew in 10 mM Mn(II) liquid culture from Little Sippewissett after about two weeks (Figure 3). The spheres were embedded in manganese carbonate crystals and under the microscope sheets of 'Merismopedia-like' cyanobacteria were visible.

One week after the first enrichment a second enrichment was prepared with inoculum from the two sites. In order to minimize the growth of sulfur reducing bacteria, the concentration of $SO4^{2-}$ was decreased from 2mM to 1mM and sediment was resuspended in $SO_4^{2-}$–free medium to obtain the inoculum. At this stage samples with 0.1-1 mM Mn(III) were included in the enrichments and the growth of eukaryotes and

cyanobacteria was inhibited by the addition of cycloheximide and DCMU. Incubations with medium at pH9 were also set up to test if higher pH aids manganese oxidation. No significant difference has been noticed, however. Also in the cultures from these enrichments, sulfate reducers were causing the formation of FeS, albeit only after about 7 days. It therefore can be concluded that the inoculum had a high proportion of these organisms. Purple and violet colonies appeared over time in agar tubes of all samples, also in controls without added metals. The pigment spectrum of these colonies best agrees with BChl*a*. All LBB tests on brownish looking colonies were negative and many colonies revealed motile red cells under the microscope.

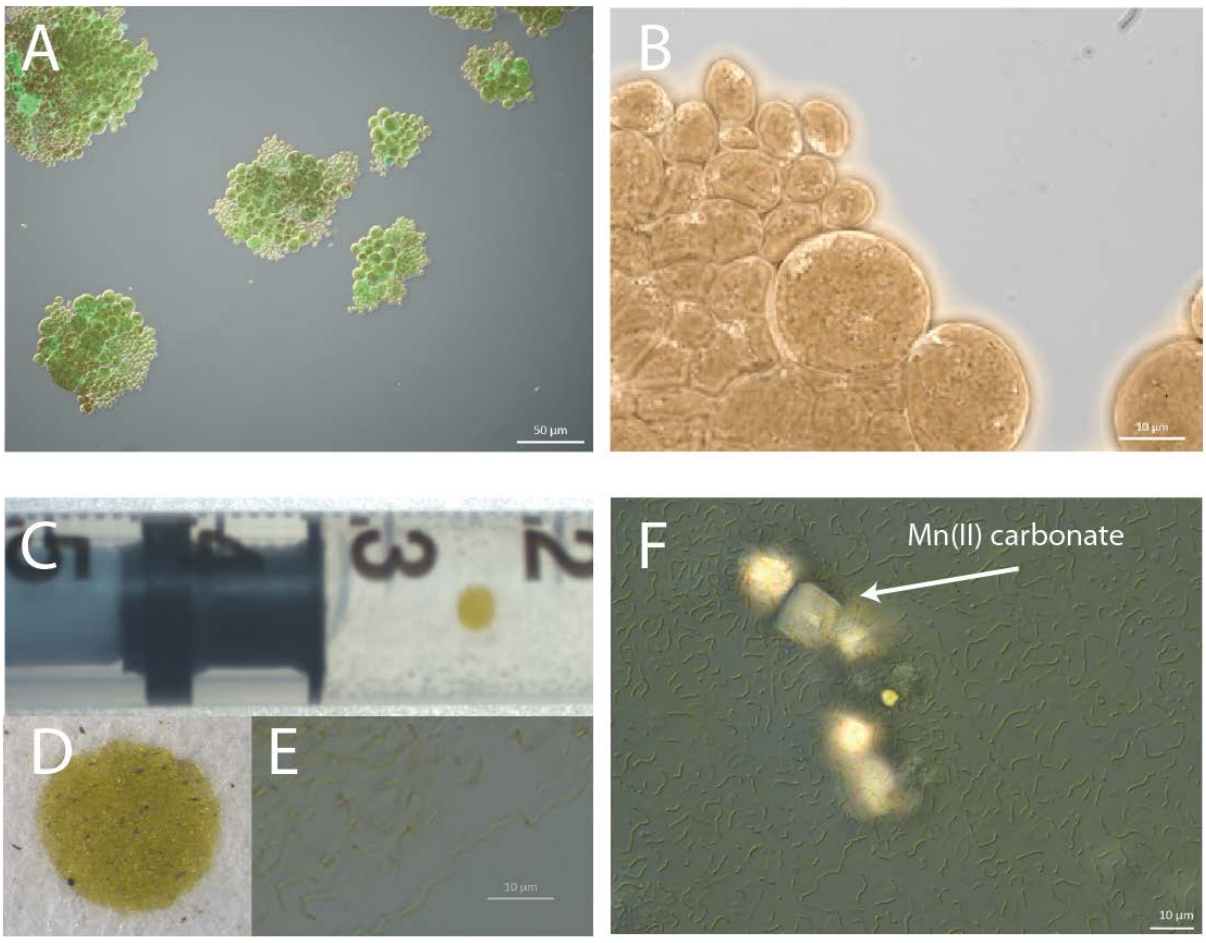


Figure 2. Microorganisms growing at 0.1M Mn(II) concentration. A) Algal colony from School Street Marsh. B) Autofluorescence image of same colony. C-F) Disk-like colonies forming within Mn(II) carbonate precipitate in liquid culture from Sippewissett.

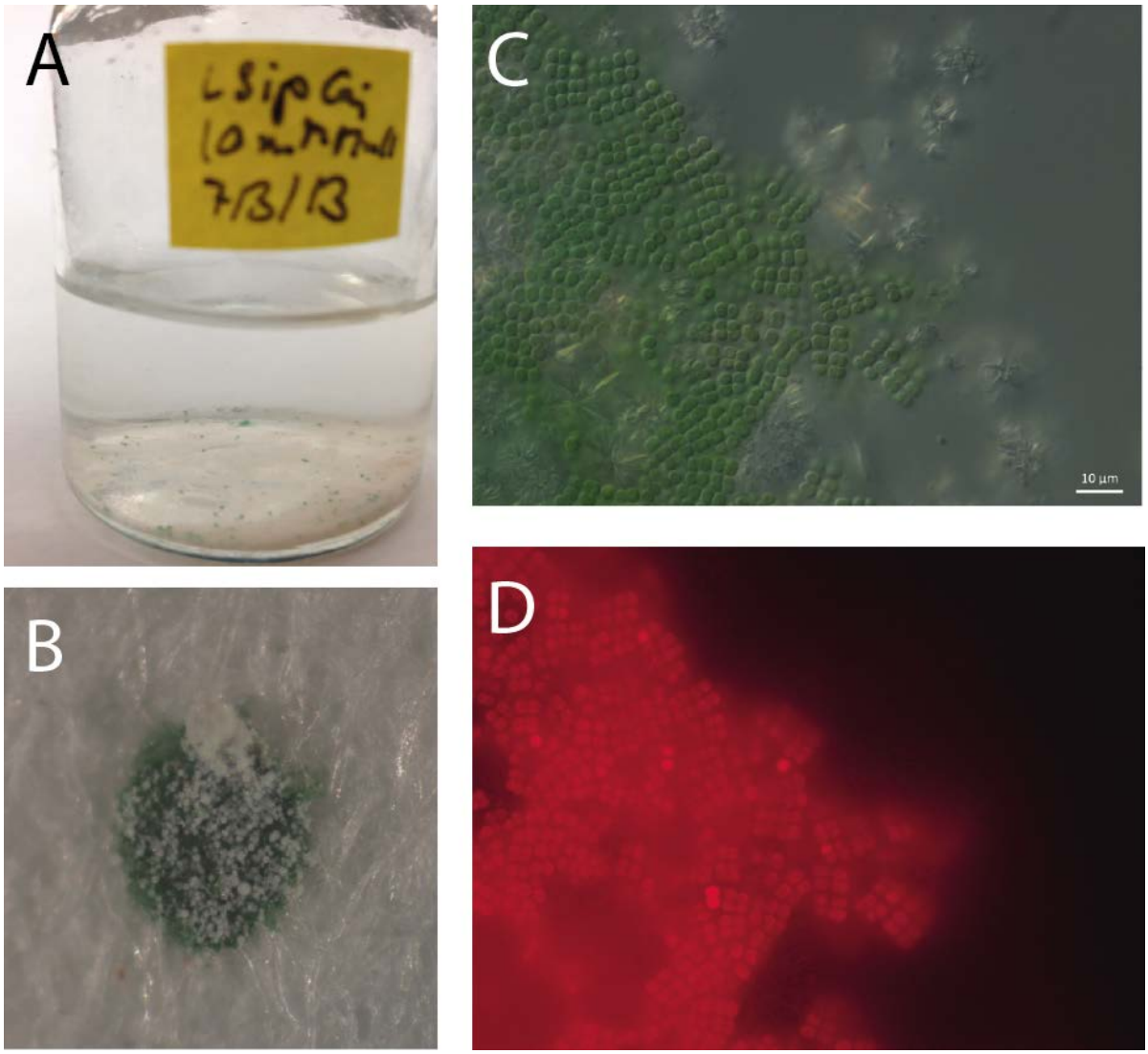


Figure 3. Cyanobacteria from Little Sippewissett growing in clumps at 10 mM Mn(II). (B) Close-up under dissecting scope showing the presence of Manganese precipitate, likely manganese carbonate, within the body of the colony. (C) DIC micrograph of colony. (D) Red autofluorescence channel.

The Mn(III) samples from Little Sippewissett showed the formation of purple color after about 10 days of incubation and the presence of purple (non sulfur) bacteria was confirmed by pigment analysis, which had its main peak at 678 nm (Figure 4). Microscopy revealed the presence of highly motile spirochetes in addition to bacteria with a relatively low degree of autofluorescence. Cells were transferred for further enrichments to serum bottles in dilutions of 1:10 to $1:10^7$ and also to agar shake tubes. In addition controls were prepared in which the protocol for Mn(III) formation was carried out without adding Mn(II) an Mn(VII) (-Mn(III) control) or Mn(II) was added instead of Mn(III). No visble growth was observed after a few days in the Mn(II) control, however significant growth has appeared in both the Mn(III) containing cultures and the –Mn(III) control, which contained added pyrophosphate and HEPES. It therefore was concluded that the growth of the enrichment was somehow based on the presence of one of these two compounds. In addition a colony isolated in an agar

share tube was tested negative in the LBB test. At the very end of the course a reddish brown colony had appeared at the light exposed side of a 1 mM Fe(II) agar shake tube from School Street Marsh. The large area of coloration likely is due to diffused pigment and not iron precipitation, but this could not be resolved carefully.


Figure 4. Enrichment cultures from Little Sippewissett obtained with 0.1mM Mn(III) and Mn(II). Top row: initial enrichment yielded a purple bacterium growing at 0.1mM Mn(III), which was transferred 1:10 into new serum bottles (bottom row). Growth was then also observed in the control including HEPES and pyrophosphate (third bottle from left).

Since FeS had also appeared in the second enrichments, a final attempt to obtain a cleaner inoculum for iron and potential manganese oxidizers was attempted using samples only from School Street marsh. In the meantime, an iron rich mat had formed. Great care was taken to use only the very top of the sediment and three sites, red brown and black sediment as well as sediment near a strong metal sheen, were chosen. $SO_4^{2-}$ was decreased to 0.5 mM and a smaller amount of inoculum was used (1:100 of supernatant of sediment slurry resuspended in $SO_4^{2-}$-free medium). After 7 days no significant growth had occurred in these samples.

## Conclusions and comments for future work

During this project no solid enrichment culture for photoferrotrophs could be obtained. It is likely that the inoculum used was not well suited for the enrichments and contained only a small amount of Fe(II)-oxidizing phototrophs previous years. School Street marsh had been flooded at the beginning of the course leading to a high

salinity and concomitantly a high content of sulfate, which had initially been added to the cultures via the inoculum. The characteristic iron rich mats only became apparent towards the middle of July.

The environment around Woods Hole provides great opportunities for the isolation of metal-oxidizers with novel and exciting features. It seems most promising to start enrichments for photoferrotrophs in the very beginning of the course in order enable subsequently higher-level analysis, for example employing sequencing-based characterization. Ideally, the iron rich and manganese rich mats used for enrichments should be identified together with an experienced colleague and characterized chemically to identify year-to-year variation. For enrichments the sulfate input should be strictly limited and parts of the sediment that contain sulfate reducing bacteria avoided as much as possible.

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