The diversity of microbial community involved in the iron cycling

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Microbial Diversity 2015

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Introduction

Iron is one of the most abundant element that can be found in the earth crust [1]. In the ancient time when the earth was anoxygenic, iron oxide minerals provide a potential source as electron donor or acceptor for the microbial respiration [2]. These processes have long known to contribute to the biogeochemical cycling. While the aerobic microbial iron-oxidation is prevalent, only recently the anaerobically iron-oxidation bacteria has been identified [3]. On the other hand, the respiration by iron-reduction has been proposed as one of the earliest form of respiration [4]. Hence, this discovery is able to complete the close circuit loop in microbial respiration involving the iron-cycle between the Fe^{3+} to Fe^{2+}. Despite the diversity of the microbial world, not only very little is known about the pathways or strategies utilized by these microorganisms but also other species that could utilize iron as part of their respiration. Only recently that the genetically tractable iron (II) oxidizing bacteria has been developed which allow the study and further understanding this metabolic process [5].

This miniproject aims to explore the enrichment of both Fe (II)-oxidizing microorganism (FOM) and Fe (III)-reducing microorganism (FRM), using various combinations of electron donor and acceptor. In particular, this project will be use to investigate the microbial diversity between the inoculum from environmental freshwater sediment samples of the Trunk River (TR) and School St marsh (SSM)

Materials and Methods

Media and culture condition. Basal medium for the Fe(III)-reducing microorganism (FRM) and Fe(III)-oxidizing microorganisms (FOM) was prepared based on the Freshwater (FW) Base Medium as follows; 990 ml of deionized water was added to 10 ml of 100X Freshwater Base (final concentration 17.1 mM NaCl, 1.97 mM $MgCl_2.6H_2O$, 0.68 mM $CaCl_2.2H_2O$, and 6.71 mM KCl). Separately, a final concentration of 5 mM NH_4Cl and 1 mM K_2PO_4 were added to the media. The sodium bicarbonate ($NaHCO_3$) was added to adjust the pH ~7. One ml of 100X Wolfe's Minerals was added to the media before autoclaving. After autoclaved, the basal media cool down for 10 minutes before transferring it into the anaerobic chamber to allow it to be de-gas overnight. One

ml of filter sterilized multivitamins solution was added to the media before aliquating the media into the autoclaved serum bottle with 48 ml each. For enrichment of the FRM, the $Fe(OH)_3$ was prepared as described previously by adding of 0.5 M $FeCl_3$ to 1M NaOH to yield the amorphic $Fe(OH)_3$ metal [6]. After washing the iron oxide with distillled water, 10 mM of $Fe(OH)_3$ were added into each serum bottle. Otherwise stated, the electron donor for this enrichments was varied with final concentration 20 mM of either Acetate, Fumarate, Ammonium, Hydrogen or 0.5 g/L Yeast extract. For the enrichment of the FOM, the basal freshwater medium was supplemented with 5 mM $FeSO_4$ as Fe(II) source. Unless otherwise stated, the sulfide is omitted from the media and 1mM Acetate was added as the carbon source. The media used for the Nitrate-dependent Iron-oxidizer, sodium nitrate ($NaNO_3$) was added to a final concentration of 10 mM. In order to enrich for the chemolitophototrophic iron-oxidizing bacteria, the acetate is omitted from the culture and replaced with CO_2.

Enrichment. The sediment sample taken from the Trunk River (TR) and School St Marsh (SSM) were used as the enrichment source. The enrichment were incubated in the dark at 30 ^oC while for the photorophic FOM was incubated at room temperature in the light. The headspace of the enrichment culture was flushed with N_2:CO_2 (80:20) to provide anoxygenic condition for the culture to grow. After two days, the rate of Fe(II) oxidation and Fe(III) reduction were analyzed using the ferrozine assay, which detect the presence of Fe(II) in the media. The enrichments were also examined by light phase microscopy to determine the presence of microorganism in the enrichment culture.

Ferrozine assay. The presence of Fe(II) were quantified by using the ferrozine assay as previously described [7]. Briefly, the 10 ul of the enrichment culture were added to the 96- well plates containing 900 ul 1M HCl. One hundred ul of ferrozine were added into the solution and allow for 5 minutes for the mixture to equilibrate. The absorbance of the mixture was taken at 560 nm using the plate reader with 4 replicated for each enrichments condition. The solution containing the media but no inoculum was used as blank for this assay. The standard curve for the absorbance and Fe(II) concentration were constructed based on the absorbance of 1 mM to 5 mM $FeSO_4$ which give a linear

correlation between the Fe(II) concentration and absorbance reading as previously described [5]. The ferrozine assay was carried out on the FRM enrichment culture every day while for the FOM with every 2 days.

Mono-fluorescent in situ hybridization (Mono-FISH). Five hundreds ul of the FRM enrichments culture with fumarate or hydrogen as electron donor were fixed with paraformaldehyde (PFA) to 4% final concentration for 8 hours at 4 ^{o}C. After the incubation the sample were sonicated three times for 10 seconds each before 20 ul of the fixed sample were filtered through a membrane filter (0.2 um pore size, white polycarbonate, 47 mm diameter; Milipore, Eschborn, Germany) using the vacuum filtration tower. The samples were first resuspended in 10 ml PBS buffer for the vacuum filtration followed by washing once with 10 ml PBS pH 7.6 buffer. The membrane filters were allowed to air-dried before embedded with the 0.1% low melting temp agar. The next step of incubation with the specific RNA reporter probes. 1 mL hybridization buffer were prepared with the following RNA probes; NON338, EUB338, Delta495, and Bet42. Each filter sample was cut into 6 sections and incubated with respective RNA probe mixed with the hybridization buffer for 2.5 hours at 46 ^{o}C [8]. The samples were washed with the Washing buffer to remove unspecific probe binding for 10 minutes followed by rinsing in distilled water before allowed to air dry. Prior to examination of the labeled-cells, the samples were counterstain with DAPI to visualize the total cells.

Energy-dispersive X-ray spectroscopy (EDS). The same fixed culture used for the FISH were concentrated on the membrane filter. Prior to imaging and EDS scanning, the sample on the membrane filter were carbon-coated with ~20 nm layer. The scanning electron microsopy (SEM) and EDS were carried out with the assistance of Marine Biological Laboratory Central Microscopy Facility.

Result and Discussion

Sediment from Trunk River (TR) and School St marsh (SSM) indicates presence of FRM. The sediment samples from both TR and SSM were inoculated in the FW basal media containing various carbon sources. In order to quantity the presence and metabolic capability of the microorganisms in these enrichment cultures to reduce the iron (III) oxide to iron (II) (Fe^{2+}), we carried out the ferrozine assay. The culture supernatant were extracted and diluted to measurable concentration to allow quantification of the Fe^{2+} in the media. Over the time, there is an increase in Fe^{2+} concentration in the fumarate, acetate, ammonium and hydrogen enrichments samples. However, the rate in which the Fe^{2+} being produce in these enrichments varies from each other (Figure 1). For example, While the initial increase rate of the Fe^{2+} between the acetate and fumarate-containing enrichments were similar after the first day, the Fe^{2+} production rate began to split in which the fumarate enrichment continue to increase its Fe^{2+} concentration up to ~1.75 mM while the acetate enrichments, began to slightly decrease its Fe^{2+} production rate followed by maximum peak at ~1.25 mM before began to tapered off after 5 days. Interestingly, the fumarate enrichments, after 5 days, the Fe^{2+} begin to stably decline over the time, which suggest that the Fe^{2+} is being depleted either FOM which are now started to grow due to abundance of Fe^{2+} or, alternatively, the Fe^{2+} began to react with the remaining iron (III) oxide forming magnetite minerals, Fe_3O_4 [9].

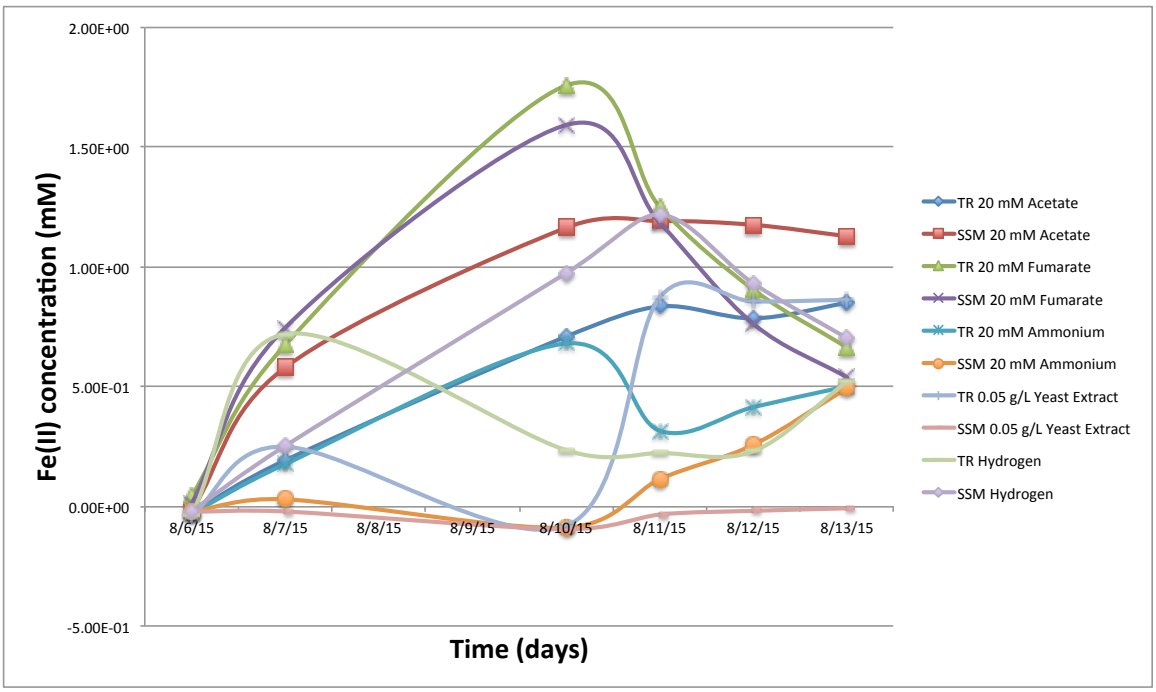


Figure 1: The concentration of Fe^{2+} in the primary enrichment sample. The enrichments culture grown with fumarate as carbon source has the highest rate of iron (III) oxide reduction.

The FRM enrichment favors the fumarate as electron and carbon source. When the primary enrichments of the FRM cultures had developed turbidity, indicating cell growth, the cultures were sub-inoculated into fresh media with corresponding electron and carbon source. Upon examination, the enrichments culture containing fumarate as the sole electron and carbon source is the first culture that shows increase in turbidity, indicating cell growth, within 2 days of incubation. However, within the same time period, other enrichment culture media remains their clear appearance and the dark orange-colored iron oxides particles remain unaltered, similar to the uninoculated control (Fig. 2). In the fumarate enrichment serum bottle, both the inoculum from the Trunk River (TR) and School St marsh (SSM) has developed black-colored minerals at the bottom instead of the dark orange the iron (III) oxides, indicating this iron (III) oxide is reduced into ferrous iron (II) oxides.

For the other enrichments, no significant turbidity were detected until after 3 days in which the enrichment with Hydrogen as electron donor began to become turbid and brown iron (III) oxide has turned into black particles, suggesting that it has been reduced to Fe^{2+}. However, this development was only observed from the inoculum

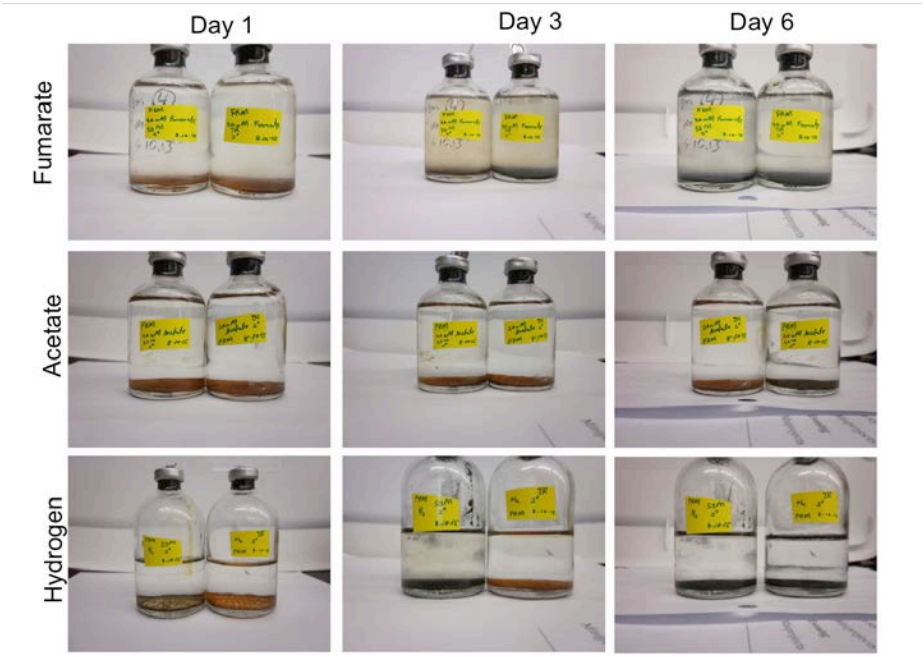


Figure 2: Secondary enrichment culture of the FRM. The growth of the enrichments culture is indicated by the turbidity and conversion of the iron (III) oxides from dark brown to black particles at the bottom.

obtained from the SSM. Eventually, after 6 days incubation, the inoculum from the TR enrichments began to show some turbidity and the ferric iron (III) oxide has turned into black particles, indicating that the iron (III) has reduced into Fe^{2+} (Fig. 2). As for the acetate enrichment, similar changes were observed only after 5 days especially from the sample inoculated from the TR. The enrichment containing the yeast extract also exhibited a very slow iron (III) oxide reduction in which only after 6 days, the Fe^{2+} was began to increase into detectable level in the media. It is possible that the inoculum may not be able to utilize this complex carbon source since they were found in oligotrophic nutrient environment and require much lower concentration of yeast extract. Perhaps, the yeast were first being utilized by the fermenters microbes and requires it to be reduced to a very low concentration before it can be utilized by the FRM. Additionally, in all of the

primary and secondary enrichments for the FRM, no gas bubbles were detected in all enrichment cultures.

The ferrozine assay on these secondary enrichment cultures is also consistent with the assay on the first enrichment in which the fumarate enrichments exhibited the highest Fe^{2+} production rate compared to the other enrichments and peaked at ~1.5 mM before started to decrease or plateu (Fig. 3). Interestingly, while the hydrogen enrichments has a lower Fe^{2+} production rate compared to fumarate, the culture are able to produce a higher concentration of Fe^{2+} at approximately 2.7 mM. It is possible that the cells in this enrichment are able to utilized iron (III) oxide efficiently as electron acceptor or this type of enrichment condition discourage the growth of FOM that would compete for the Fe^{2+} in the media.

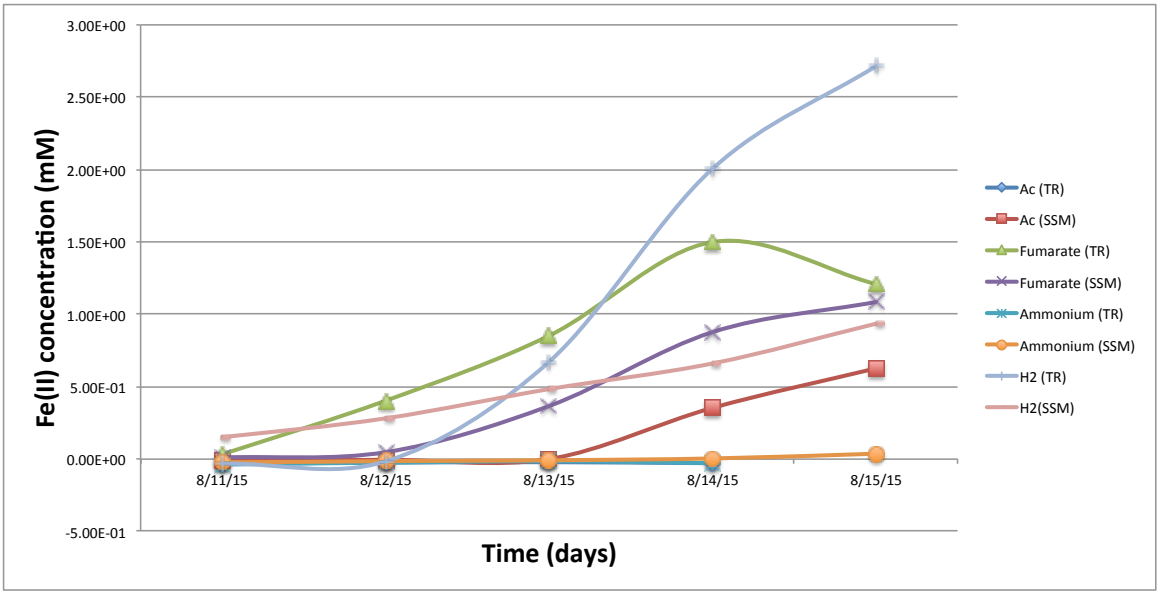


Figure 3: Fe^{2+} concentration in the secondary enrichment culture. The increase in Fe^{2+} is more prominent in the hydrogen enrichment while the fumarate enrichment has the highest Fe^{2+} production rate

The Deltaproteobacteria are the major bacterial group presence in the Trunk River Fumarate enrichment. While the light microscopy confirms the presence of microbial growth in the enrichments culture, based on the cell morphology alone, it is not possible to distinguish or determine the microorganisms that thrive in these enrichments conditions (Fig. 4). In particular, in determining if these microorganisms were in fact FRM or microbes with other class of microorganisms such as sulfate-reducing/oxidizing or acetogenic bacteria. While the 16S rRNA sequencing is the best tool to identify the microorganisms that are present in these enrichments cultures, due to the time constrain of this project, the mono-fluoresecent in situ hybridization (mono-FISH) was utilized to determine at least the phylum of these cells. Four 16S rRNA probes were selected to represent the likely phyla that can be found in this enrichment; NONSENSE (nonspecififc probe), EUB338 (Bacteria probe), Delta495a (Deltaproteobacteria probe), and BET42 (Betaproteobacteria probe). We specifically selected the Deltaproteobacteria probe since it has been previously reported that one of the well known bacterial species that are capable of coupling the oxidation of organic material with iron (III) oxide reduction, *Geobacter metallireducans*, belong to this specific phylum [10].

For this experiment, we only focus on the fumarate and hydrogen enrichments culture since based on the secondary enrichments, these are the one that exhibit robust and high activity of iron (III) reduction. The EUB338 probe indicates that the majority of the microorganism that grew in these enrichments are bacteria. When the Delta495 probe was used against these enrichments samples, only the TR fumarate enrichment is labeled with this probe but not the SSM fumarate enrichment nor the SSM hydrogen enrichment (Fig 5). While it is possible there are other bacterial phylum that may carry out the iron-reduction, we cannot rule out the possibility that the probe that we use did not hybridize well with its target. As for the other probe for betaproteobacteria

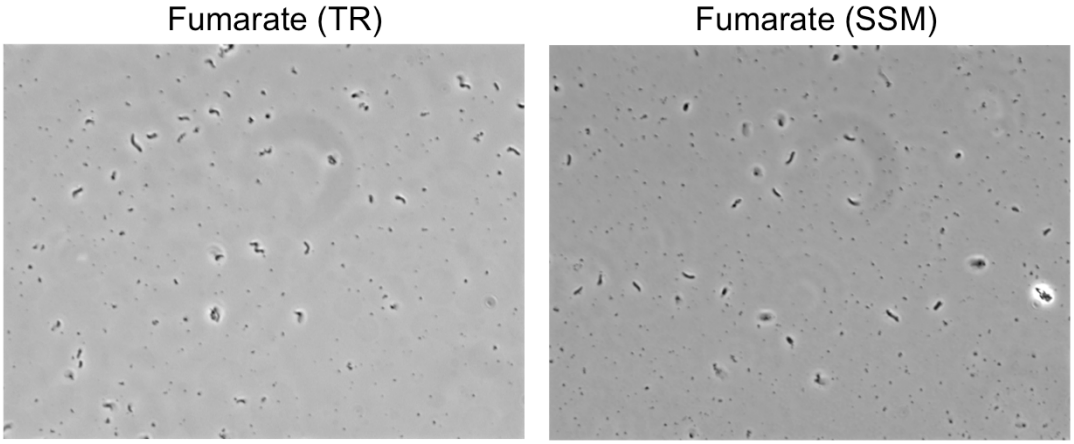
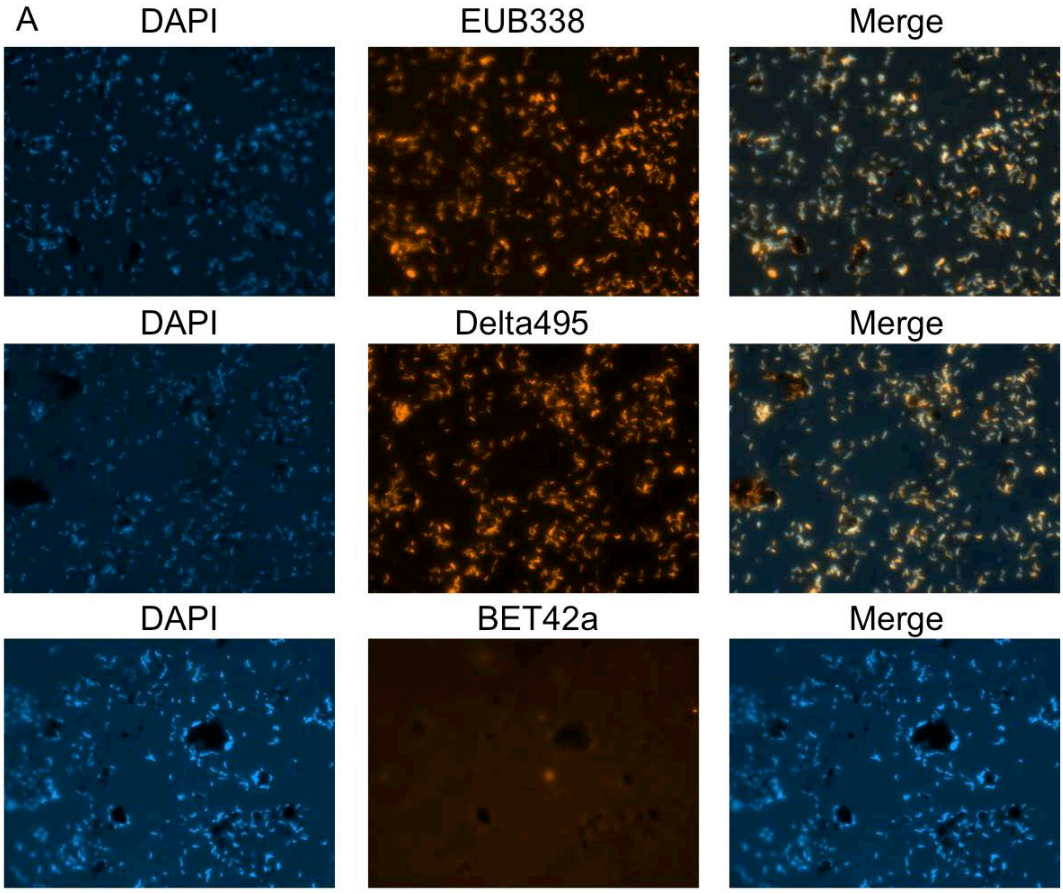


Figure 4: Light microscopy of FRM enrichment culture. The cells from rod and vibrio-like morphology which is not readily distinguishable between the Trunk River and the School St marsh sample.

(BET42a), we also did not detect the presence of bacteria in thus phylum in our enrichment samples (Fig. 5). Similar to the Deltaproteobacteria probe (Delta495), this could be due to failure in hybridization of the probes to the target. Optimization of the hybridization of these probes may be required followed by replicate experiment to confirm the results. As the result stand now, we can firmly conclude that the majority of TR fumarate enrichments contain the Deltaproteobacteria phylum.

The cells aggregates and attach to filamentous superstructure in the iron-reduction enrichments. While examining the DAPI-stained fumarate enrichment, we observed the formation of filamentous superstructure in which the bacterial cells adhered. Hence, it is interesting to determine if this superstructure is made of iron particles in which the cells

adhere to allow efficient electron transfer to the iron (III) oxides as electron sink. In order to determine the element of this structure, we carried out the energy-dispersive X-ray spectroscopy (EDS), which allow us to determine its elemental composition. The EDS revealed that this filamentous superstructure consists mainly carbon compound and has very low amount of elemental iron (Fig. 6). Hence, we can deduce that it not made up of the iron particles and instead, possibly, polysaccharides secreted by these bacteria to aggregates and form the biofilm structure. It would be very interesting to investigate further the formation of this biofilm and to dissect its importance in the electron transfer of these iron-reducing microorganisms.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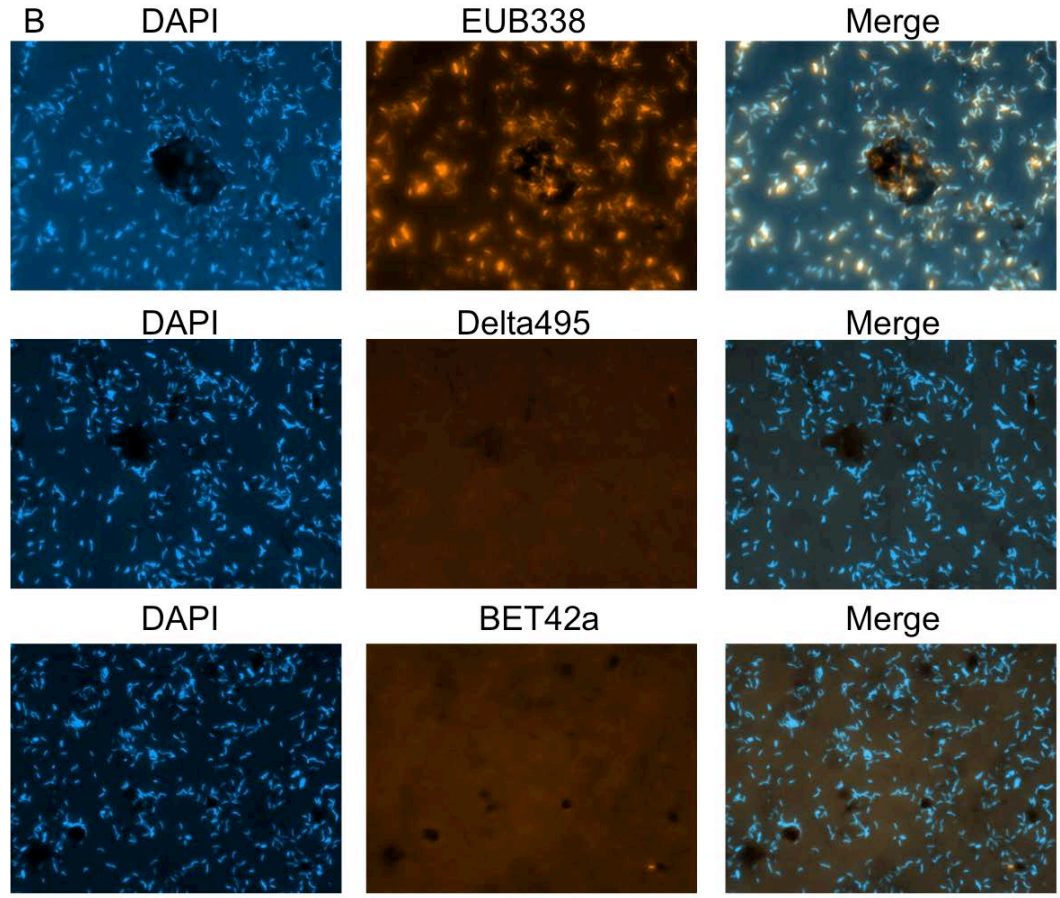
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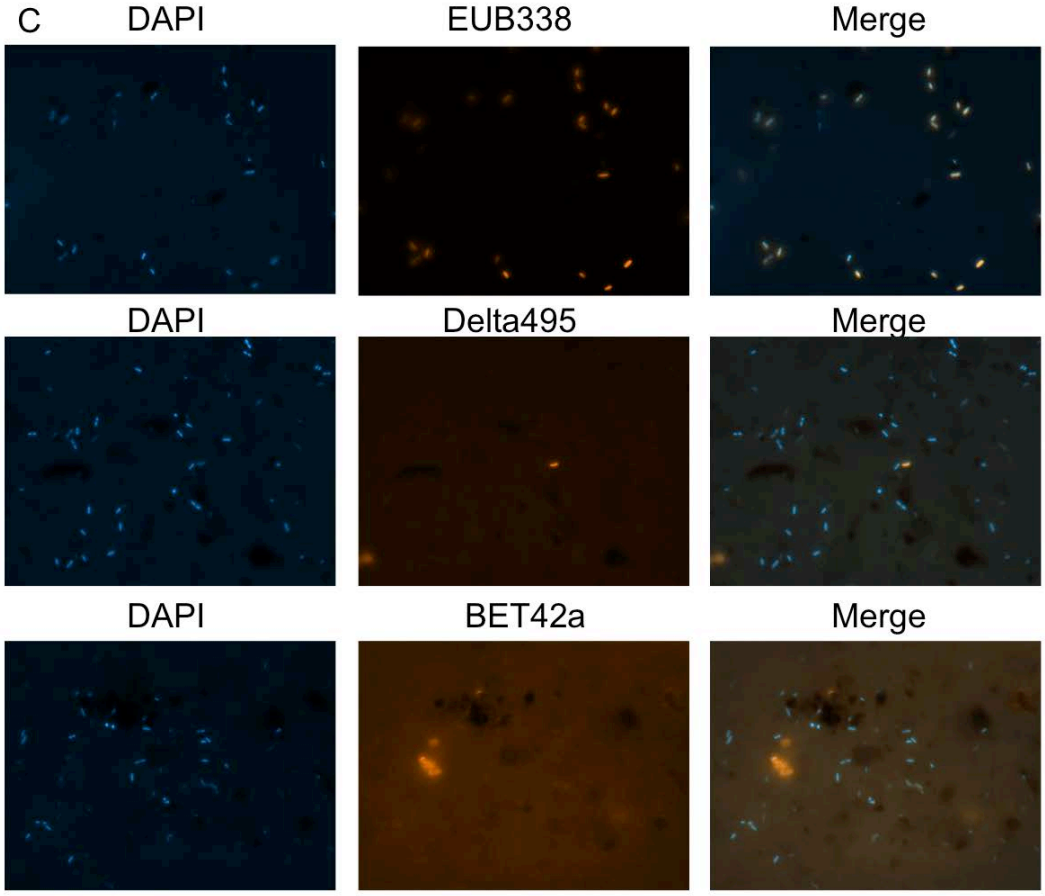

Figure 5: The mono-FISH assay using the FRM enrichments using DAPI, eubacteria , deltaproteobacteria and betaproteobacteria probes. The sample examine is from the Trunk River fumarate enrichment (A), SSM fumarate enrichment (B), and SSM hydrogen enrichments (C).

Enrichment of iron-oxidizing microorganism (FOM). In addition to the FRM enrichment, we are also interested in enriching the FOM from both the Trunk River and School St marsh sample. Using the ferrozine assay, we are able to determine which enrichments condition that yield growth or successful oxidation of the Fe (II). The enrichments with the nitrate as electron acceptor has the most activities, especially when acetate is provided as the carbon source. We also able to enrich for the nitrated-dependent FOM phototrophs that is able to fix CO_2 as its carbon source (Fig 7). Nonetheless, mores test needs to be carried to examine these enrichments culture. The decrease in Fe^{2+} in the

enrichment may due to nonspecific abiotic effect in which the media could be accidently exposed to the atmospheric oxygen during the sampling process.

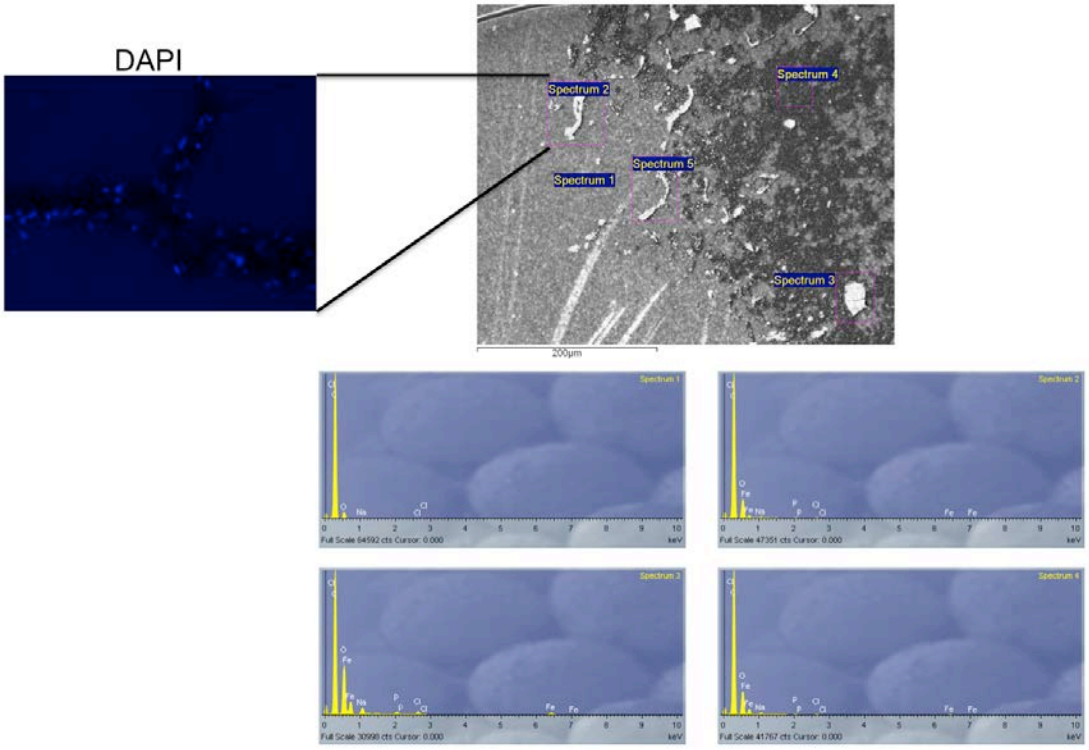

Figure 6: Energy-dispersive X-ray spectroscopy (EDS). The EDS reveals that the filamentous superstructure form by the FRM were consist of main carbon compound instead of iron.

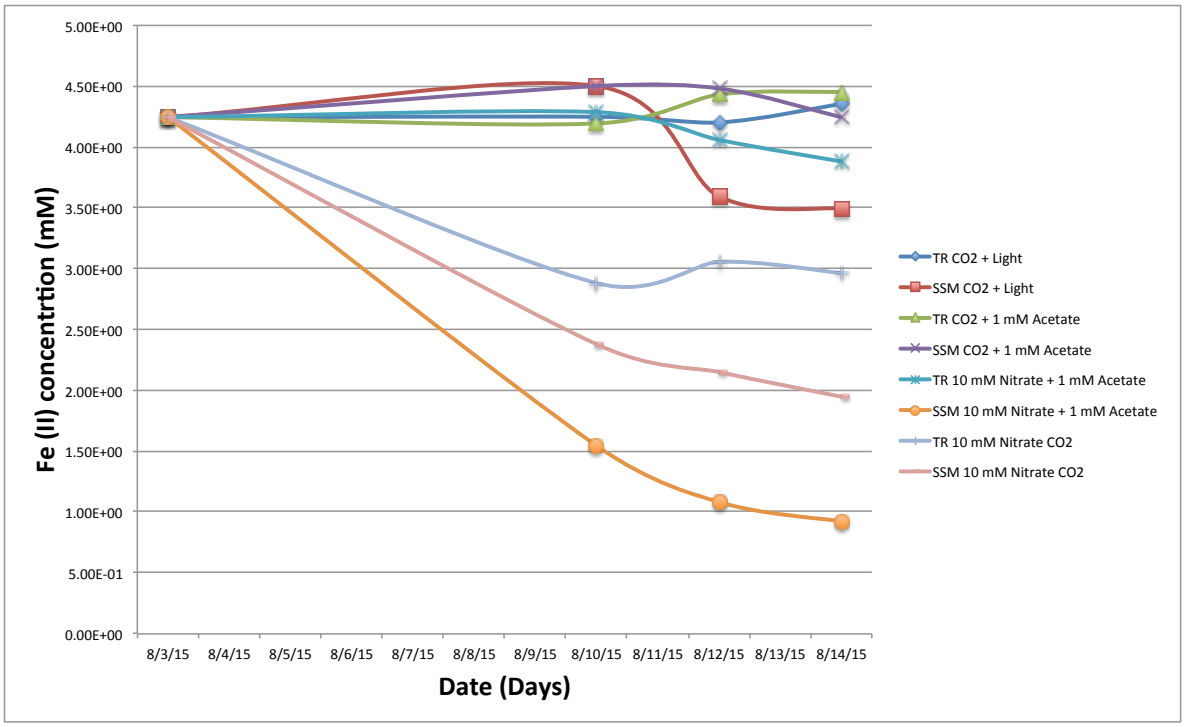

Figure 7: Fe^{2+} concentration in the Fe(II)-oxidizing microorganism (FOM) enrichment culture. The increase in Fe^{2+} is more prominent in the nitrogen enrichment.

Acknowledgement

Special thanks to Sebastian Kopf for help with the anaerobic chamber, cultures, and ferrozine assay. Dianne Newman and Lina Bird for their suggestions in shaping up the miniproject. Thank you also to the TAs and class members of Microbial Diversity 2015 for being generally helpful.

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