ENRICHMENT AND ISOLATION OF IRON-OXIDIZING BACTERIA FROM AN ANCIENT EARTH ANALOGUE

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ENRICHMENT AND ISOLATION OF IRON-OXIDIZING BACTERIA FROM AN ANCIENT EARTH ANALOGUE

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LIST OF SYMBOLS AND ABBREVIATIONS

16S	16S ribosomal RNA
ATP	Adenosine triphosphate
BIF(s)	Banded Iron Formation(s)
CH_4	Methane
CO_2	Carbon dioxide
DAPI	4’6-diamidino-2-phenylindole
DNA	Deoxyribose nucleic acid
Fe^{2+}	Ferrous Iron
Fe^{3+}	Ferric Iron
FeS	Ferrous sulfide
Ga	Billion years ago
GOE	Great Oxidation Event
Mn^{3+}	Manganese (III)
MWMM	Modified Wolfe’s Mineral Media
NH_4^+	Ammonia
NO_2^-	Nitrite
NO_3^-	Nitrate
O_2	Molecular Oxygen
PAL	Present atmospheric levels
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SO_4^{2-}	Sulfate

SUMMARY

Fe^{2+} was an abundant component of ancient anoxic oceans and could have acted as a respiratory electron donor. The overall goals of this study were to test whether anaerobic microbial growth could occur with Fe^{2+} as the electron donor in Fe^{2+}-rich sediments from an ancient ocean analogue (Lake Matano, Indonesia) and to determine the taxonomic identity of the bacteria.

Sediments were incubated with Fe^{2+} sulfide as the electron donor in a nitrogen:carbon dioxide (90/10%) atmosphere. Manganese (III), nitrate, nitrite, and oxygen were provided as electron acceptors. With Mn^{3+} as the electron acceptor, cultures showed some evidence of growth near the middle of the gradient tube. However, orange Fe^{3+} oxides were absent, suggesting that anaerobic Fe^{2+} oxidation had not occurred. Ferric oxides were also absent in tubes containing nitrate and nitrite. A white precipitate was present in cultures with Mn^{3+}, which indicated that the microbes reduced Mn^{3+} to Mn^{2+}. The precipitate was not present in uninoculated controls. With oxygen as the electron donor, a layer of orange Fe^{3+} oxide minerals formed near the water-air interface, indicative of growth of microaerophilic Fe^{2+}-oxidizing bacteria. This layer did not form in uninoculated controls. Our preliminary results suggest that anaerobic Lake Matano enrichments are capable of Fe^{2+} oxidation using oxygen but not alternative electron acceptors.

After subsequent transfers of the enrichments that showed growth of microaerophilic Fe^{2+}-oxidizing bacteria, the bacteria were isolated and their 16S rRNA gene was sequenced. Sequences were most similar to the Betaproteobacteria genus *Comamonas* and the Alphaproteobacteria genus *Skermanella*. Some species of

Comamonas are known to oxidize Fe^{2+}, while the exact mechanism of the metabolism of *Skermanella* are not well known. The presence of microaerophilic Fe^{2+} oxidizing bacteria from Lake Matano, Indonesia serves as a link between understanding the transition from an anoxic to an oxic world.

CHAPTER 1

INTRODUCTION

Biogeochemical cycling of iron

Iron (Fe) is a chemical element found throughout the Earth's surface and core (Ehrlich et al., 2016). Fe is a by-product of volcanic activity, and natural physical processes of hydration, oxidation, and fragmentation of Fe-containing rocks and minerals contribute to the accumulation of Fe on Earth. Fe is an incredibly reactive element and exists in oxidation states ranging from 2- to 6+ (Ilbert and Bonnefoy, 2013). The three most common oxidation states are: 0, 2+ and 3+. In nature, biotic and abiotic processes readily convert Fe^{2+} to Fe^{3+} and vice versa; however, Fe^{3+} is not soluble in neutral to alkaline conditions, thus Fe^{2+} is generally considered to be more soluble and thus more bioavailable than Fe^{3+} (Ehrlich et al., 2016).

Banded iron formations (BIFs) are globally distributed deposits or alternating iron-rich minerals and iron-poor, siliceous mineral layers that were deposited from seawater between 3.8 to 1.8 Ga (Posth et al., 2011). The composition of a BIF is typically made up of Fe^{2+} and Fe^{3+}-containing minerals, chert, magnetite, hematite, Fe-rich silicate minerals, carbonate minerals, minor amounts of sulphide (in the form of pyrite and pyrrhotite) (Li et al., 2013). BIFs may have formed via Fe^{2+} oxidation either by O_2 (biotic or abiotic), by anoxygenic photoferrotrophic bacteria (Crowe et al., 2008), or other anaerobic microbial Fe^{2+} oxidation pathways (Kappler et al., 2005) (**Figure 1**). The height of BIF formation was 2.7 – 2.4 Ga – a timeframe that overlaps with the estimated rise of oxygen on the Earth (Anbar et al., 2007)(Posth et al., 2011).

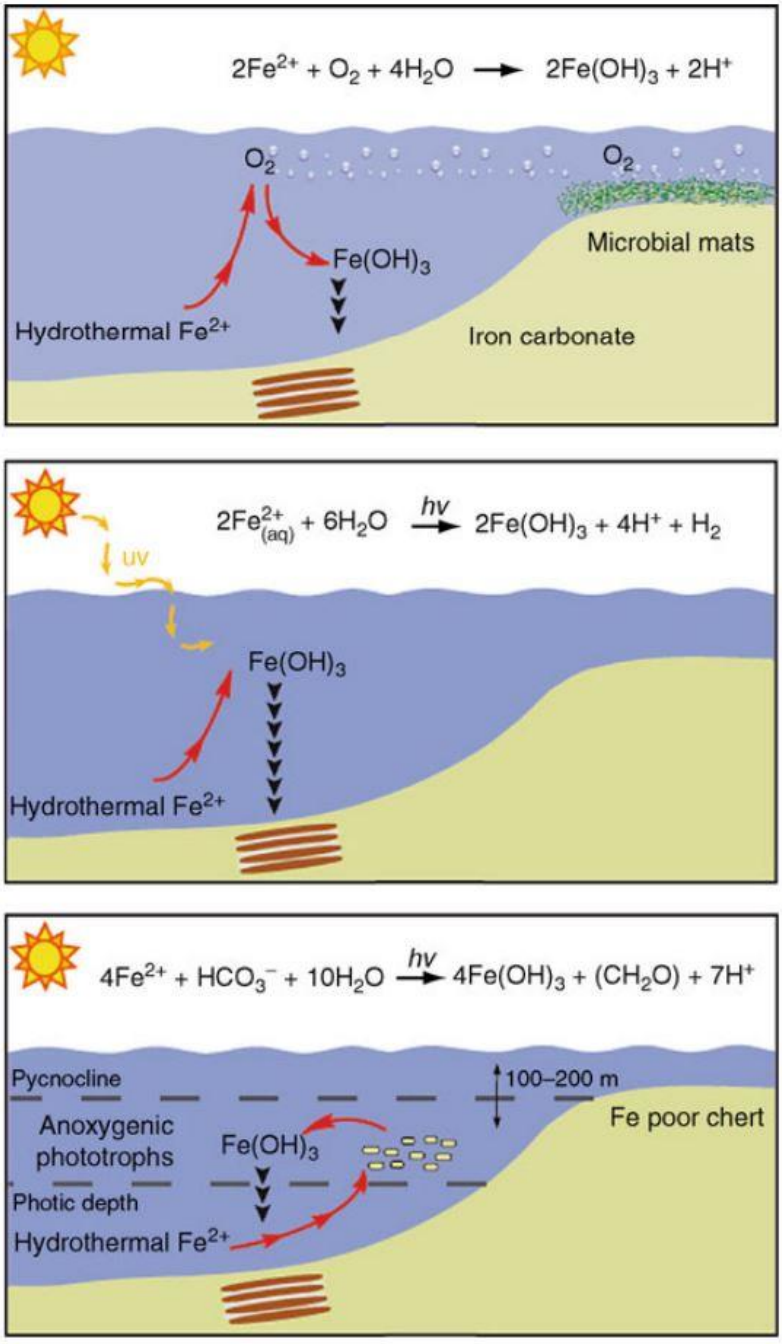

Figure 1. Banded Iron Formations deposition models from Posth et al. (2011): (Top): O_2 produced by cyanobacteria released into water column to react with hydrothermal dissolved Fe^{2+}; (Middle): abiotic Fe(II) photooxidation by UV light; (Bottom): direct microbial Fe^{2+} oxidation via anoyxgenic Fe(II)-oxidizing phototrophs.

A Transition to an Oxic World and Lake Matano

Although there exists some debate about the exact timing and mechanisms of the oxygenation of Earth, isotope fractionation of sulfur isotopes has indicated that before 2.4 Ga O_2 was likely scarce in Earth's early history (Farquhar and Johnston, 2008) with a concentration $<10^{-5}$ times the present atmospheric level of 21% (Anbar et al., 2007)(Lyons et al., 2014). This indicates that the oxygenation of the Earth, referred to as the GOE, was likely to have occurred between 2.5 – 2.1 Ga (**Figure 2**). The most significant source of free oxygen on the Earth's surface is oxygenic photosynthesis (Lyons et al., 2014), but there exist questions about the timing and evolution of this metabolism (i.e. could the emergence of O_2 in the atmosphere have been prior to or coincident with the GOE (Lyons et al., 2014). The significance of the GOE is the influence the rapid increase in atmospheric oxygen had on the evolution of other biochemical metabolisms. Specifically, the oxygenation of the Earth's atmosphere led to the oxidation and precipitation of Fe^{2+} (Ilbert and Bonnefoy, 2013).

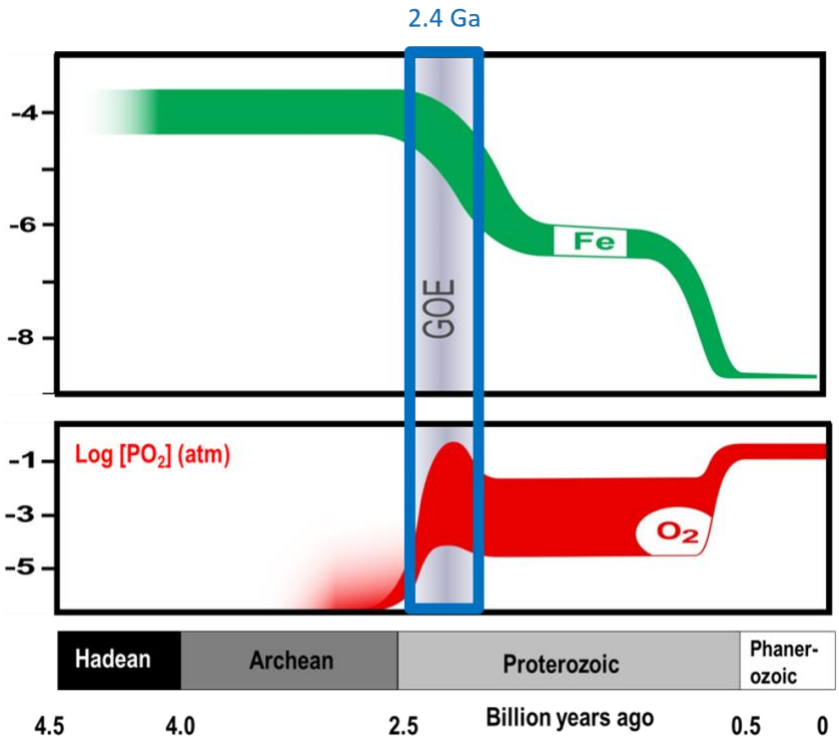

Figure 2. Evolution of Earth's atmospheric oxygen concentration relative to dissolved Fe^{2+} in Earth's oceans over time. It is estimated there was a sharp increase in the atmospheric concentration of O_2 ~2.4 Ga, followed by a decrease in atmospheric O_2 concentration before arriving at PAL of ~21% (Lyons et al., 2014). Modified from (Okafor et al., 2018).

Lake Matano is a modern environment with comparable biogeochemical characteristics of the ancient ferruginous oceans from which BIFs are assumed to have deposited (Crowe et al., 2008)(Crowe et al., 2014). The significance of the oxidation of Fe in low O_2 environments is that photoferrotrophic and microaerophilic organisms could have played a role in the production of Fe^{3+} in BIFs before the evolution of oxygenic photosynthesis. Lake Matano has a depth of over 590 m (Crowe et al., 2008) with oxic waters above ~100 m and anoxic waters below ~100 m (**Figure 3**). Lake Matano serves

as an excellent analogue for the Archean and early Proterozoic oceans because of the high Fe^{2+} concentrations below 100 m, low sulfate concentrations, deep light penetration (which allows for photoferrotrophy at the oxic-anoxic interface), and relatively stable temperature (Crowe et al., 2008).

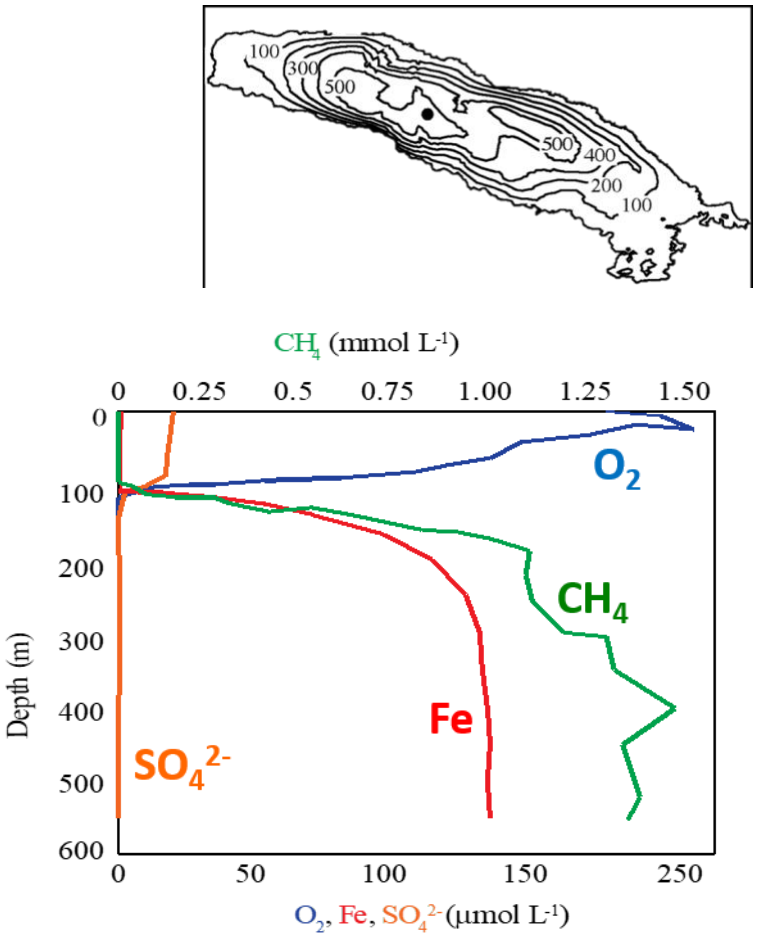

Figure 3: Bathymetry and chemical profile of Lake Matano. (Top): Bathymetry of Lake Matano with indications of relative depth (m) marked. Modified from Crowe et al. (2008). (Bottom): Chemical profile of Lake Matano with evaluation of CH_4 (mM), SO_4^{2-} (µM), Fe^{2+} (µM), and O_2 (µM) concentrations relative to depth (m) of the lake.

Biological Importance of Iron

Energy creation via redox chemistry

In the process of respiration, one chemical species is reduced while a complementary chemical species is oxidized. This redox process creates an electrochemical gradient that is used to create ATP. **Equation 1** shows the general reaction in which oxidized species 1 (Ox_1) gains an electron to become reduced species 1 (Red_1), and a reduced species 2 (Red_2) loses an electron to become oxidized species 2 (Ox_2).

$$Ox_1 + Red_2 \leftrightarrow Red_1 + Ox_2 \quad \textbf{(Equation 1)}$$

As electrons are transferred, the change in the free energy between the electron acceptor (oxidized species) and the electron donor (reduced species) can be used to predict how thermodynamically favorable a respiratory process is. **Figure 4** depicts potential electron donors that microbial life may use.

Figure 4. Examples of organic and inorganic electron donors that may be used by microorganisms in respiration.

Iron redox chemistry and life

Fe is an important element in almost all organisms across all three domains of life. Biologically, Fe is a component of proteins and enzymes. Specifically, it is a key cofactor in several proteins for large scale metabolic processes, such as: (1) transport, storage and activation of molecular oxygen via hemoglobin and myoglobin; (2) activation and decomposition of peroxides (Ehrlich et al., 2016); (3) nitrogen fixation via nitrogenase; (Ilbert and Bonnefoy, 2013); and (4) electron transfer via a range of redox potentials (**Table 1**).

Table 1. Redox potentials of iron and non-iron coupled compounds.

Reduction pair	Eenv (volts)
O_2/H_2O (pH 2)	+1.12
ClO_4^-/Cl^- (pH 7)	+0.873
O_2/H_2O (pH 7)	+0.8
Fe^{3+}/Fe^{2+} (pH 2)	+0.77
$Fe(SO_4)_2^-/Fe^{2+}$ (pH 3)	+0.72
$Fe(SO_4)_2^-/Fe^{2+}$ (pH 1)	+0.697
ClO_3^-/Cl^- (pH 7)	+0.616
NO_3^-/NO_2^- (pH 7)	+0.42
Fe(III)-citrate/Fe(II)-citrate (pH 7)	+0.385
Fe(III)-NTA/Fe(II)-NTA (pH 7)	+0.372
$Fe(OH)_3/Fe(II)_{aq}$ (pH 7)	+0.014
γ-FeOOH $_{lepidocrocite}$/ $Fe(II)_{aq}$ (pH 7)	−0.088
$FeOOH/FeCO_{3\ siderite}$ (pH 7)	−0.05
α-FeOOH $_{goethite}$/ $Fe(II)_{aq}$ (pH 7)	−0.274
α-$Fe_2O_{3\ hematite}$/ $Fe(II)_{aq}$ (pH 7)	−0.287
$Fe_3O_{4\ magnetite}$/ $Fe(II)_{aq}$ (pH 7)	−0.314

Table 1 is reproduced from Ilbert and Bonnefoy, 2013. Not all species in this table are referred to in this document, but all of these redox potentials are of interest to understand the variability of Fe^{2+}/Fe^{3+} dependent on the ligand and pH of the environmental conditions.

A diverse range of microorganisms can utilize the redox active Fe, which can easily cycle between its 2+ and 3+ oxidation states. Fe^{2+} can serve as an electron and energy source for oxic, microaerophilic, nitrate-reducing, and phototrophic bacteria (Ehrlich et al., 2016). Meanwhile, Fe^{3+} can serve as a terminal electron acceptor in the electron transport chain or coupled to the oxidation of organic and inorganic compounds

during anaerobic respiration. When an environment has a neutral pH and a very little amount of oxygen present (i.e. microoxic), O_2 can serve as the terminal electron acceptor for the oxidation of Fe^{2+}. Organisms that have this kind of metabolism are dubbed microaerophilic Fe^{2+} oxidizers (Ehrlich et al., 2016). Once conditions become anoxic, these Fe^{2+}-oxidizing organisms use alternate electron acceptors, such as NO_3^-.

Research Objectives

The overall goal of this study was to understand the growth conditions of microbes from an ancient ocean analogue (Lake Matano, Indonesia). To meet this overall aim, the specific objectives of this thesis were to test whether anaerobic microbial growth could occur with Fe^{2+} as the electron donor in Fe^{2+}-rich sediments from an ancient ocean analogue (Lake Matano, Indonesia) and to determine the taxonomic identity of the bacteria.

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CHAPTER 2

DESIGN OF AN ENRICHMENT TECHNIQUE FOR ISOLATION OF MICROAEROPHILIC ORGANISMS

Introduction

Microaerophilic organisms oxidize Fe^{2+} at circumneutral pH (5-8) conditions using atmospheric O_2 as the terminal electron acceptor. **Equation 2** outlines the skeletal reaction of this metabolism (Ehrlich et al., 2016):

$$4\ Fe^{2+} + 10H_2O + O_2 \rightarrow 4Fe(OH)_3 + 8H^+ \qquad \textbf{(Equation 2)}$$

This biotic reaction readily competes with the abiotic oxidation of Fe^{2+}, thus microaerophilic organisms are typically found in environments where there exists a high concentration of Fe^{2+}. Microbial Fe^{2+} oxidation occurs most favorably in the presence of organic ligands because of their ability to stabilize Fe^{2+} and slow the chemical reaction by competing with O_2. The kinetics of the abiotic and biotic oxidation of Fe^{2+} is kept balanced by the concentration of Fe^{2+} in the habitat; the partial pressure of O_2; the abundance of the microaerophilic organisms; the temperature; the pH; the availability of organic ligands; and the formation of Fe^{3+} minerals (Ehrlich et al., 2016).

Most microaerophilic organisms are unicellular, and under light microscopy, these organisms have been classified to produce extracellular biomineral filaments (Ehrlich et al., 2016). These filaments are believed to help the cells avoid the formation of a hard crust on the structure of these organisms by directing the Fe^{3+} minerals that form away from the cells. The microaerophilic organisms in marine environments, such as Lake Matano, are typically found in iron flocs/mats that are associated with pools of nutrients and trapped metals, which provide for optimal growth conditions.

Per mol, the Fe^{2+} oxidation metabolism does not produce much energy, when compared to the oxidation of glucose, which produces 36-38 molecules of ATP per completed reaction. However, studying microorganisms that have this metabolism as a source of energy is critical to understanding the transition that organisms across all domains of life may have endured from an anoxic, ferruginous Earth to an oxic world.

Materials and Methods

Growth Conditions

To evaluate the role that three different electron acceptors played in the oxidation of Fe^{2+}, gradient tubes (**Figure 5)** with two distinct layers were created by adapting the protocol from (Emerson and Floyd, 2005). The bottom layer, consisting of FeS, provided the iron source for the microbes to utilize in their metabolic process. The carbon source in the bottom layer was high-melt agarose. The top layer was a variation of Modified Wolfe's Mineral Media (MWMM) for freshwater neutrophilic, microaerophilic organisms and also contained low-melt agarose, 10 mM sodium acetate, sodium bicarbonate, and Wolfe's trace minerals.

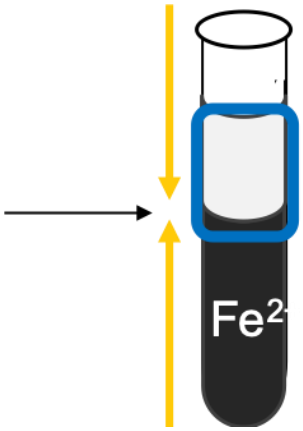


Figure 5. Schematic diagram of the gradient tubes used in the growth and enrichment of the microbes in this experiment. The blue box indicates the top layer; the yellow arrows indicate the proposed movement of the Fe^{2+} and the electron acceptors (Mn^{3+}, NO_3^-/NO_2^-, and O_2); and the black arrow indicates the interface where the redox reaction is supposed to occur, and microbial growth is observed.

A total of 18 gradient tubes were set up with different electron acceptors provided in the top layer. Six of the gradient tubes had a solution of NO_3^-, NO_2^-, and NH_4^+ with total concentration of 10mM added to the top layer. Six of the gradient tubes contained 10 mM Mn^{3+} suspended in liquid media added to the top layer. These 12 gradients were all inoculated with sample (**Table 2**) in an anoxic chamber to avoid any influx of O_2. After inoculation, a red stopper was added to the top, and it was crimped closed. Six of the gradient tubes had atmospheric O_2 allowed to influx into the top layer (**Figure 6**). O_2 was added to the sample by placing a piece of autoclaved aluminum foil around the top of the tube. There was one control tube not inoculated with Lake Matano sample.

Table 2. Samples from Lake Matano used in this study.

Current Sample Number	1	2	3	4	5
Original Sample Name (N. Szeinbaum)	1 (sediment)	160524 (sediment-free enrichment)	Core 1 7.5 – 10.0 cm (sediment)	Tr9 (sediment-free enrichment with Mn^{3+})	5 (sediment-free enrichment)

The cultures that did utilize O_2 as the electron acceptor conducted in this study were performed in an anoxic chamber consisting of a nitrogen:carbon dioxide (90/10%) atmosphere and a minimum of 3 % hydrogen, so as to ensure as little inference from O_2 in the microbe respiration as possible.

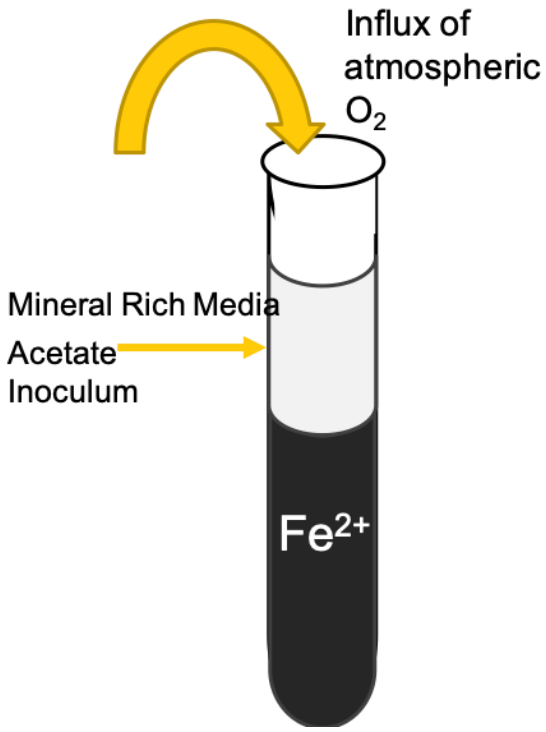


Figure 6. Schematic diagram of the gradient tubes that presented microbial growth with Fe^{2+} oxidation coupled to O_2 reduction.

Results and Discussion

The samples were initially allowed to grow for approximately one month. During this month, microbial growth was observed, which is documented in **Figure X** from 13 selected cultures. The expectation that Fe^{2+} would be oxidized likely occurred given the variation in the control of the vials containing the different electron acceptors near the middle of the gradient tube – at the interface of the top and bottom layers. Based on the initial observations, all five of the inoculated samples with Mn^{3+} in the top layer and all five of the inoculated samples with O_2, were transferred to new gradient tubes of the same composition as the initial conditions. The respective control culture of the Mn^{3+} and O_2 cultures were also repeated, for a total of 6 gradient tubes of each electron acceptor. Following the first transfer of the inoculum to new gradient tubes, the most measurable microbial growth was observed in the tubes using O_2 as the electron acceptor, thus they were continued to be monitored for their growth. Tubes using O_2 as the electron acceptor were subject to three transfers (**Figure 7**) of the inoculum from the original sample outlined in **Table 1**.

Figure 7. Selected cultures evaluated in this study with oxidized nitrogen, Mn^{3+}, and O_2 as electron acceptors

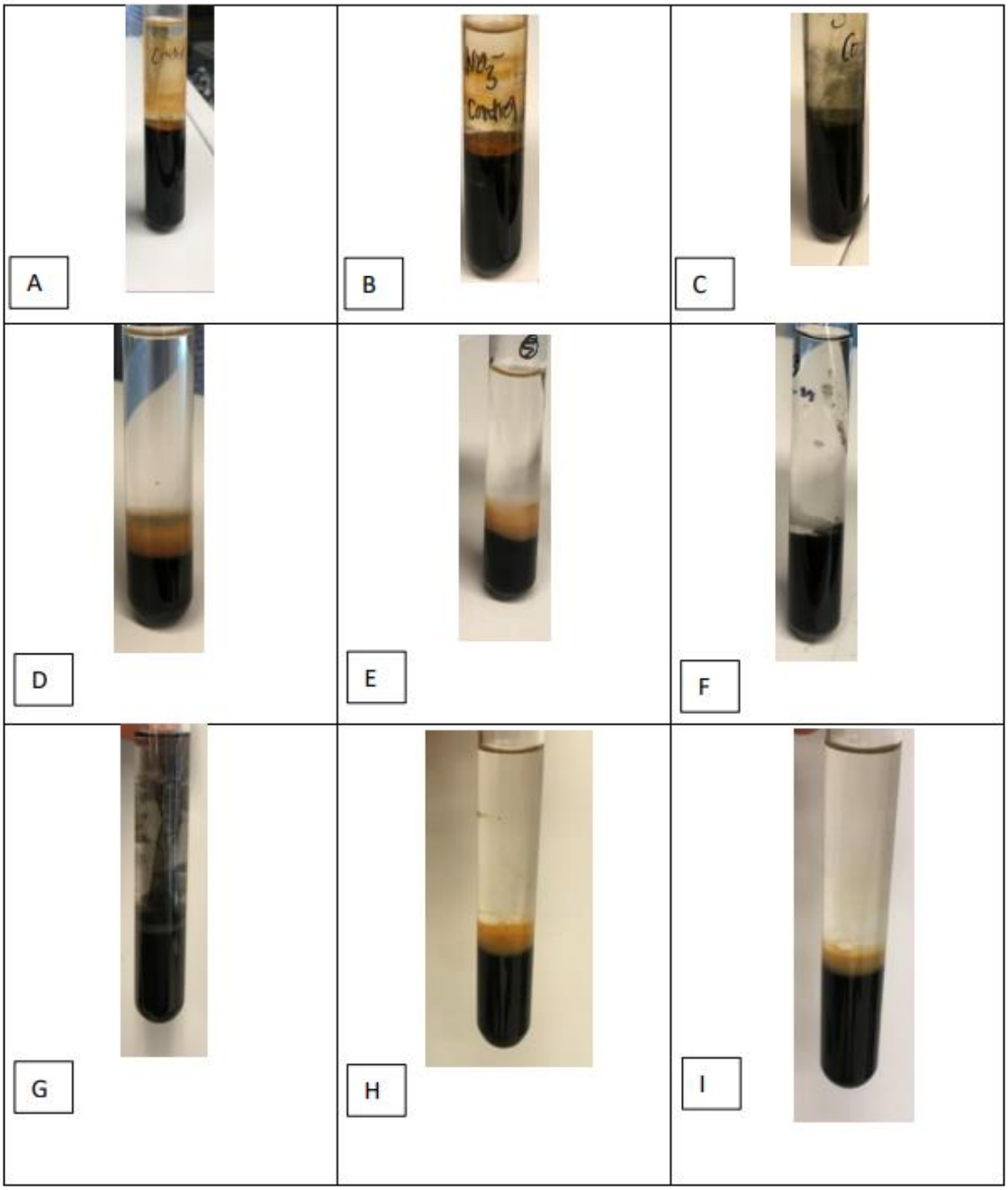
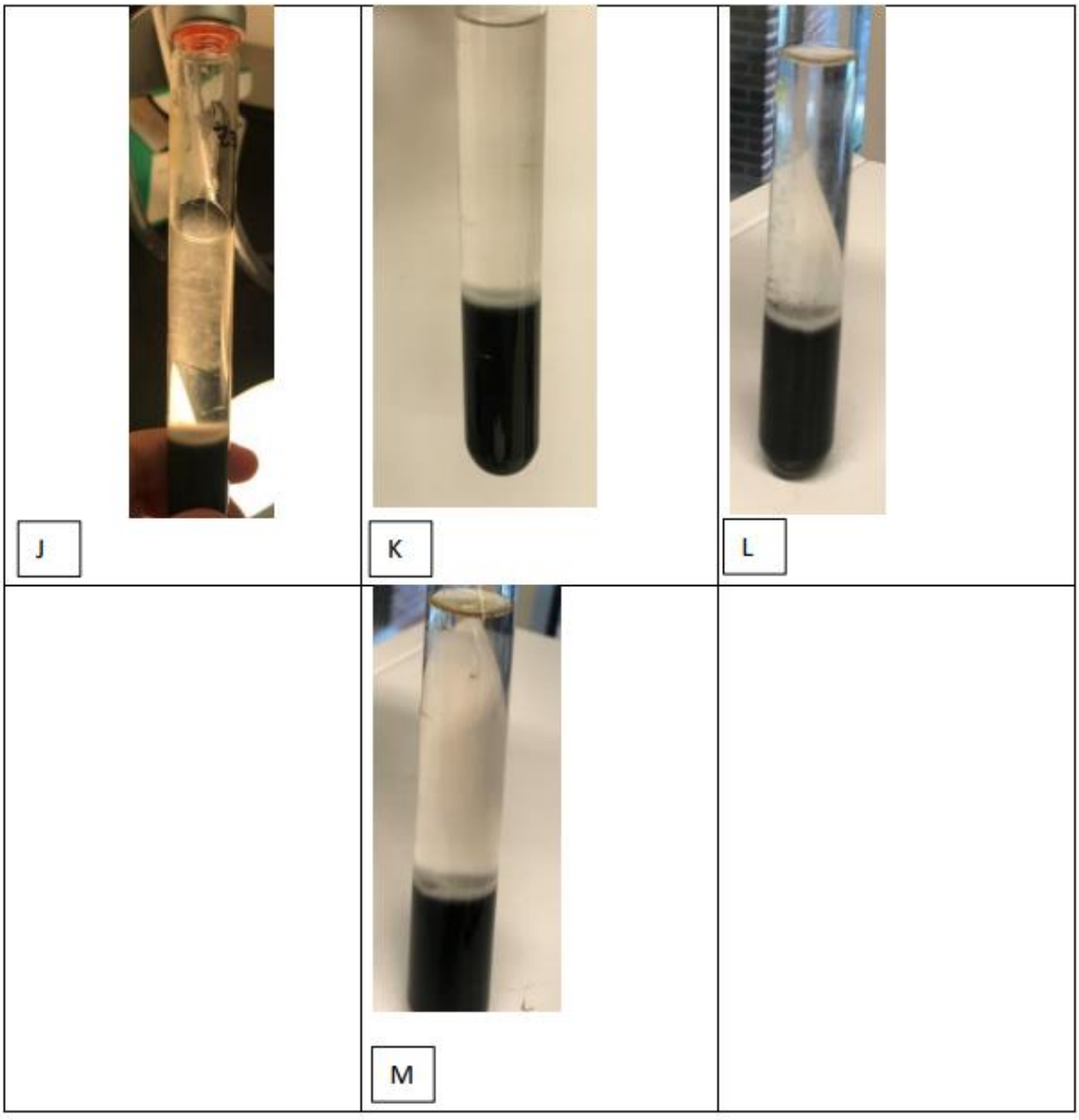


Figure 7 (continued). Selected cultures evaluated in this study with oxidized nitrogen, Mn^{3+}, and O_2 as electron acceptors



A - *The oxygen control vials for the enrichment and isolation of iron-oxidizing bacteria following one week of the initial inoculation*.

B- *The N-solution control vials for the enrichment and isolation of iron-oxidizing bacteria following one week of the initial inoculation.*

C- *The manganese(III) control vials for the enrichment and isolation of iron-oxidizing bacteria following one week of the initial inoculation.*

D -*Following four weeks past the inoculation, Sample 1 from Lake Matano with oxygen as the electron acceptor*.

E- *Following four weeks past the inoculation, Sample 5 from Lake Matano with oxygen as the electron acceptor*.

F- *Following four weeks past the inoculation, Sample 3 from Lake Matano with manganese(III) as the electron acceptor.*

G- *Following four weeks past the inoculation, Sample 4 from Lake Matano with manganese(III) as the electron acceptor.*

H- *Two days following the first transfer of microbes, a sample was taken from the bottom of the top layer of Sample 1 from Lake Matano with oxygen as the electron acceptor.*

I- *Two days following the first transfer of microbes, a sample was taken from the top of the top layer of Sample 1 from Lake Matano with oxygen as the electron acceptor.*

J- *Two days following the first transfer of microbes, a sample was taken from the bottom of the top layer of Sample 1 from Lake Matano with manganese(III) as the electron acceptor*.

K- *Two days following the first transfer of microbes, a sample was taken from the top of the top layer of Sample 1 from Lake Matano with manganese(III) as the electron acceptor.*

L- *Two days following the first transfer of microbes, a sample was taken from Sample 2 from Lake Matano with manganese(III) as the electron acceptor.*

M- *Two days following the first transfer of microbes, a sample was taken from Sample 3 from Lake Matano with manganese(III) as the electron acceptor*

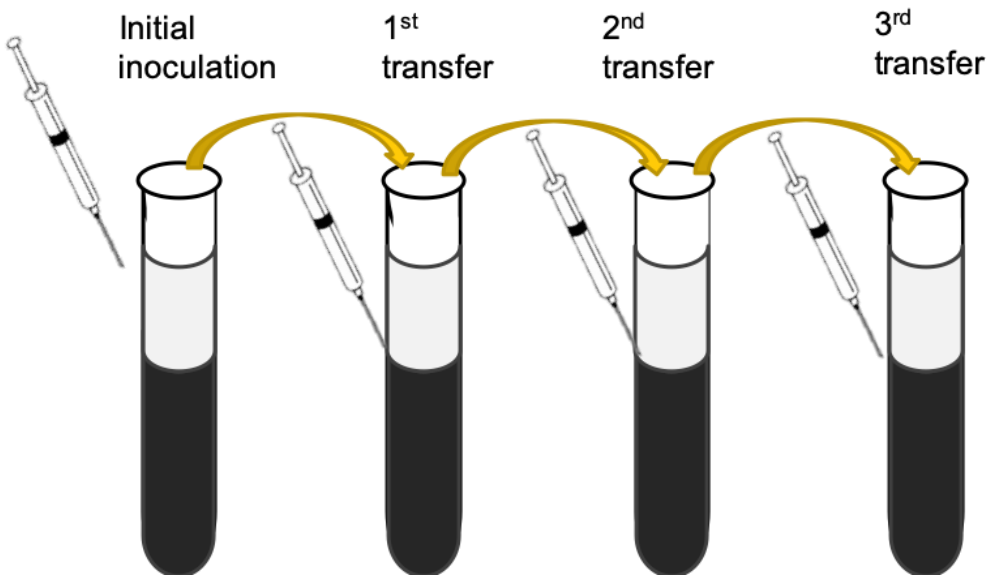


Figure 8. Schematic diagram outlining the process of the transfer of microbial inoculum leading to the enrichment and subsequent isolation of the organisms from the original sediments.

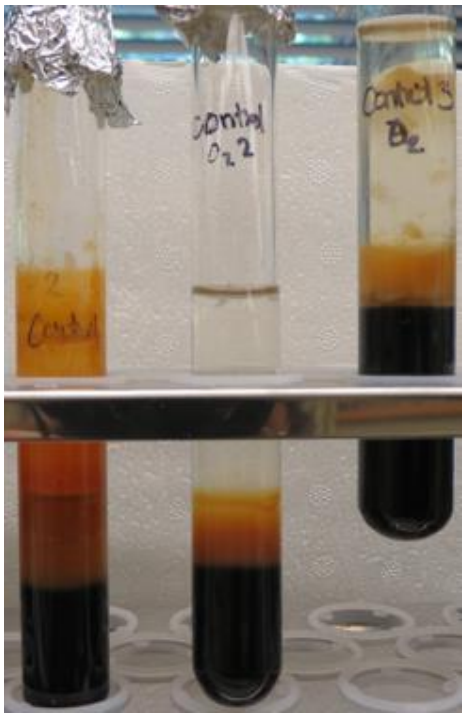


Figure 9. Control gradient culture tubes with O_2 as the electron acceptor and no inoculum.

CHAPTER 3

GENETIC SEQUENCING OF ISOLATES

The growth of the microbes between the months of June 2018 to August of 2018 were observed, and it was determined that the culture tubes where O_2 was the electron acceptor demonstrated evidence of microbial Fe^{2+} oxidation from each sample inoculum outlined in Table 1 (**Figure 10**). Between September 2018 to December 2018, the same experimental process outlined in Chapter 2 was repeated for the cultures until observations determined that the microbes should be subject to 16S sequencing to help determine their identity and characterize their metabolisms.

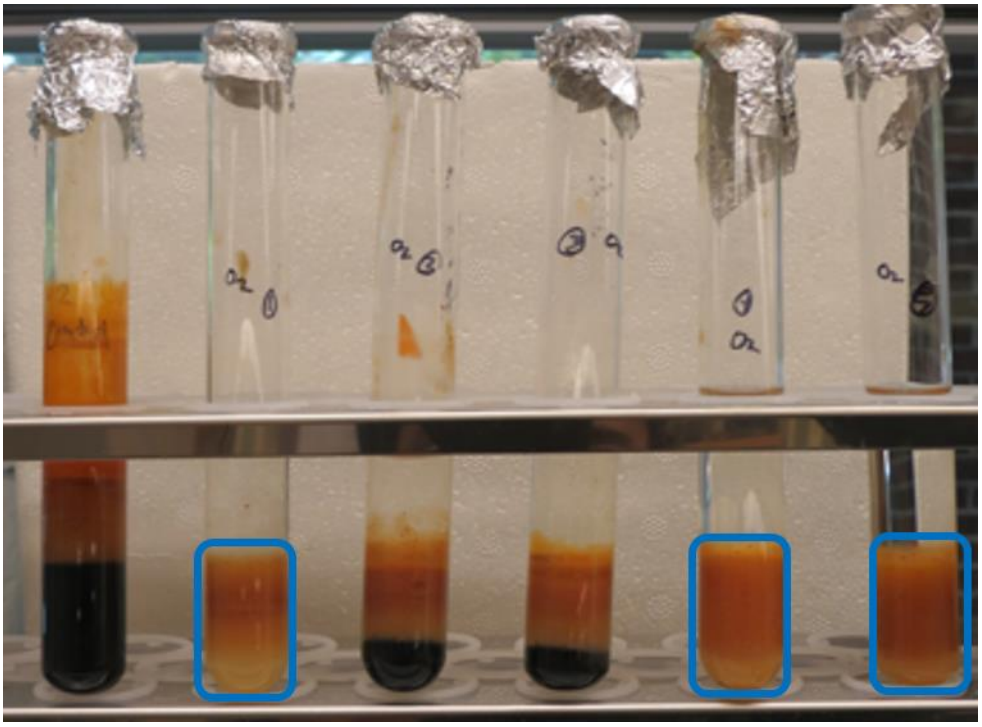


Figure 10. Six gradient tube cultures from the initial inoculation in June 2018 with Lake Matano samples from **Table 1** with O_2 as the electron acceptor. The blue boxes indicate complete microbial Fe^{2+} oxidation.

Materials and Methods

DNA Isolation and Extraction

DNA was obtained from the biomass of the enrichment cultures outlined in Chapter 2. Appendix 1 outlines the procedure followed.

PCR Amplification

16 rDNA was amplified using universal primers U1 corresponding to the hypervariable 16S DNA gene region to obtain approximately 200 base pair length amplifications of the gene. Prior to performing the PCR, the DNA concentrations of each sample were quantified. Appendix 2 outlines the procedure followed. Primers 8F and 1489R with 60 °C annealing temperature were used.

Phylogenetic Analysis of Sequence Data

The phylogenetic trees were constructed in the open source software, MEGA7 after the sequences obtained were analyzed using the open source, web-based platform nBLAST using the nucleotide collection and the megaBLAST option.

Results and Discussion

The nBLAST results for the 16S rRNA sequencing of Sample 4 from Table 1 indicated the isolated species is most closely related to the genus of the betaproteobacteria *Comamonas*. *Comamonas* are a member of the family Comamonadaceae, and these microorganisms are known Fe^{2+} oxidizers (Emerson et al., 2015). Figures 10 depicts the constructed phylogeny of the isolate, named "*IsolateFeOxD."*

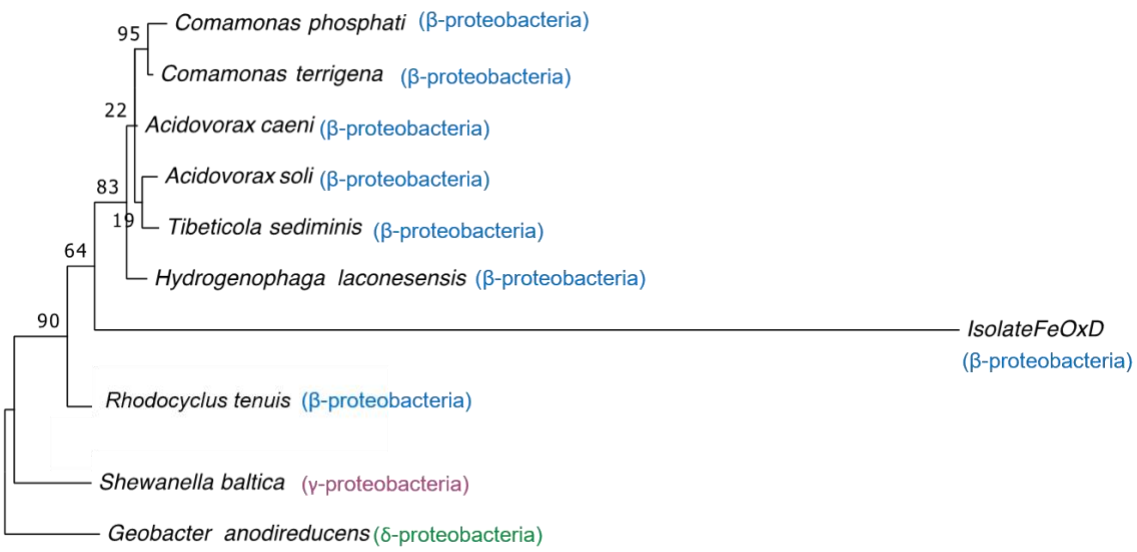


Figure 11. Phylogenetic tree of the potential novel betaproteobacterium isolated from the enrichment cultures in this experiment. *G. anodireducens* is the out-group. *IsolateFeOxD* identifies as a potential new species of betaproteobacterium most closely related to the genus of *Comamonas*.

The nBLAST results for the 16S sequencing of Sample 5 from **Table 1** indicated the isolated species is most closely related to the genus of the Alphaproteobacteria genus *Skermanella.* The genus *Skermanella* is not known to oxidize nitrate/nitrite; these

microorganisms have been reported to oxidize arsenite and may be a key organism in understanding the mechanism of this metabolic pathway (Luo et al., 2012).

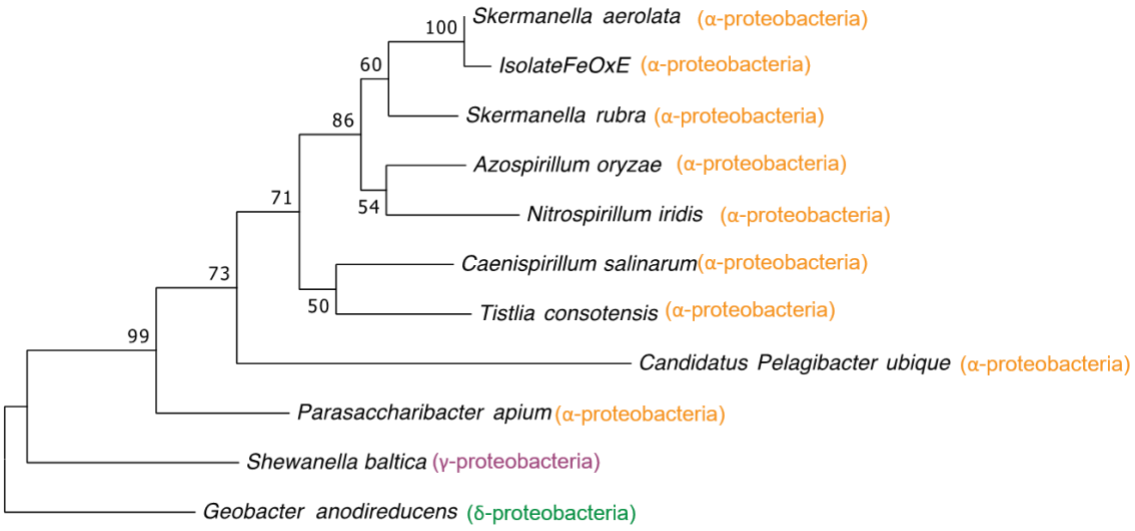


Figure 12. Phylogenetic tree of the alphaproteobacterium isolated from the enrichment cultures in this experiment. *G. anodireducens* is the out-group. *IsolateFeOxE* identifies as the species of *Skermanella* isolated in this experiment.

CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

Microaerophilic organisms play a crucial role in biogeochemical cycling of Fe, O_2 and other elements, and they may have played a role in the transition of microbial metabolisms from an anoxic and ferruginous world to an oxic one. The work of this dissertation investigated the microbial metabolisms of microorganisms from Lake Matano, Indonesia.

The study revealed that there is evidence of Fe^{2+} oxidizing microorganisms from an ancient lake that is anoxic below 100 m. The experimentation allowed for the isolation and enrichment of these microaerophilic organisms from Lake Matano, and the 16S rRNA gene sequencing identified one of the isolates as a potential novel betaproteobacteria and the other as an alphaproteobacterium with a potential arsenic metabolism.

The significance of this study and further research is understanding how these microorganisms can respire in anaerobic environments and shed light on how life came to exist in the manner that it does today. Likewise, this research can have more practical applications in the future for researching life in space.

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APPENDIX 1: DNA EXTRACTION PROTOCOL

General Considerations. Unless otherwise stated, manipulations were performed under aerobic, sterile conditions created by the use of a flame. RNA and DNA isolation reagents were provided in the purchased RNA PowerSoil Total RNA Isolation Kit and RNA PowerSoil DNA Elution Accessory Kit from MO Bio Laboratories Inc (Maryland, USA). DNA concentration was determined by the use of a Qubit 3.0 Fluorometer and its reagents from ThermoFisher (Waltham, MA). All data was collected in the Glass Research Laboratory at the Georgia Institute of Technology in Atlanta, GA.

Preparation for Extraction. From previous experiments, Fe^{2+} enriched gradient tubes that demonstrated microbial growth in the upper layer had a 100 μL aliquot transferred to a separate bead tube. The samples having their DNA extracted are outlined in Table 1. 2.5 mL of bead solution, 0.25 mL of Solution SR1 and 0.8 mL of Solution SR2 were mixed together in the tube. Each tube then had 3.5 mL of phenol: chloroform: isoaryl alcohol (pH 6.5 – 8.0) (Sigma-Aldrich) added to it while under a fume hood. The bead tubes were vortexed at maximum speed for 15 minutes and then centrifuged at 2500 rpm for 10 minutes at rt. The top layer was transferred to a clean 15 mL collection tube (MO Bio Laboratories Inc.), and 1.5 mL of Solution SR 3 was added. The solution was vortexed to ensure mixing, and then incubated at 4 °C for 10 minutes. The samples were then centrifuged at 2500 rpm for 10 minutes at rt. The supernatant was transferred to a new 15 mL collection tube, where 5 mL of Solution SR4 was added. The solution was inverted and allowed to incubate at room temperature for approximately 30 minutes. The solutions were again centrifuged at 2500 rpm for 30 minutes at rt. The supernatant was decanted, and the 15 mL collection tube was inverted on a paper towel for two minutes.

The 15 mL collection tube was turned right side up, and 1 mL of Solution SR5 was added. The solution was repeatedly pipetted to disperse the pellet formed.

Running the Column. Five RNA Capture Columns (MO Bio Laboratories Inc.) were prepared for each RNA Isolation Sample. Remove the cap of a new 15 mL Collection Tube for each sample and hang the RNA Capture Column inside of the collection tube. Add 2mL of Solution SR5 to the column and allow it to flow through the tube into the collection tube, but do not allow the column to run dry. Add the RNA isolation sample prepared and dispersed and allow it to run through the column. Wash the column with 1 mL of Solution SR 5 and allow it to flow through the column.

Elution of the RNA and DNA. Transfer the RNA Capture Column to a new 15 mL Collection Tube and add 1 mL of Solution SR8 to the column to elute the DNA into the collection tube. Transfer each eluted DNA sample to a 2.2 mL Collection Tube and add 1 mL of Solution SR4. Invert to mix. Incubate for 10 minutes at -20 °C. Centrifuge at 13,000 rpm for 15 minutes to pellet the DNA. Remove the supernatant and air dry the pellet on a paper towel. Resuspend the DNA in 100 μL of Solution SR7. Keep solution in the freezer until next prepared use.

APPENDIX 2 : PCR AMPLIFICATION PROTOCOL

Table 1. Standard volumes of reagents to create PCR tubes

Reagents	**Volumes for one tube (μL)**	**Volumes for Working Solution of ten tubes (μL)**
Mix (premixed w/DNTP, Buffer)	4	40
Pr 1 F8 (10 μM)	0.4	4
PR 1492 R (10 μM)	0.4	4
DNA	2	2 μL/tube
dH_2O	14	140
Polymerase (taq)	0.2	2

*For the samples from the Lake Matano isolations of the gradient tubes, used 10 μL since DNA concentration calculated was very low.

Table 2. Adapted standard volumes of reagents to create PCR Tubes for Lake Matano Isolates of the gradient tubes

Reagents	**Volumes for one tube (μL)**	**Volumes for Working Solution of six tubes (μL)**
Mix	4	24
Pr 1 F8 (10 μM)	0.4	2.4
Pr 1492 R (10 μM)	0.4	2.4
DNA	5	5 μL/tube
dH_2O	10	60
Polymerase (taq)	0.2	1.2

Protocol

1. Before performing PCR, quantify the DNA concentration in the samples following the DNA extraction.
2. Obtain a container of ice to ensure the solution in the PCR-specific tubes are maintained at a low enough temperature to not promote the reaction.
3. Allow solutions of Mix, Pr 1 F8, Pr 1492 R and polymerase to briefly thaw before use.
4. Create a working solution, adapted to the number of samples present and needed to be analyzed.
5. Add the appropriate volume of working solution followed by DNA solution to the PCR tube. Make sure to label tubes appropriately.

*Note: total volume in the PCR tubes should be 20 μL

6. Open the appropriate file on the Quantitative PCR Instrument and allow the reaction to run.
7. Remove the PCR from the instrument and keep in the freezer of the 4°C refrigerator until the sample is ready to be sent off for sequencing.

VITA

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GHAZI was born in Atlanta, Georgia. She attended public schools in Alpharetta and Johns Creek, Georgia, received a B.S. in Chemistry with a minor in French Language and a research option certificate in Earth and Atmospheric Sciences from the Georgia Institute of Technology in Atlanta, Georgia in 2019. In September of 2019, Ghazi will pursue a Doctorate in Philosophy of Ocean, Earth and Atmospheric Sciences with a concentration in Ocean Ecology and Biogeochemistry at Oregon State University in Corvallis, Oregon under the advisement of Dr. Julie Pett-Ridge. When she is not working on research, she enjoys reading and hanging out with her cat, Louie.