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Characterization of Metal-Reducing Microorganisms in Walker Lake: A Terminal Saline Desert Lake

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CHARACTERIZATION OF METAL-REDUCING MICROORGANISMS IN WALKER LAKE: A TERMINAL SALINE DESERT LAKE

By

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ABSTRACT:

Metal-reducing microorganisms are increasingly being recognized as an essential component of aquatic microbial ecosystems involved in decomposition of organic matter. Alkaliphilic microbial reduction from alkaline lakes is a little studied field and one particular ecosystem, Walker Lake, presents the opportunity to investigate alkaliphilic metal reducers in their native ecological setting. Walker lake is a terminal, saline, desert lake with pH 9.4, Walker Lake samples of surface and deep sediments and water column samples from 0, 10, 5, 15, 17.5, 19 m depths were cultured by serial dilution in synthetic Walker Lake medium and supplemented separately with Hydrous Ferric Oxide (HFO), Fe Citrate (Fe CR), Fe nitrilotriacetic acid or Fe NTA a chelating agent), and manganese oxide (MnO_2) as terminal electron acceptors. Dilutions were duplicated and the highest cell activity was produced in sediment samples, ranging from 10^5-10^7 cells/ml, when inoculated with HFO, Fe Citrate, and Fe NTA and 10^3-10^4 cells/ml were observed in the sediment samples with Mn(IV) as the terminal electron acceptor. Microbial isolation, using overlay technique and serial dilution, was attempted with moderate success. Comparative data analysis of Walker Lake physical parameters collected in 2013-2014 and data collected in previous Walker Lake studies in 2008 indicated a lake with decreasing water depth, increasing salinity (2.5 mg/l to 18 mg/l) and a subsequent loss of stratification. Alkaliphilic metal-reduction in Walker Lake is still a developing story with upwards of 10^5-10^7 cells/ml of Fe-reducing microbes in the sediment.

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Section I: Introduction and Literature Review

A. Alkaliphiles and Alkaline Habitats

Extreme environments are contrasted with moderate environments by the high or low distribution of pH, temperature and salinity (Sorokin, 2014). Microorganisms in such environments are often specifically adapted to the conditions; organisms that display optimal growth at pH in excess of 8, usually between 9 and 10, can be defined as alkaliphiles (Grant et al., 1990). Alkaline lakes, for example, can be host to unique alkaliphilic microorganisms, a particular subset of these microbes in anaerobic areas are the alkaliphilic metal reducers. Microbial metal reduction is the use of metals as terminal electron acceptors in place of oxygen in cellular respiration. A particular subset of metal reduction is Fe (III) and Mn (IV) reduction (Nealson & Myers, 1992). This section will elaborate on alkaliphilic environments, specifically alkaline lakes, and the characteristic metal reduction, Fe (III) and Mn (IV), that can be anticipated from such environments.

i.) Alkaline Lakes

Alkaline lakes, also known as soda lakes, and soda deserts represent the majority of stable alkaline environments in the world (Ulukanli, 2002). Naturally occurring, stable alkaline lakes fall into two categories: 1) The environment contains high Ca^{2+} concentration often found in groundwater in the form of $Ca(OH)_2$ and 2) the environment has low Ca^{2+} concentrations, commonly found in soda lakes and soda deserts (Grant et al., 1990). Alkaline lakes are defined by high concentrations of Na_2CO_3 and depleted Mg^{2+} concentrations; the presence of the CO_3^{2-} helps to develop a buffering capacity in the lake waters and an increase in pH (Sorokin, 2014). The formation of alkalinity in soda lake environments is established with a combination of geographical, topographical and climatic conditions; geological conditions must favor the formation of alkaline drainage

waters, topography must restrict surface outflow, and climatic condition should favor evaporative concentrations (Jones et al., 1998). An anomaly of soda lakes is their unusually high concentration of dissolved organic carbon (DOC), dissolved inorganic phosphorus, and high standing crops of bacteria (Humayoun et al., 2003).

ii.) Alkalphilic Fe (III) and Mn (IV) Reduction

Alkaline metal-reducing microorganisms were the focus of this research, which was conducted on water column and sediment samples from a saline and alkaline (pH~9.4) terminal lake, Walker Lake, NV. Studies mentioned in this section are relevant to characterizing alkaliphilic microbes and were conducted in aquatic systems ranging to Walker Lake (e.g. Mono Lake, CA, Soap Lake, WA). An alkaliphilic, metal-reducing bacterium, characterized and isolated from leachate ponds at the U.S Borax Company in Boron, CA, grew on a series of metal complexes such as Fe (III)-Citrate, Fe (III)-EDTA, Co (III)-EDTA, or Cr (VI) as the respiratory terminal electron acceptor (Ye et al., 2004). QYMF, the representative microbe, is a strict anaerobe that grows over a range of pH values from 7.5 to 11.0, with optimal growth at pH 9.5 (Ye et al., 2004).

A variety of alkaliphilic metal reducers from saline or hypersaline environments (e.g. *Geoalkalibacter ferrihydriticus, Holomonus, Bacillus spp.,* etc.) are able to tolerate a wide range of pH and NaCl concentrations, may be fermenters and are facultative or obligate anaerobes (Pollock et al., 2007, VanEngelen et al., 2008, Zavarzina et al., 2006, Zhilina et al., 2008). Briefly, *Geoalkalibacter ferrihydriticus* strain Z-0531 is the first alkaliphilic representative of the family *Geobacteraceae* and was isolated from a soda lake in Russia, Lake Khadyn. This bacterium is a Gram-negative rod, obligate alkaliphile

(pH 7.8-10.0), capable of using Fe (III), Mn (IV) and elemental sulfur, among other metals as the terminal electron acceptor (Zavarzina et al., 2006).

An alkaliphile from the genus, *Holomonas*, isolated from a chemically-stratified Washington State soda lake, Soap Lake, is able to grow at pH 9, and reduces Cr (VI) and Fe (III) (VanEngelen et al., 2008). In this study, researchers observed a positive correlation between microbial reduction of various metals and increasing amounts of Fe (III), indicating a crucial role of Fe (III) in the reduction of Cr (IV) metals, (VanEngelen et al., 2008). A fermentative alkalithermophile, *Anaerobranca californiensis*, is an obligate anaerobe isolated from a hypersaline hot spring in Mono Lake (California, USA) (Gorlenko et al., 2004). *Bacillus spp.* strain SFB is an Fe (III)-reducing bacterium that was also isolated from Soap Lake, Washington. It is a halotolerant, fermentative, facultative anaerobe that can grow in medium with 15% by mass NaCl (Pollock et al., 2007). A Fe (III)-reducing peptide fermenting bacterium, *Alkaliphilus peptidofermentans*, isolated from Verkhnee Beloe (Buryatia, Russia) can tolerate NaCl concentration of 50 g/l (Zhilina et al., 2009).

B. Dissimilatory Metal Reduction

Dissimilatory metal reduction in microorganisms is a developing field of microbiology and is increasingly being recognized as an environmentally-significant process. A particular significance resides in the ability of metal reducers to affect the speciation of a metal by cycling it between soluble and insoluble forms, a process that also impacts the mobility of the metal (Gadd, 2004). The importance of understanding the characteristics of metal-reducing microorganisms stretches beyond their environmental context.

Microorganisms are the only members of the biosphere that can use elements such as Fe, Mn, Cr, As, S, to name a few, as terminal electron acceptors (TEA) for energy transduction in the absence of oxygen. In stratified lakes, where anaerobic conditions persist and soluble or insoluble sources of metals can substitute for oxygen, studying microorganisms in aquatic systems can reveal information about the interaction of metals and the microbial community and highlight both the biotic and abiotic processes of metal cycling.

i. Studies and Significance of Dissimilatory Metal Reduction

Microbial metal reduction as a field of study gained momentum in the late 1980s. Spearheading the research were scientists like Kenneth H. Nealson, Charles R. Myers and Derek R. Lovely. These microbiologists were the initial experts on anaerobic microbial metal reduction and characterized metal-reducing bacteria that today serve as examples. Derek Lovley has done extensive work on *Geobacter metallireducens*, which was first isolated from Potomac River sediments in Washington D.C. in 1987 (Pollock et al., 2007). Kenneth Nealson characterized a strain known as MR-1, now known as *Shewenella oneidensis* (Venkateswaran et al., 1999) in 1988 from Oneida Lake in New York (Myers & Nealson, 1988). Originally, microbe-metal interactions were studied under aerobic conditions. Aerobic microbes use O_2 as their terminal electron acceptor, coupling the oxidation of organic compounds to the reduction of oxygen while assimilating metals into cellular components (Lovley, 1993). In dissimilatory metal reduction, the distinction lies in microorganisms using metals as terminal electron acceptors in the absence of oxygen in anaerobic environments. In the process of metal reduction, electrons from organic compounds or H_2 are transferred to the oxidized forms

of metals such as iron, manganese, uranium, selenium, or chromium. In the presence of oxygen, iron is oxidized to its ferric Fe (III) state (Lovley, 1993). In anoxic environments, ferric Fe (III), iron may be microbially reduced to the ferrous Fe (II) state. Iron is one of the most abundant metals available in the earth's crust; the reduction of Fe (III) to Fe (II) is a geochemically significant reaction in sedimentary environments and deposits of iron (Nealson & Myers 1990).

ii.) Formation and Significance of Fe (III) and Mn (IV) Oxides

Fe (III) and Mn (IV) have redox potentials near that of nitrate and above that of sulfate, making them both thermodynamically and kinetically favorable as terminal electron acceptors (Weber et al., 2006). However, Mn (IV) and Fe (III) form insoluble oxides and oxyhydroxides with different minerals that may limit a complete redox potential; the crystalline solid-phase of Fe (III) oxide minerals, for example, include ferrihydrite, goethite, hematite, and magnetite (Nealson & Myers, 1992). A key difference between Fe and Mn is the formation of insoluble iron sulfide precipitates, while manganese sulfide rarely precipitates (Myers & Nealson, 1988).

As stated, microbial iron redox cycling in sediments can lead to the degradation of organic matter, and along with Mn (IV), nitrate, sulfate, and carbon dioxide, may be the primary terminal electron acceptor for the decomposition of organic matter (Nealson & Myers, 1992). The majority of microbial metal reduction occurs in stratified environments and sediments where there is a high input of organic carbon (e.g. bottom of a lake with decomposing materials) (Nealson &Myers, 1992).

iii.) Diversity of Fe (III) and Mn (IV)-Reducing Microbes

Dissimilatory Fe (III)-reducing microorganisms include a wide phylogenetic diversity from both the Archaea and Bacteria. These microorganisms can tolerate a variety of physical conditions (Pollock et al., 2007)). Microorganisms in the family Geobacteraceae of the δ-Proteobacteria are among the most common and well studied in modern subsurface environments at circumneutral pH (Lovley et al., 2004). *Shewenella spp.* are γ-proteobacteria that have been isolated as Fe (III)-reducing and Mn (IV)-reducing from diverse sedimentary environments and still growing evidence suggests that β-proteobacteria may also take part in Fe (III) reduction (Fredrickson & Gorby, 1996). Other diverse dissimilatory Fe-reducing bacteria include Acidobacteria, fermentative microorganisms, and methanogenic microorganisms (Weber et al., 2006).

iv.) Metal Cycle Model in a Stratified Lake

An example of a metal cycle in a stratified lake, with a separated lower anaerobic hypolimnion, might begin upon microbial oxidation of a metal and formation of a solid phase mineral. For example, Fe (III) oxide minerals such as hematite, goethite, or magnetite are crystalline solid-phase electron acceptors. These minerals are electron sinks for iron-reducing microbes and are subsequently reduced to Fe (II), a soluble state of iron. Fe (II) diffuses upward through the anoxic hypolimnion until it makes contact with oxygenated epilimnion, oxidizes and precipitates to the sediment once again (Lovley, 1993, Weber et al., 2006). Characterizing the key players in the distribution and use of the soluble and insoluble forms of a metal will ultimately enhance knowledge about biogeochemical processes of aquatic systems.

v.) Metal Microbe Interactions

Now that the metal redox cycle and introduction of dissimilatory Fe and Mn reduction has been established, it is important to highlight how microorganisms may come into contact with and undergo reduction of metal oxides. Metal-microbe interactions can be divided into three distinct processes that can contribute to the distribution of the metal in aquatic systems: intracellular interactions, cell-surface interactions and extracellular interactions (Ford & Ryan, 1995). An intracellular interaction with metals implies assimilation of the metal for various functions, ranging from detoxification (e.g. methylation of mercury (Ford & Ryan, 1995)) and enzymatic prosthetic group functions.

Cell surface interactions with metals are perhaps the most influential factor in the distribution of the metal in natural waters (Ghiorse, 1984). Although the cell surface interactions are different depending on the microorganism, principally the process is the same: a negatively charged cell-surface interacts with the positive metal ion. The cell wall of Gram-negative bacteria have lipopolysaccharides and phospholipids with phosphoryl groups that give an abundantly electronegative charge suitable for metal binding and Gram-positive bacteria have teichoic acids and peptidoglycan, composed of phosphoryl and carboxyl groups, potential sites for metal binding (Ford & Ryan, 1995).

Extracellular interactions with toxic metals are either direct or indirect and heavily impact the mobilization of the metal. This process is most relevant to dissimilatory metal reduction. Extracellular interaction with metals is done through the production of acidic metabolites that form extracellular polysaccharides (EPS) (Ghiorse, 1984). EPS polymers strongly bind metals and their interactions are a direct consequence of the negatively charged functional groups in the exopolymer, namely pyruvyl,

phosphoryl, hydroxyl, succinyl, and uranyl groups (Ford & Ryan, 1995). In addition to EPS, microorganisms may also secrete siderophores and chelating agents to coordinate bonds with the metal ion at a higher affinity and aid in iron sequestration (Gadd, 2004). Siderophores are commonly known to coordinate Fe (II) but they can also bind metals such as Mg, Mn, Cr (III), Ga (III), and radionuclides like plutonium (IV) (Gadd, 2004). Metal-reducing microorganisms mainly interact through intracellular interactions, using metals as terminal electron acceptors, immediately resulting in the solubilization and subsequent mobilization of the metal.

C. Walker Lake: Formation of Alkaline Lake

i. Present-Day Walker Lake: Affects of Alkalinity

The Walker River flows into Walker Lake and the diverting of the river, whether due to anthropomorphic or topographical changes, has resulted in a decrease in lake water levels (Benson et al., 1991, Stine, 1990). The lake declined 48 m between 1882 and 2010 due to upstream diversion (USGS, 2005).

Figure 1: Walker Lake Google Earth image.

Walker Lake (38.99°N Long. -119.84°W Lat.) is oval shaped with a north-south trending long axis (Fig. 1). The minimum elevation of the bottom is 1,173 m above sea level near the center of the lake, and the lake bottom is steepest near the western shore, shallowest at the northern and southern ends. Due to anthropomorphic diversion of the river, the lake continues to desiccate resulting in an increase of the evaporative concentration of the total dissolved solids from 2,500 mg/L in 1882 to 18,000 mg/L in 1994 (USGS, 1995). As a

terminal lake, with no outlet for surface water, the only outflow of the lake is evaporation from the lake surface. The increase in salinity is having a dire effect on the ecosystem and particularly the survival of the Lahontan Cutthroat Trout, an endemic species protected under the US' Endangered Species Act (USGS, 2005).

ii. Preliminary Research

My previous research at the Desert Research Institute attempted to isolate metal-reducing microorganisms from Walker Lake. I conducted a preliminary study from December 2012-Febuary 2013 of the metal-reducing microorganisms in archived water samples collected from Walker Laker. Walker Lake stratifies during warmer weather causing a hypolimnion, an anoxic layer which may harbor unique metal-reducing microorganisms. My research focused on isolating metal-reducing microbes from archived sediment and water column samples collected in summer of 2008 by the Moser Lab.

In my research I enriched for Fe-reducing microorganisms with HFO as the terminal electron acceptor and characterized putative isolates. The abundance of Fe-reducing microorganisms increased proportionally with sediment depth and serial dilutions. Cell activity at lower depths was determined using Thoma cell counting chamber (Blaubrand, Wertheim, Germany) and measured on average, 10^7 cells/mL. However, there was no cell activity in the enrichments of the samples from the water column of the hypolimnion. This may have been due to 1) oxygen contamination of stored water column samples, and 2) the 3-year gap between sampling (2008) and inoculation (2012), which may have resulted in the death of the microorganisms. Furthermore, putative isolates were 99% genetically similar to partial uncultured

sediment bacteria of the genera, *Clostridium* and *Bacillus*. The preliminary research was conclusive in determining relatively significant levels of Fe-reducing bacteria in the sediment samples, providing the evidence necessary to continue research for these microorganisms at Walker Lake, an aquatic system with high solute concentration, and alkalinity.

Studies done by Brucker et al. (2010) on samples collected by the Moser Lab in 2008 used Fe NTA and Fe CR only as sources of iron-based terminal electron acceptors. At the time of sampling the lake was stratified and results from the serial dilution cultivation of iron-reducing microorganisms yielded an increasing number of Fe-reducing microbes from the lower water column depths in addition to the sediment samples. The Fe NTA samples produced a steady increase in cell activity, reaching 10^5 cells/mL in the deeper sediment as well as from the anoxic 22 m water depths. Fe Citrate cultures also yielded cell growth in 17.5, 18, 19, and 22 m water depths and a maximum 10^4 cells/mL in the deep sediment samples.

From the study conducted by Bruckner et al., (2010) which enriched microorganisms with different forms of iron oxide terminal electron acceptors (Fe CR and Fe NTA) and my preliminary results from archived samples of 2008 using HFO as the terminal electron acceptor, it became evident that Walker Lake, in both sediment and stratified water column depths, has the potential to yield iron-reducing microbes and that these microbes may be novel alkaliphilic metal-reducers.

Section II: Material and Methods

A. Sample Collection

i. Field Site

Samples used for this research were collected from Walker Lake on 02 November 2013 and again on 04 September 2014 using a 15’ Boston Whaler Boat. The sampling site was a mid-lake location known as the WL3 station and had a depth of 19.4 m in 2013 and is located 38°42'00.0"N, 118°43'17.9"W. In 2013, sampling time was early in the morning with fair weather conditions, however strong winds persisted and gradually picked up, preventing thorough sampling of various water depths. Samples collected on 04 September 2014 used similar sampling techniques; however, samples of five different water column depths were obtained. Comparatively, Walker Lake parameters during a sampling in September 2008 are provided in Table 1 and 2. Sampling in September 2008 was also conducted at WL3 by the Moser lab.

ii. Sample Collection Protocol

In 2013, collection of only three water column samples at depths of 0, 10, and 19 m and two separate sediment samples of surface and deep sediment were collected using a Ponor Grab and determined as the surface and deep layer by coloration. In 2014 five water column depths (0, 10, 15, and 17.5 m) along with sediment samples distinguished as surface (oxic) and deep (anoxic) sediment were collected. Walker Lake was determined to be isothermal at the time of sampling using 6920-V2 multi-parameter sonde (YSI, Yellow Springs, OH). Relative physical parameters included measurement of pH, total dissolved solids (TDS), and dissolved oxygen (DO); the Moser lab measured the parameters of the lake. Samples were collected in N_2-flushed vials for anaerobic culturing.

Water samples from precise depths, based on real time profile data, were collected using peristaltic pumps (Geotech, Inc. and Masterflex E/S) with sterile platinum-cured silicone tubing attached to the sonde. Water samples were filtered using a 100 µm prefilter to remove debris before injecting anaerobically into capped nitrogen flushed serum bottles. Sediment samples were collected using a PONAR dredge and observed to have a gradient of lighter color at the surface and darker in the deeper anoxic layer. As such the color was used to distinguish the surface and deep sediment layers and collected in 50 mL polypropylene tubes, Figure 2. Samples were stored at 4°C and inoculated within approximately 48 hours.

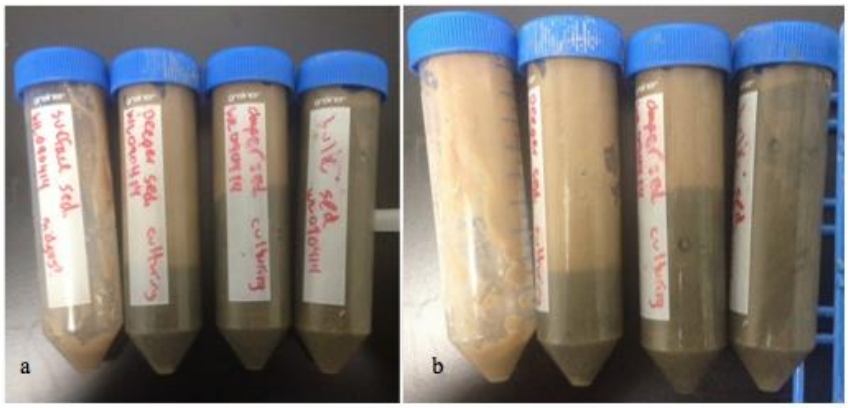

Figure 2: Sediment samples collected from Walker Lake, a. from left: surface sediment, deeper sediment, deeper sediment, bulk sediment, b. close-up of figure 2a samples: difference in surface to deeper sediment indicated by lighter and darker coloration.

Water chemistry including metal and ion analysis was conducted by Desert Research Institute's Water Laboratory, Reno, NV. for samples collected in 2008 and 2013 and by ACZ Laboratories (Steamboat Springs, CO) Inc. for samples collected in 2014. Measured parameters included ions, dissolved metals, and alkalinity among others.

iii. Media Preparation

The most recent water chemistry of Walker Lake compiled by the Moser lab was used to design a synthetic Walker Lake Basal Medium and was modified with metal. All microbial enrichments and cultivations were acquired using Walker Lake medium consisting of, per liter, 1.0g $MgSO_4 \cdot 7H_2O$, 1.04g $MgCl_2 \cdot 6H_2O$, 0.0735g $CaCl_2 \cdot 2H_2O$, 0.197g K_2HPO_4, 0.121g NH_4Cl, 0.6g KCl, 7.3g NaCl, 4.68g Na_2SO_4, 2.385g $NaHCO_3$, 1.073g Na_2CO_3, and 1 mL trace mineral supplement (ATCC), and 1mL vitamin supplement (ATCC) adjusted to 9.4 using NaOH. Formate, lactate, and acetate (FLA) were the sources of carbon added at 500 µM of basal salts. Citrate was an indirect addition of carbon added as a complex in the form of Fe (III) Citrate.
Samples collected on 04 September 2014 were inoculated with a modified Walker Lake medium that replaced the 4.68g Na_2SO_4 with 4.68g of NaCl.

iv. Metal Oxide Dissimilatory Electron Acceptors.

The metal electron acceptors were prepared and added to the Walker Lake basal salts media at concentrations of 4.17 µM HFO, 2.5µM Fe Citrate, 2.5µM Fe NTA, and 4.14 µM MnO_2 (Kostka & Nealson, 1998).

HFO was prepared by dissolving tetrachloroferrate anion $(FeCl_4)^-$ in distilled water to form $FeCl_3$ and neutralized with 10 M NaOH. The solution was allowed to settle, was decanted, and the precipitate was centrifuged and washed several times with milliQ water before anaerobically preparing for use. Fe Citrate was prepared by dissolving 0.25 M Fe Citrate to 250mL distilled water and adjusting pH to 7 with 10 M NaOH, the solution was autoclaved before anaerobic preparation (Kostka & Nealson, 1998). Fe (III) NTA was prepared by dissolving equimolar amounts of Nitrotriacetic acid and $FeCl_3 * 6H_2O$ in distilled water and adjusting the pH to 7 before preparing anaerobically

(Kostka & Nealson, 1998). Colloidal Mn O_2 was prepared aseptically by combining 100 ml of both $Na_2S_2O_3$ and $KMnO_4$ separately in distilled water and mixing for final concentration of 0.25M; stored anaerobically (Kostka & Nealson,1998).

Additionally, a sulfate-free media was used for cell culturing with the 2014 samples. Sulfate-free media was used in an effort to limit the presence of sulfate-reducing bacteria or abiotic side reaction to HS.

v. Overlay Technique

Overlay plates were used for each separate terminal electron acceptor for isolation of enrichment dilutions. Overlay plates were prepared by combining Walker Lake basal medium with autoclaved agar. This was poured first as the supporting-layer, cooled and set aside. The procedure was repeated but modified with 2% soft agar solution and combined with (Kostka & Nealson, 1998). Once prepared, the basal salts soft agar solution was aliquoted into four separate 500 mL bottles and the terminal electron acceptors (HFO, Fe Citrate, Fe NTA, and MnO_2) and their respective highest dilution cultures, both from sediment and water column samples were added. Overlay isolation techniques were not applied to 2014 sediment and water column cultures due to time constraints.

B. Enrichment Cultivation

Walker Lake samples were inoculated following serial dilution in synthetic Walker Lake Basal Medium within 48 hours after sampling and anaerobically inoculated (Hungate, 1969). The samples were labeled as sediment surface (SS), sediment deep (SD) as determined by difference in coloration (Fig. 2), water depths were classified as 0 m, 10

m, and 19 m. Each sample was individually inoculated with the four terminal electron acceptors (HFO, MnO_2, Fe NTA, Fe Citrate) in duplicate. The SS sample was serially diluted to 10^{-9} in Walker Lake Basal Salts media described above with HFO as the terminal electron acceptor and duplicated; the entire process was then repeated with MnO_2, Fe NTA, and Fe Citrate. The same method was applied to the dilution of the remaining samples SD, 0 m, 10 m, and 19 m.

Determination of cell abundance and pure culture observations were conducted by microscopy using a Thoma cell counting chamber (Blaubrand, Wertheim, Germany) with an Axioskop2 Plus microscope (Zeiss, Thornwood, NY). The method of characterization involved using Axioskop2 Plus microscope (Zeiss, Thornwood, NY) and included mobility, cell structure and spore formation.

Samples were transferred three times to fresh media from serial dilutions at an initial volume of 1 mL and subsequently 50 µl after cell activity was detected using microscopy and color change of the metal was observed. Simultaneously, overlay plates were inoculated with the highest enrichment dilution of each sample using the procedure described above. Plaque formation on the overlay plates were selected and transferred to liquid media for further isolation.

Reduction of metals was determined by the color change of the media. Fe-based TEA turned black when reduced and MnO_2 turned white when reduced. During transition from oxidized to reduced medium, plaque formation was used to confer microbial growth. Further microbial activity was determined by biofilm formation and turbidity. Microscopy was used to determine the morphology of the cells in the cultures.

Section IV Results

A. Walker Lake Parameters

i. Physical Parameters

Throughout this research, cultivation of metal-reducing microorganisms has been attempted for samples collected from Walker Lake by the Moser Lab in 2008, 2013 and 2014. The physical parameters relevant to this study include depth, temperature, pH, dissolved oxygen (DO), and total dissolved solids (TDS) of the lake. These parameters are recorded in Table 1 the data were compiled by Katie Willever, graduate student working on the Walker Lake Project at the Moser lab.

Table: 1 Physical Parameter of Walker Lake

Date	Depth (m)	Temp (°C)	pH	DO mg/L	TDS g/L
2008 Sept	0	22.9	9.41	6.62	14.5
	10	21.3	9.49	6.48	14.4
	22*	14.7	9.64	0.1[a]	13.8
2013 Nov	0	14	9.34	8.78	18.5
	10	13.9	9.36	8.79	18.5
	19.4*	13.9	9.36	8.72	18.5
2014 Sept	0	22.8	9.38	7.26	19.7
	5	22.8	9.38	7.19	19.7
	10	22.8	9.38	7.14	19.7
	15	22.8	9.38	7.11	19.7
	17.5*	22.8	9.38	7.11	19.7

*maximum depth of lake during time of sampling
[a] Indication of an anoxic layer

In 2008, the lake was stratified and a hypolimnion was observed at a depth of 22 m with a temperature of 13.9°C and DO of 0.1 mg/L. The pH at 22 m was 9.64 with TDS at 13.8 g/L. In 2013, the lake depth decreased from 22 m in 2008 to 19.4 m and further decreased when sampled in 2014 to 17.5 m. Stratification of the lake was not observed during sampling of the lake in 2013 and 2014. A consistent DO of ~8 mg/L was observed

at all depths of the lake in 2013; whereas, in 2014 the DO levels were ~7.1 mg/L. A pH of 9.4 was measured during 2013-2014 sampling. The TDS in the lake in 2008 was ~14g/L, in 2013 ~19g/L, and in 2014 ~20g/L.

ii. Iron and Manganese Analysis

The presence of dissolved iron and manganese was measured at various depths in 2008, 2013 and 2014 and are recorded in Table 2. Dr. Jim Bruckner, former postdoc at the Desert Research Institute who worked on the Walker Lake project and Katie Willever compiled the data listed in Table 2. Trace amounts of dissolved Fe and Mn (Fe was measured in µg/L). In 2008, the highest measurement of dissolved Fe was 40 µg/L at 10 m, in 2013 50 µg/L in both 10 m and 19 m depths, in 2014 60 µg/L of dissolved iron was measured at 10 m and <100 µg/L was measured in 17.5 m depths. Dissolved Mn in Walker Lake during all three years of sampling was not observed above 30 µg/L. In 2008, the highest dissolved Mn measurement was 23 µg/L at 22 m. In 2013, a consistent measurement of <20 µg/L was observed at all depths (0,10,19 m). The most recent measurement from the 2014 sampling saw the highest measurement of <30 µg/L at depths of 5 m and 17.5 m.

Table 2: Fe and Mn Concentrations in Walker Lake

Year	2008	2008	2013	2013	2014	2014
	Fe(µg/L)	**Mn(µg/L)**	**Fe(µg/L)**	**Mn(µg/L)**	**Fe(µg/L)**	**Mn(µg/L)**
0	35	6	40	<20	<200	<5
5	31	6	n/s	n/s	<100	<30
10	40	6	50	<20	60	<10
15	n/s	n/s	n/s	n/s	<200	<5
17.5	30	9	n/s	n/s	<100	<30
18	24	11	n/s	n/s	n/a	n/a
19	22	15	50	<20	n/a	n/a
22	17	23	n/a	n/a	n/a	n/a

n/s: not sampled
n/a: not applicable

B. Enrichment Cultivation

Results from duplicate serial dilution cultivation from samples, collected in 2013 and 2014, are represented in Figures 3-6. Growth from each metal is represented separately and with a side-by-side comparison of growth from 2013 and 2014.

i. HFO

HFO as the terminal electron acceptor (TEA) produced maximum growth in the samples from surface (oxic) and deep (anoxic) sediments (Fig. 3) at 10^7-10^8 cells/mL. Water column samples from 2013 yielded maximum growth at 0 m and 10 m with 10^2 cells/mL. Water column samples from 2014 had growth of 10^4 cells/mL at 15 m.

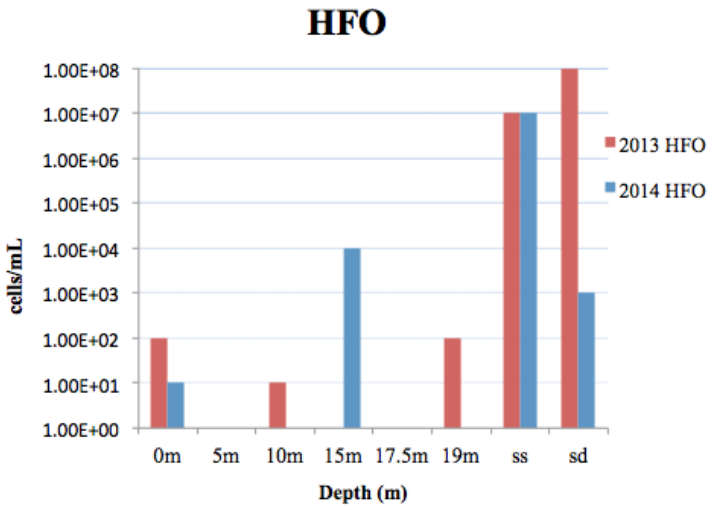

Figure 3: Dilution cultivation characteristics for samples inoculated with HFO as the terminal electron acceptor (average of duplicates), red (November 2013 samples), blue (September 2014 samples).

ii. Fe NTA

Cultures with Fe NTA (Fig. 4) as the TEA had similar growth as HFO, the highest dilution with cell activity was at 10^7-10^6 cells/mL from the sediment surface (oxic) and deep (anoxic) samples. Serial dilutions of water samples were consistently around 10^1-10^2 cells/mL.

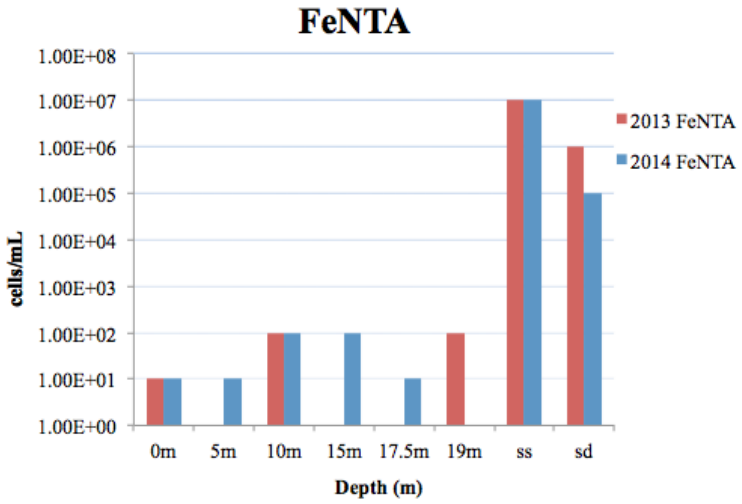

Figure 4: Dilution cultivation characteristics for samples inoculated with FeNTA as the terminal electron acceptor (average of duplicates), red (November 2013 samples), blue (September 2014 samples).

iii. Fe Citrate

Cultures inoculated with Fe CR as the TEA (Figure 5) produced the highest water sample dilutions ranging from 10^4 and 10^6 cells/mL. Sediment enrichments were consistently high reaching dilutions 10^7 cells/mL from 2013 samples.

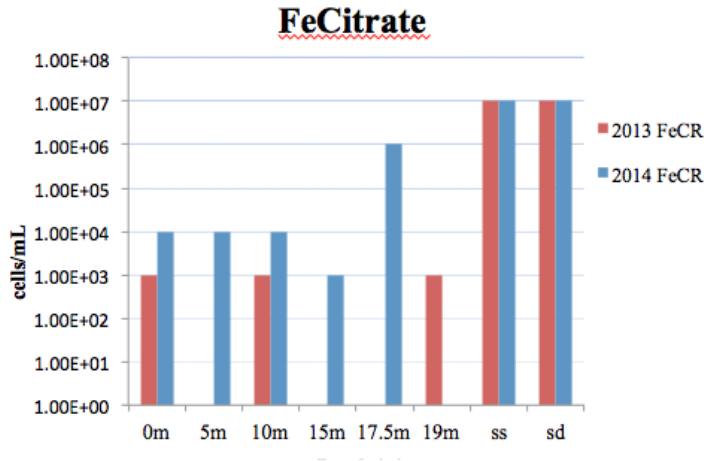

Figure 5: Dilution cultivation characteristics for samples inoculated with FeCitrate as the terminal electron acceptor (average of duplicates), red (November 2013 samples), blue (September 2014 samples).

iv. MnO_2

There was no cell activity observed in the water column cultures with MnO_2 as the TEA from 2013 and 2014 samples (Figure 6). The highest activity of 10^4 cells/mL was observed only in the sediment cultures, which was similar in both surface and deep sediments.

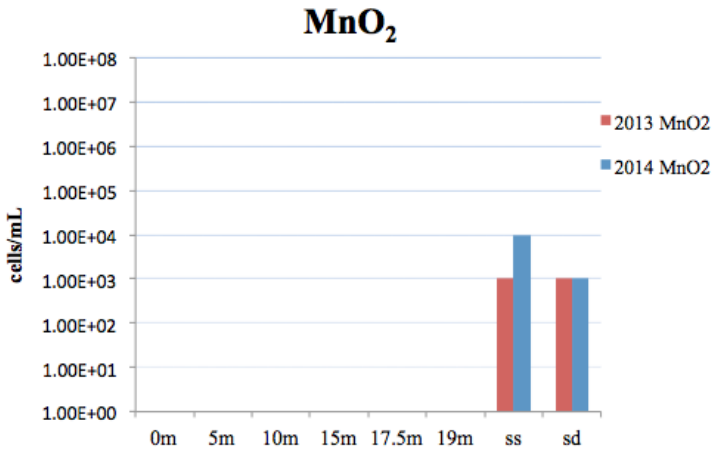

Figure 6: Dilution cultivation characteristics for samples inoculated with MnO_2 as the terminal electron acceptor (average of duplicates), red (November 2013 samples), blue (September 2014 samples).

C. Growth Characteristics

i. Dilution Characteristics

The change in the transition state and the corresponding color change in the medium of oxidized and reduced metals are shown in Figures 7-10. After inoculation from the sample, the color change occurred as a plaque-like formation until the entire medium was reduced. HFO, Fe CR, and Fe NTA changed from an oxidized state of rust (HFO, Fe NTA) and green (Fe CR) to a partially-reduced state of black (Figures 7a, 8a, and 9a). MnO_2 changes from an oxidized black state to a reduced white Fig 10(b-d); areas of brownish rather than white color change indicate intermediate level of reduction (Figure 10b). MnO_2 serial dilutions shown in the Figure 10a were attempted isolation from serial dilution conducted in samples taken in November 2013.

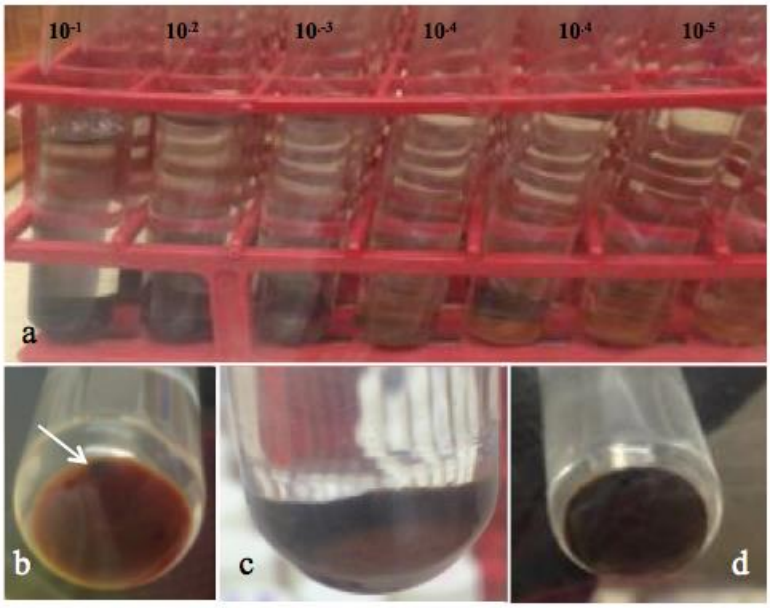

Figure 7: HFO serial dilution from 2014 samples **a.** HFO Serial dilutions 14 days after culturing of surface sediment samples **b.** Oxidized (Fe^{3+}) HFO medium with colonies of reduced iron (Fe^{2+}) indicated by arrow **c.** perimeter of reduced HFO with oxidized center. **d.** completely reduced HFO, medium changes to black.

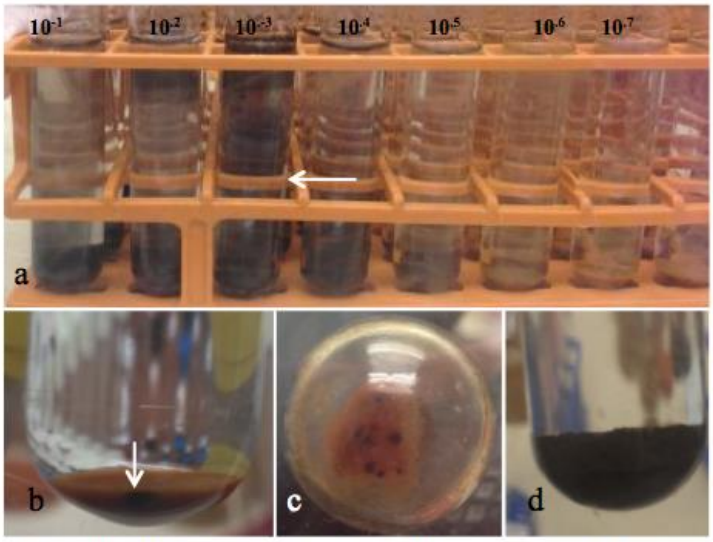

Figure 8: FeNTA serial dilution from 2014 samples **a.** FeNTA serial dilutions of surface sediment samples 14 days after inoculation, formation of biofilm in reduced FeNTA in 10^{-3} dilution indicated by arrow **b.** Oxidized (Fe^{3+}) FeNTA medium with colony of reduced iron (Fe^{2+}) indicated by arrow **c.** test tube positioned horizontally to view further colony formation. **d.** completely reduced FeNTA, medium changes to black.

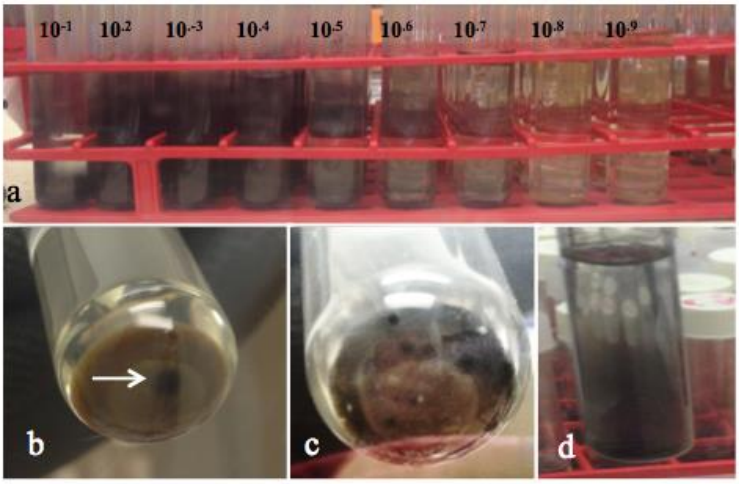

Figure 9: FeCitrate serial dilution from 2014 samples **a.** FeCr serial dilutions of sediment samples 14 days after inoculation **b.** Oxidized (Fe^{3+}) FeNTA medium with colony of reduced iron(black spot) (Fe^{2+}) indicated by arrow **c.** further colony formation. **d.** completely reduced FeCR medium changes to black, formation of biofilm.

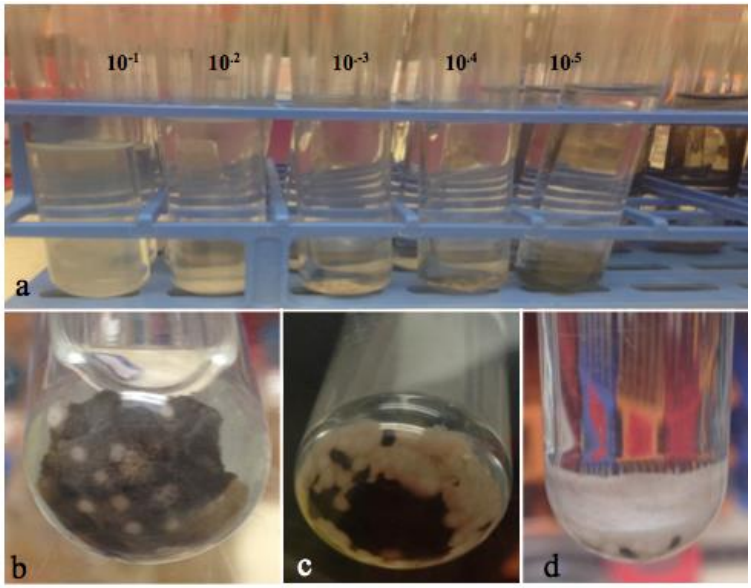

Figure 10: Dilution cultivation for isolation using Mn(IV) as TEA with 2013 deep sediment samples. **a.** Third transfer of MnO_2 serial dilutions after 15 days **b.** Oxidized (Mn^{4+}) MnO_2 medium with colony of reduced manganese(white) (Mn^{2+}) **c.** irregular shape of Mn-reduction indicates microbial reduction. **d.** completely reduced MnO_2 medium changes to white with pockets of oxidized MnO_2 indicated by arrow.

ii. Isolation Technique

The highest dilution from the initial serial dilution was subjected to further isolate in liquid and overlay media. Cultures from September 2013 samples were used. HFO

serial dilutions of 10^{-7} from the sediment samples were transferred to new liquid media twice before extinction or isolation of a single morphological microbe. Isolation attempts using the overlay technique failed to produce black colonies with HFO samples; opaque irregular shaped colonies formed and after picking a core using Pasteur pipette, did not transfer successfully to HFO liquid medium.

Putative isolates were not obtained from sediment and water column transfers of Fe NTA and Fe CR. Both Fe NTA and Fe CR overlay plates did not yield formation of black plaques. Isolation technique in liquid media for Fe NTA and Fe CR samples were not attempted due to time constraints. MnO_2 cultures transferred successfully for three months in liquid media. Under microscope observation six different morphologies were observed: spirochete, coccus, bascillus, coccobacillus, corkscrew form, and streptobacilli. These six morphologies have persisted in a series of isolation attempts. Overlay technique on MnO_2 cultures were also successful, however when transferred to liquid media, no detectable cell activity nor color change in the medium was observed.

V. Discussion

Walker Lake is an increasing saline lake as indicated from physical parameters in 2008 (~15 g/L), 2013 (~19 g/L) and 2014 (~20 g/L). The alkalinity of Walker Lake was consistent at pH 9.4 at all depths in sampling years of 2013 and 2014 (Table 1). Even with trace amounts of Fe (<100 µg/L in 2013,<30 µg/L in 2014) at the deepest part of the lake, there are still approximately 10^6-10^7 cells/mL of iron-reducing microorganisms in the sediment samples. With even lower concentrations of Fe recorded at lower depths, in the HFO serial dilutions, the most representative form of colloidal Fe in the lake, there are approximately 10^2-10^4 cells/mL of iron-reducing microorganisms. The maximum

amount of dissolved manganese recorded in the lake is <30 µg/L at 5 m and 17.5 m in 2014, and at these concentrations there were still 10^3-10^4cells/mL of manganese-reducers. Regardless of similar concentrations of dissolved manganese in the water samples, no cell activity was observed in the water column samples collected in either 2013 or 2014.

Comparatively, dilution cultivation conducted by Brukner et al. (2010) with samples collected in 2008 yielded growth of 10^4-10^5 cells/mL in both the 22 m water column and the deeper sediment (Figure 11). In addition there was an increase in microbial activity from 17.5, 18, 19, to 22 m of water depth. However, during the time of sampling in 2008, the lake was stratified and as such the lower depths were part of the hypolimnion. When samples were collected in 2013 and 2014, no hypolimnion was detected; the lake was mixed. An anoxic environment therefore was only present in the sediment and accordingly the greatest amount of growth was also observed in the sediment samples.

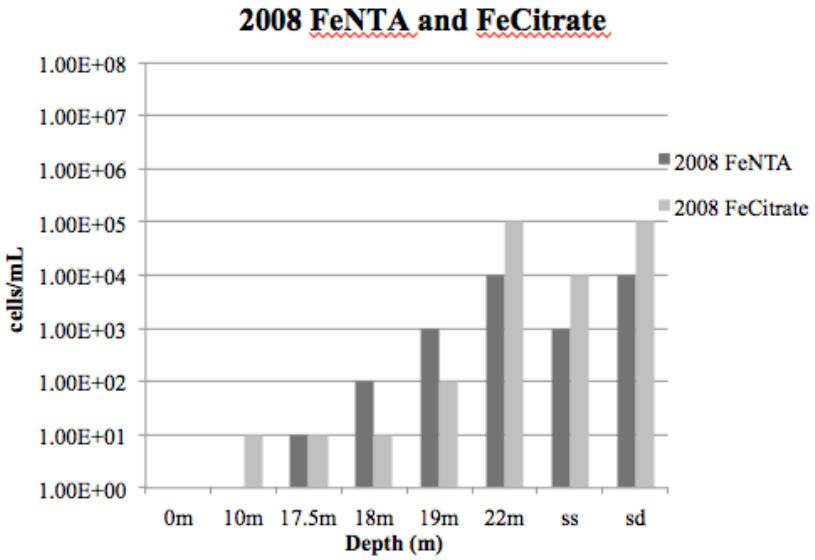

Figure 11: Dilution cultivation data from samples collected in 2008 (stratified lake environment), chart modified from Bruckner et al., 2010 data.

A sulfate-free medium was prepared for serial dilution cultivation of samples collected in 2014. This further defined the medium to select for alkaliphilic metal-reducing microbes. The change in media essentially confirmed that the TEA in MnO_2 cultures was indeed Mn(IV). Further confirmation of microbial manganese reduction is determined by the plaque formation in the medium as observed in Figures 7-10. Regardless of a change in media from 2013 that eliminated sulfate, the microbial activity of iron and manganese-reducing microorganisms in 2014 cultures stayed relatively the same.

Isolation techniques using further dilution methods and overlay plates were not altogether successful. Although MnO_2 plates yielded success in both plates and media transformation, the six morphologies observed were not separated even with additional attempts.

A. Lessons Learned and Future Goals

One of the main goals for this study was to isolate and characterize Fe (III)- and Mn (IV)-reducing microorganisms; however, the isolation technique used in this study did not successfully obtain a putative isolate. Overlay technique and recipe need to be modified and perfected. The future of this study rests upon obtaining isolates of the various samples grown on different terminal electron acceptors. DNA Extraction of 16s rRNA genes can then be applied to isolates and phylogenetic DNA analysis can characterized. Alternatively, amplified gene library can be established of mixed enrichments if isolates are not obtained. Alkaliphilic metal reduction is a field of study that is significant for its insight into the biogeochemistry of aquatic systems. However, conducting research of alkaliphilic metal reducers in Walker Lake as it continues to

undergo chemical and topographical changes can lead to insight about the role and presence of microbial metal reduction.

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