Sulfate, Nitrate and Selenium Reduction in Mining Wastewater Brine using Anaerobic Bacteria

by

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Abstract

The mining industry is a water usage intensive industry that generates large volumes of wastewater. This wastewater is technically difficult to treat when it is very saline. Large quantities of reagents are needed for chemical treatment, which is very expensive. The current thesis explores the possibility of using anaerobic bacteria to treat highly saline wastewater, as an alternative and more cost effective technology. Specifically, the concentrations of sulfate, nitrate and selenium were monitored, as these are constituents of concern at many mine sites. Samples of brine at different stages of a reverse osmosis treatment process were received from a mining company. Growth media for anaerobic bacteria were made according to the concentrations of chemicals present in the bine samples with certain amendments. Three sediment samples collected from different mine environments were tested as inocula for the experiment. Three growth conditions were also designed in order to determine the best suitable treatment conditions: condition #1 contains additional ammonia and iron salt as nutrients, condition #2 has only additional iron salt as nutrient and condition #3 has only zero-valent iron as an alternative additional iron source. DNA samples were extracted from culture sediments and analyzed using qPCR. Based on the results obtained, it was found that different combinations of inocula and growth condition were suitable for removing the most amount of sulfate, nitrate or selenium separately. In order to remove all three constituents at the same time, the best treatment was using inoculum collected from Mount Polley and adding only iron (II) chloride salt as nutrient besides carbon sources (condition #2).

Preface

This dissertation is an original work of the author, J. Liu, under the guidance of Professor S. A. Baldwin.

Raw zero-valent iron scraps were kindly provided by Connelly GPM, Inc. for research purposes. Experimental preparation and execution for the R.O. brine wastewater treatment using the adapted culture were assisted by D. Korvin.

Results and discussion related to selenium reduction in the experiment (Chapter 4 and 5) were based on analytical data provided by both ALS Environmental and civil engineering lab located at the University of British Columbia. The DNA extraction protocols included in Appendix D were designed and compiled by J. Taylor.

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List of Symbols and Abbreviations

#	Number
%	Percentage
ANCOVA	Analysis of Covariance
BC	British Columbia
bp	Base Pair
CDC	Center for Disease Control and Prevention
CO_2	Carbon Dioxide
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic Acid
GE	Gel Electrophoresis
H^+	Hydrogen Ion
H_2O	Water
H_2S	Hydrogen Sulfide
HS^-	Bisulfide
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
MIW	Mining-Influenced Water
M.O.E.	Ministry of Environment
qPCR	Quantitative Real-Time Polymerase Chain Reaction
R.O.	Reveres Osmosis
rpm	Revolutions Per Minute
rRNA	Ribosomal Ribonucleic Acid
S^{2-}	Sulfide
SMWW	Standard Methods for the Examination of Water and Wastewater
SO_4^{2-}	Sulfate
SR	Sulfate Removal
SRB	Sulfate Reducing Bacteria
SRR	Sulfate Reduction Rate
TDS	Total Dissolved Solids
UASB	Up-flow Anaerobic Sludge Blanket
USEPA	United States Environmental Protection Agency
v/v	Volume per Volume
w/v	Weight per Volume
WHO	World Health Organization
ZVI	Zero-Valent Iron
Δ	Change

List of Units

°C	Degree Celsius
cm	Centimeter
Copies/ng	Number of DNA Copies per Nanogram of DNA
g	Gram
g/L	Gram per Liter
M	Molar
m^3/hr	Cubic Meter per Hour
mg	Milligram
mg/L	Milligram per Liter
mL	Milliliter
ML/d	Mega Liter per Day
mL/L	Milliliter per Liter
mM	Millimolar
ng	Nanogram
ng/μL	Nanogram per Microliter
nm	Nanometer
ppm	Parts per Million
V	Volt
μg/L	Microgram per Liter
μL	Microliter
μm	Micrometer

Acknowledgments

1 Introduction

Looking at a typical office or school setting, many of the essentials that people use today are made from products either directly or indirectly coming from the mining industry. Mines exploit ores at different places all over the world and process them in order to look for small fractions of elements useful to peoples' daily lives. However, most of what is left over from the processing of these ores is not used otherwise and is left sitting at the mine sites. The left over is usually in the form of a mixture of water and fine particles that is called 'tailings' in general (U.S. Environmental Protection Agency August, 1994). There are also waste rock piles at mine sites and water flowing through these landforms can leach out some of the metals and other chemical compounds in the waste rock. Seepage containing concentrations of these constituents above the British Columbia (BC) water quality guidelines must be contained or treated before release to the environment.

Even though the water associated with tailings can be separated by settling out the fine particles, it usually contains high concentrations of total dissolved solids (TDS), sulfate, nitrate and heavy metals(Shao, et al. 2009). Whether the water is reused in the mineral processing process or released into the environment, it needs to be treated first. Otherwise, a large amount of tailings stored on site may raise environmental concerns when accidents happen (Hoekstra 2014).

There are already some existing technologies being implemented by the mining industry.For example, flocculation and membrane separation are used in order to produce cleaner water to be reused in the process(Zinck and Griffith, Review of Mine Drainage Treatment and Sludge Management Operations 2013). However, many technologies

encounter difficulties with the high TDS, salinity and high concentrations of contaminants present in the mining wastewater(Shao, et al. 2009). As a result, larger operating costs are spent to purchase essential chemicals required and to replace spent equipment parts, such as fouled membranes. Among all the different technologies available, biological treatments generally require less capital and operating costs (Zinck and Griffith, Review of Mine Drainage Treatment and Sludge Management Operations 2013). Since the discovery of sulfate reducing bacteria (SRB), much research and many studies have been performed in order to understand theirgrowth requirements and potential for use in mine wastewater treatment (Yi, et al. 2009, Bai, et al. 2012, Van Den Brand, et al. 2014). One particular trait of SRB that raises the interest among all the researchers is its tolerance in very saline environment and its potential of reducing metals that are difficult to separate in other treatment processes(Stam, et al. 2010, Zinck and Griffith, Review of Mine Drainage Treatment and Sludge Management Operations 2013).

Treatments have also been used in different combinations in order to improve the overall efficiency of contaminants removal (Hayrynen, et al. 2008). For example, reverse osmosis (R.O.) can be used to remove TDS first from the mine water and biological treatments can then be used to remove specific contaminants from the R.O. brine, such as selenium. However, it is not known whether bacteria capable of selenium reduction can tolerate and be active in such highly concentrated solutions. Therefore, one of the objectives of this thesis will be to examine the hypothesis that bacterial selenium reduction and removal is possible in highly saline mine-affected water effluents.

It was found that some anaerobic bacteria are already present in mine tailings water seepage collection ponds. These ponds are often populated with volunteer plants and associated microbial communities. It is possible thatthey are particularly acclimated to high TDS and are already slowly cleaning up the wastewater (Bordenave, et al. 2010). This thesis will investigate the effectiveness of using these anaerobic bacteria in treating mining wastewater by comparing inocula derived from different sediment samples collected from the local environment and mine tailings seepage ponds. Specifically, concentrations of sulfate, nitrate and selenium will be observed throughout the experiment as indicators of the performance of the anaerobic bacteria. These are three constituents of the most concern at some very large mine sites in British Columbia.

2   Background and Literature Review

In order to understand the significance of the study and the mechanisms of the anaerobic bacteriabetter so that the experiments can be properly designed, a literature review was performed.

2.1   Environmental and Health Effects of Mine-influenced Water

Based on a review (Zinck and Griffith, Review of Mine Drainage Treatment and Sludge Management Operations 2013)conducted by Natural Resources Canada, it was found that when the final effluent from the mining industry was discharged, in most cases, it was discharged into sensitive environments with aquatic life in the local area. If mine wastewater is not treated sufficiently and released into the environment, it will affect the local aquatic and terrestrial wildlife species changing their population sizes and may also cause long-term ecosystem effects (Zinck and Griffith, Review of Mine Drainage Treatment and Sludge Management Operations 2013). Among all the different kinds of contaminants present in the mining wastewater, the environmental effects of sulfate, nitrate and selenium will be examined closely in this thesis.

2.1.1 Environmental and Health Effects of Sulfate

Sulfur can be found in many different forms in the environment and there are various species of bacteria that use sulfur as a source of nutrient. For example, as shown in the figure below, sulfate is the most oxidized form of sulfur and sulfide is the most reduced

form of sulfur. Sulfate can be reduced to sulfite first and then to sulfide in anaerobic

environment by sulfate reducing bacteria (SRB). Sulfide can also be oxidized back to

sulfate in aerobic environment by sulfur oxidizing bacteria (SOB)(Postgate 1984).Based

on the type of pathway that is taken by the bacteria, the type of genes associated with the

pathways will be different as well. One of the pathways used by the SRB to reduce sulfite

to sulfide is using the *dsrAB* gene(KEGG: Kyoto Encyclopedia of Genes and Genomes

2014). In most cases, sulfate is soluble in water and exists in the environment in aqueous

phase. When it needs to be separated from the solution, it can be transformed into

elemental sulfur or reacted with metal ions to form precipitates. Then it can be easily

filtered out using membranes (Lorax Environmental 2003).
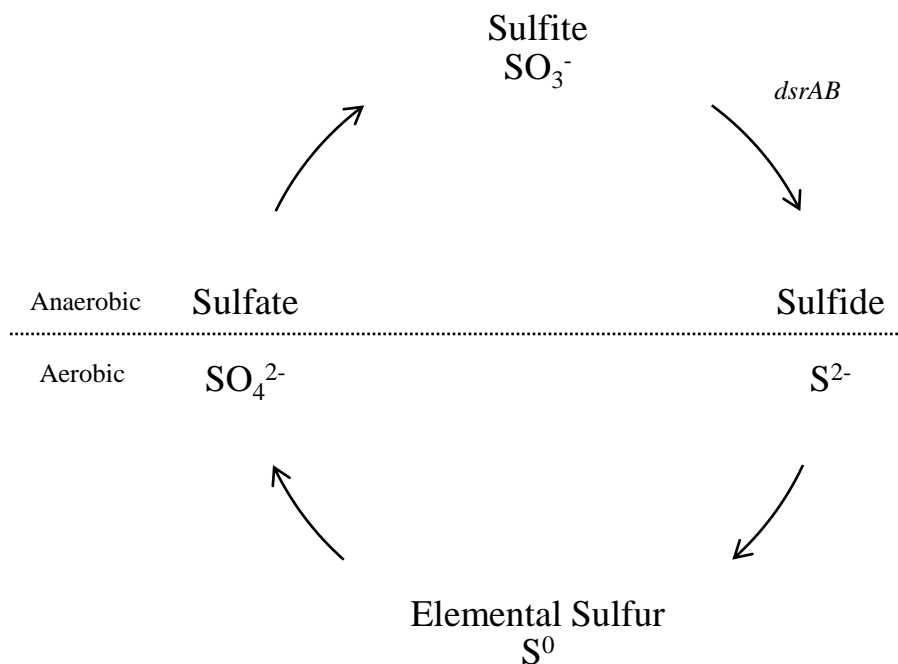
Figure 2-1 The sulfur cycle

Several research studies have been conducted by various sources. Based on a compiled report by the World Health Organization (WHO), the major environmental effect sulfate has is the potential of increasing the possibilities of 'acid rain'(The World Health Organization 2004). It is known to react with calcium carbonate that is commonly found in construction materials and increases the corrosion rates of buildings, bridges and other types of infrastructures. Thus, maintenance cost for this infrastructure increases due to frequent replacement of materials. It also makes the companies or the governments that are dealing with the impacts of acid rain carry more burdens financially. If acidic sulfate mine wastewater is released into rivers or lakes, it may decrease the pH of the water. The natural response of fish to acidic environment is to produce more mucus that is used to protect the gills. This will increase the possibilities of suffocating the fish and the reduction of the fish population in the local environment (Ljung, et al. 2009).

Compared to the environmental effects of sulfate, the health effects of sulfate cause more concern and debate. Several research studies have been conducted over the years both on human and animals. One study introduced different concentrations of sulfate in water to human infants (Backer, et al. 2001). Another used piglets as the test subject (The World Health Organization 2004). In both studies, it was suspected that if too much sulfate is consumed, it causes a non-pathogenic diarrhea. Even though laxative effects were observed, the United States Environmental Protection Agency (USEPA)and the Centers for Disease Control and Prevention (CDC) debates that it was unknown whether it was really caused by excessive sulfate consumption and what the threshold of sulfate in drinking water should be. More data need to be collected in order to determine if dehydration from diarrhea is a potential hazard(The United States Environmental

Protection Agency September, 1998). As a result, sulfate remains unregulated. The only concern for the general public right now is the distinct acidic smell of water if the sulfate concentration exceeds 350 mg/L on average. Based on the type of metal ions present in the water along with sulfate, the level of sulfate concentration threshold changes (The World Health Organization 2004). Regulation regarding the sulfate concentrations in effluents to be discharged to the environment is being implemented in British Columbia. The maximum allowable concentrations depend on the hardness of the receiving environment water and range from 128 mg/L to 429 mg/L (Meays and Nordin 2013).

2.1.2 Environmental and Health Effects of Nitrate

Nitrogen is an essential nutrient for all organisms since it is an important element in deoxyribonucleic acid (DNA) building. It can be found in many different forms in the environment so that it is readily available for the living organisms. The common forms of nitrogen include nitrate, nitrite, ammonium and nitrogen gas. Based on the type of nitrogen available in the local environment, bacteria go through either reduction or oxidation processes to make the nitrogen biologically available for reproduction (Harrison 2003). For example, nitrogen in the gaseous form is very stable and relatively inert. One of the pathways taken by the nitrate-reducing bacteria is called the nitrogen fixation. In this process, nitrogen gas is reduced into ammonia that can then be used easily by the bacteria. The type of gene associated with this process is called the *nifH* gene. Another pathway called the denitrification uses nitrate as an electron acceptor and reduce it to nitrite first and then to nitrogen. This process usually happens when the concentration of oxygen is very low or in anaerobic environment. The gene associated

with the final step in this pathway, to form nitrogen gas, is called the *nosZ* gene (KEGG: Kyoto Encyclopedia of Genes and Genomes 2014).
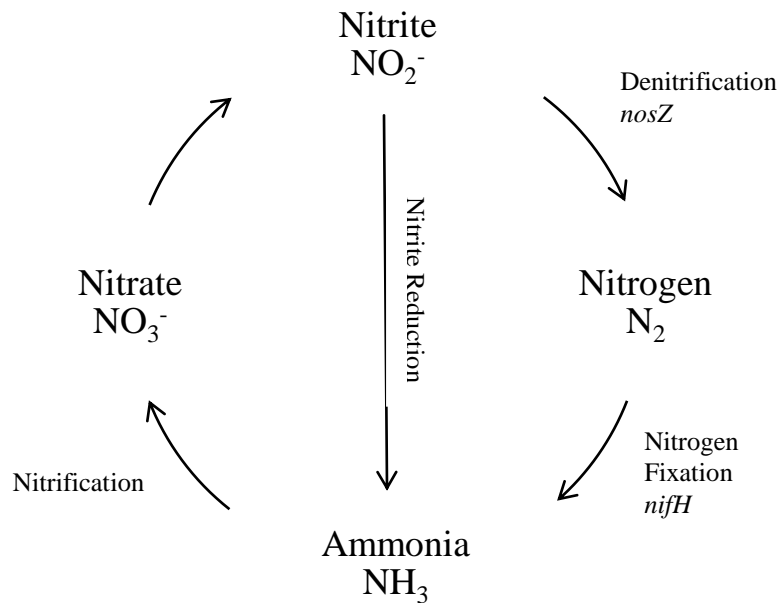
Figure 2-2 The nitrogen cycle

Nitrate is commonly observed in fertilizers and explosives, the latter being widely used on mine sites for ore blasting. When excessive amounts are used, nitrate dissolves in water and is transported for very long distances (Hayrynen, et al. 2008). If nitrate is transported through surface or ground water, some can be taken up by local vegetation as a nutrient. Excess nitrate may reach local water sources, such as estuaries, rivers and lakes. High nitrate concentrations may cause a significant imbalance in local aquatic species. For species that are nitrogen tolerant, the population will increase rapidly. However, if there are species that are very sensitive to the amount of nutrients present in the environment, they may quickly decline, which may lead to their extinction(Smith, Tilman and Nekola 1999). Such changes are most significant in microorganisms, such as

phytoplankton. If more nutrients are available suddenly, the population of phytoplankton may increase exponentially, which is commonly known as the 'red tide'. When phytoplankton die and start to decay, dissolved oxygen becomes unavailable for other types of aquatic life. This may cause death of fish due to suffocation (de Jonge, Elliott and Orive 2002). A bloom in a toxic type of phytoplankton species is another deleterious outcome of nitrate pollution. If fish or shellfish consume these toxic species, they may not be edible to human any more which may lead to significant financial losses for framers (Smith, Tilman and Nekola 1999).

The health effects of nitrate to human areless of a concern compared to its environmental effects. Based on studies compiled by the WHO, most of the nitrate consumed by human can be absorbed by regular metabolism or converted into nitrite in saliva and stomach for food digestion. Excess amount will be excreted in urine. The toxicity level for nitrate was determined to be low to moderate. Water quality guidelines for fresh water aquatic life stipulated by BC Ministry of Environment (M.O.E.) shows that nitrate should not exceed an average of 40 mg/L NO_3^--N or a maximum of 200 mg/L (Nordin and Pommen 1986). However, nitrite was found to be more toxic than nitrate. When excess amount was injected into animals, it caused adrenal hypertrophy if the concentration of nitrite exceeds 100 mg/L for potassium nitrite. It was also found that the hypertrophy effect could be recovered after 60 days (The World Health Organization 2011). The regulated nitrite concentrations are much more stringent. The average value is around 0.02 mg/L NO_2^--N for the province of BC (Nordin and Pommen 1986).

2.1.3 Environmental and Health Effects of Selenium

Among all the different kinds of heavy metals present in mining wastewater, selenium raises the most concern. It is similar to sulfur in terms of chemical properties. It exists in aqueous solutions as oxyanions, selenite and selenate. The most reduced form of selenium is selenide. When it is combined with hydrogen, it is gaseous and very toxic similar with hydrogen sulfide (Matheson Tri-Gas Inc. 2014).Sulfur is known to be an essential element in some proteins. If selenium is used in place of sulfur, it may cause malfunction in the proteins, specifically failures in re-productivity and teratogenic developments in embryos (Lemly, Environmental Implications of Excessive Selenium: A Review 1997). Similarly with sulfur and nitrogen, there are many species of bacteria that use selenium as an electron acceptor. However, in most environments, the population of these bacteria is very small and it is very difficult to study their mechanisms. Two types of genes were designed in the lab and their performance was tested in this thesis: *srdB* and *serA*(Krafft, et al. 2000, Kuroda, et al. 2011). Both of these genes are associated with the selenium reduction process, but different pathways and potentially different species are associated with these two genes.

Selenite
SeO_3^{2-}

serA
srdB

Reduction Selenate   Selenide

Oxidation SeO_4^{2-} Se^{2-}
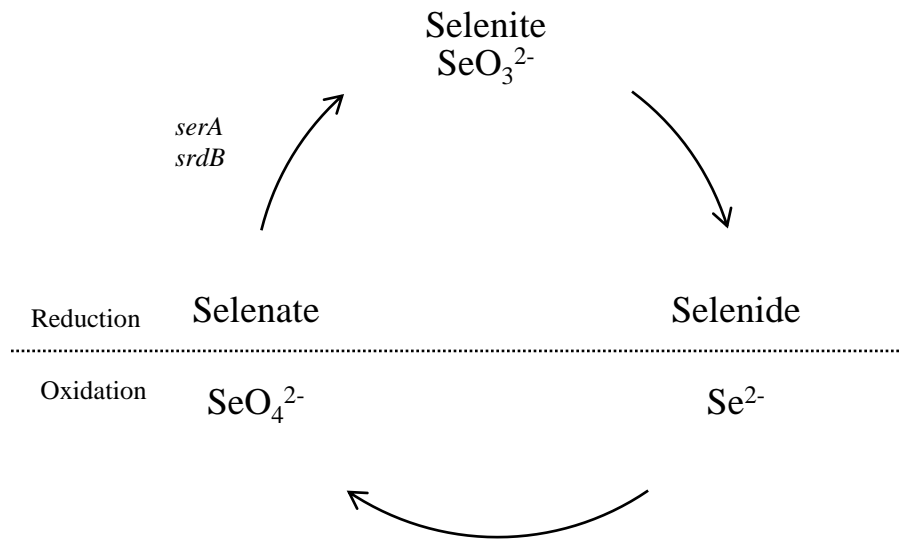
Figure 2-3 The selenium cycle

Based on a study performed by Dr. Lemly specifically for the mining industry, excessive amounts of selenium caused birth defects and very significant deformations in fish. When the larva develop into juvenile fish, the yolk may not be completely absorbed by the time the fish fully develops, which causes a swollen abdomen. In mature fish, deformed spines in both vertical and horizontal directions have been observed as a result of selenium toxicity. Deformed spines will decrease the swimming speed of fish and increase their chances of being caught by their predators. This may cause a decrease in one species' population that may lead to a chain effect of other population changes, such as their predators(Lemly, Aquatic Hazard of Selenium Pollution from Mountaintop Removal Coal Mining April, 2009).

The environmental effects caused by selenium may last for a long period of time even after the sources of release have stopped. This is because selenium can bio-accumulate in

the food chain. As a result, its effects may be carried on by offspring. Its effects may be more observable in the higher levels of the food chain (such as in fish) causing greater impact (Lemly, Environmental Implications of Excessive Selenium: A Review 1997).

For health effects of selenium, studies have shown that selenium deficiency correlates with Keshan disease and Kaschin-Beck disease. It was also suggested that it is a possible anticarcinogen. Often it is needed as a nutrient supplement. However, the diseases mentioned above have not been completely understood by people and their correlation with selenium still need to be proven. So far, selenium is more of a hazard towards animals than to human beings (The World Health Organization 2011).

2.2 Existing Selenium Removal Technologies

Since selenium has such a great hazardous potential to the local environment, a literature review on existing removal technologies was conducted in order to understand their benefits and challenges. The work described in this thesis is built on previous knowledge with the aim to improve treatment options. A review of selenium treatment options was compiled by CH2MHILL in 2010 (Sandy and DiSante June, 2010). In this review, advantages, disadvantages and capital and operating costs were listed in detail. All the technologies can be categorized into three groups: physical removal, chemical removal and biological removal. In this section of the thesis, an example technology from each category will be explained and related to the current research.

2.2.1 Physical Removal – Reverse Osmosis

One of the processes discussed in the review is called reverse osmosis (R.O.). For the current research, two different samples of wastewater were received from Teck Metals Limited to gain a better understanding of the type of water that would need biological treatment. The first sample was concentrated brine collected directly after an R.O. processwas used to clean the mining-influenced water (MIW). The second sample was concentrated R.O. brine after a sulfate removal (SR) process.

In a R.O. process, the feed water is pressurized so that the solvent (water) is forced to penetrate a semi-permeable membrane and can be released into the environment or reused. The solute, which is called the concentrate or the reject brine, is the residual containing the concentrated contaminants. This can be further processed, such as with biological treatment, to remove the contaminating chemical compounds(Sandy and DiSante June, 2010). The volumetric flow rates of raw wastewater being produced by the mining industry are challengingfor effective biological treatment. Thus physico-chemical treatment processes are often more favorable. However, the residualsfrom the physico-chemical processes are toxic and are expensive and hazardous to store. Frequently these residuals must be treated further. The volume of the wastewater being produced at mine sites can be as large as 170 m^3/hr or approximately 4 Million L/d (Shao, et al. 2009). The total dissolved solids (TDS) and pH of raw mine wastewater are in the ranges of 600 to 7000 mg/L and 2.5 to 10 respectively (Zinck and Griffith, Review of Mine Drainage Treatment and Sludge Management Operations 2013). One study found that some nutrients could be captured in the concentrate, which reduces the harshness of the

environmental conditions for the following biological treatment. It is better to implement a volume reduction process first, such as R.O., in order to remove some suspended solids and reduce the salinity of the wastewater (Hayrynen, et al. 2008). This process is favored by many companies because it requires less space relatively and it usually has a very high percentage of removal for TDS. It has also been demonstrated by various pilot plants and industrial scale operations that selenium can be reduced to less than 5 μg/L simply by this one treatment process, which is less than the standard regulated by the BC government (Sandy and DiSante June, 2010, Ministry of Environment, BC 1991).

On the other hand, the challenges of the process include membrane selection, fouling of the membrane and high maintenance costs. In order to select a suitable membrane, many parameters, such as operating pressure andfeeding temperature etc., need to be considered and optimized(Bartels, Franks and Andes 2010). Membranes used for R.O. processes have the smallest pore size compared to other types of membrane filtration processes. The particulates present in the wastewater may build up on the surface and plug the pores of the membrane. Microorganisms may also grow on the membrane and this decreases the efficiency of the R.O. process. Consequently, membranes need to be washed and replaced regularly (Malaeb and Ayoub 2011). This may increase the operating cost of the R.O. process on top of the already more expensive capital cost compared to other types of wastewater treatment systems (Zinck and Griffith, Review of Mine Drainage Treatment and Sludge Management Operations 2013).

Table 2-1 Concentrations of chemicals in the sample wastewater before and after the SR

process

	Pre-SR	Post-SR
Unit	mg/L	mg/L
Bromide (Br)	<5.0	<1.0
Chloride (Cl)	96.0	113.0
Fluoride (F)	<2.0	<0.4
Nitrate (as N)	56.9	235.0
Nitrite (as N)	<0.1	<0.02
Sulfate (SO4)	60300	1730
Aluminum (Al)	<1.0	<0.2
Arsenic (As)	<1.0	<0.2
Cadmium (Cd)	<1.0	<0.1
Calcium (Ca)	76.0	913.0
Cobalt (Co)	<1.0	<0.01
Copper (Cu)	<1.0	<0.01
Iron (Fe)	<1.0	<0.03
Lead (Pb)	<1.0	<0.05
Magnesium (Mg)	55.0	51.1
Manganese (Mn)	<1.0	<0.005
Molybdenum (Mo)	<1.0	<0.03
Nickel (Ni)	<1.0	<0.05
Potassium (K)	10.0	21.9
Selenium (Se)	7.0	1.87
Silicon (Si)	2.0	0.207
Sodium (Na)	35490	38.8
Zinc (Zn)	<1.0	<0.005

Since the brine obtained after the R.O. process contains very concentrated contaminants,

an additional SR process was implemented before the biological treatment. Wastewater

samples before and after the SR process, being tested by Teck, were sent to ALS

Environmental for analyses on the compositions of the brine (Table 2-1). By comparing

the two samples of brine, the concentrations of most contaminants were decreased, for

example, sulfate, iron and selenium. The concentrations of other contaminants, such as

nitrate, calcium and chloride, increased after the SR process. Even though magnesium stayed relatively the same, it can be consumed by microorganisms in the biological treatment. Overall, the brine obtained after the SR process is less saline. Most of the contaminants remained are essential nutrients for microorganisms (Postgate 1984). Less additional nutrients would be required to treat this brine and reduce the potential additional cost of the treatment process.

2.2.2 Chemical Removal – Zero-Valent Iron

Zero-valent iron (ZVI) is a common name used for iron or iron scraps in the form of nanoparticles. This significantly increases the contact area with other chemicals and consequently the reaction speed(United States Environmental Protection Agency 2012). Because of its reactive characteristics, ZVI has been studied and used in various wastewater, groundwater or soil treatments (Feng, et al. 2014). In order to use ZVI for wastewater treatment, the pH of the wastewater needs to be adjusted first to create an acidic environment. When the pH value is between 4 to 7, ZVI starts to oxidize and form green rust. The amount of green rust present in the process essentially determined how efficient the process would be. Green rust can combine with negatively charged ions in the wastewater and cause them to precipitate. During the process, dissolved metals in the wastewater can also be separated. For example, when ZVI is added to the wastewater, selenium can be reduced from the soluble form, which is selenate or selenite, to the insoluble from, which is elemental selenium. At the end of the process, precipitation can be relatively easily removed from the water through either sedimentation or filtration (Sandy and DiSante June, 2010).

This technology is still under research because various different reactions can happen throughout the process. The optimum reaction conditions still need to be fine-tuned. When the pH is decreased at the beginning of the process,ZVI can also form hydrogen gas with the acid in addition to green rust. When treating mining wastewater, there is usually nitrate and sulfate present in the water. One potential intermediate product that could be produced in the process is sulfite. If sulfite is reacted with hydrogen gas, hazardous gas, hydrogen sulfide, can be produced. This will increase the difficulties in managing the treatment process. Nitrate can also compete with selenium for electrons, especially when the concentration of selenium is very small compared to nitrate. It is possible that most nitrate ions get reduced into ammonia, where the concentration of selenium stays relatively the same. This can be troublesome for some companies if they have a limit on the amount of ammonia can be discharged in their effluent (Sandy and DiSante June, 2010).

Ferrous iron can also be created during the same process and stays soluble throughout the treatment. As a result, the pH needs to be adjusted again at the end of the process to precipitate ferrous iron out. Alternatively, aeration can be used to oxidize ferrous iron into the less soluble ferric iron. This may significantly increase the operation cost of the process both chemically, due to pH adjustment, and electronically, due to intensive air pumping. The ZVI also gets consumed over time and particles can deposit on the surface of ZVI. Additional costs may be associated with replacing and disposing the spent ZVI that has potential environmental hazardous materials, such as selenium. The current resolution to increase the efficiency and decrease the cost of the process is by having longer residence time(Sandy and DiSante June, 2010).

However, these are not considered as potential challenges for the current study because the main objective of the study is to reduce the concentrations of sulfate, nitrate and selenium in the wastewater. It actually is beneficial for the current study if the concentrations of the three species can be reduced simultaneously by ZVI. Having a biological treatment after the ZVI process was examined before. Results were shown that this orientation of treatment processes is feasible (Zhang, et al. 2005).The potential of using ZVI in biological wastewater treatment was also investigated by other researchers(Karri, Sierra-Alvarez and Field 2005, Shin and Cha 2008, Bai, et al. 2012, Feng, et al. 2014). By adding ZVI into the treatment process, it was found that reduction rates of nitrate and sulfate were increased separately during the same observing period. However, reducing sulfate and nitrate simultaneously needs to be further investigated. Higher operating temperature, which is suitable for bacterial growth, also favors the formation of green rust. When the pH value is between 7 and 9, it was found that not all selenium dissolved in the water would be fully reduced to elemental selenium. Part of it will be reduced partially from selenate to selenite and adsorb to the surface of ZVI (Sandy and DiSante June, 2010). This part would also be biologically available for any microorganisms present in the water and be further reduced.

2.2.3 Biological Removal - Wetlands

The most popular biological treatment process for MIWis, so far, to use constructed wetlands in which large amounts of vegetation is planted in relatively shallow wastewater. Wastewater is re-circulated through the wetland until the effluent meets the discharge standards. Wetlands do not require,or require very few, additional resources, which

decreases the operating costs significantly compared to other types of biological treatments for which carbon sources and other nutrients essential for microbial metabolisms need to be added.In a constructed wetland, natural death and decomposition of the native vegetation usually provides enough carbon substrates for microorganisms living in the wetlands. These microorganisms are found naturally in the environment and the types vary based on the different species of plants used in the wetland. Particular groups of microorganisms living in wetland sediments are largely responsible for reducing the concentration of contaminants, such as nitrate, sulfate and metals, rather than the plants themselves (Sima, et al. 2009). Some plants can also accumulate metals from water running through the wetland. This may be desired as in the case of phytoremediation, if the metals can be recovered from the plants and do not render the plants toxic to wildlife that may graze on them. Hyper metal accumulating plants can be avoided if this presents a problem.

There are several different configurations that can be used to construct wetlands: surface flow, subsurface flow and vertical up-flow. Even though subsurface flow and vertical up-flow configurations showed better removal efficiencies, they either still need to be tested in industrial scale or need to be optimized due to energy intensive pumping associated with the process. Otherwise, combinations of the three configurations can be used for optimum reduction of the contaminants. However, the system is prone to clogging that needs to be cleaned regularly or even reconstruction of the wetland (Sandy and DiSante June, 2010). Similar with all other biological treatments, operating temperatureis an important parameter that affects many of the design considerations. Especially when the wetland is constructed in relatively cold environment, the rate at which contaminants are

removed from the wastewater is much slower (Sima, et al. 2009). In order to obtain a sufficient amount of retention time in the wetlands, the size of the wetland increases proportionally. The requirement of large piece of land for the treatment may not be available all the time that may pose challenges to certain industries. This treatment process also exposes the wastewater to local wildlife. If it is not managed properly, even larger environmental effects can be afflicted to the local environment. Any associated corporation may also be prosecuted by the local government (Sandy and DiSante June, 2010).

Based on the review compiled by CH2MHILL, the technology of utilizing anaerobic bacteria in bioreactors is not a mature technology. For example, one possible configuration of the bioreactor is called the up-flow anaerobic sludge blanket (UASB) bioreactor(Sandy and DiSante June, 2010). In the UASB bioreactors, a dense layer of microorganisms is seeded at the bottom that forms granules naturally. When the wastewater is fed into the bottom of the reactor at a relatively high speed, it keeps the sludge suspended in the reactor so that the contact area can be maximized. On the top of the reactor, there is a gas, liquid and solid separator. It separates the treated water from the gas produced from microbial activities and the sludge that is entrained by the gas bubbles. This configuration provides better mixing between the wastewater and the granular sludge and the granules help the sludge settle within the reactors to prevent washout. Even though it has been studied in many different areas of wastewater treatment and showed promising results, challenges and many parameters still need to be resolved and tested (Seghezzo, et al. 1998, Li 2009).

When the UASB bioreactor is used to treat selenium, it was found that the reactor is prone to short-circuiting because gas produced by the microorganisms gets trapped below all the biomass. As a result, it needs to be taken offline and fixed. It was also found that the treatment process is very sensitive to temperature changes and the effluent does not meet the discharge standards all the time. Followup additional treatment process was required sometimes. Since the granular sludge is made of microorganisms, it may also require a long time to reach a steady state for the start up process (Sandy and DiSante June, 2010). However, the high efficiency provided by this configuration of the bioreactor attracted many researchers to study the process and try to optimize it (Li 2009). The results obtained from this research can also be potentially applied to the design of a UASB bioreactor at the mine site following the R.O. process. This may also improve the efficiency of the bioreactor in treating selenium since the wastewater being treated would have more nutrients and less salinity (Seghezzo, et al. 1998).

2.3 Sulfate Reducing Bacteria

Even though the raw mining wastewater has been treated with the R.O.and the SR processes, the salinity and some of the components' concentrations are still very high. In order to find a suitable group of microorganisms that can survive in this type of environment, the composition of the treated wastewater is compared to seawater first since seawater is commonly considered very saline water.

Table 2-2 Comparison between the composition of post-SR R.O. brine and the seawater

	Post-SRBrine	Seawater
Unit	mg/L	mg/L
Anions and Nutrients		
Chloride (Cl)	113	19.345
Nitrate (as N)	235	-
Sulfate (SO$_4$)	1730	2.701
Calcium (Ca)	913	0.416
Dissolved Metals		
Iron (Fe)	<0.03	-
Magnesium (Mg)	51.1	1.295
Phosphorus (P)	<0.3	-
Potassium (K)	21.9	0.39
Selenium (Se)	1.87	-
Sodium (Na)	38.8	10.752
Zinc (Zn)	<0.005	-

Based on the table above, many elements have high concentrations that need to be reduced, especially sulfate, nitrate and calcium.Therefore, suitable microorganisms that are selected to treat the mining wastewater need to be able to reduce sulfate in very saline environment using some metals and possibly nitrate as nutrients.

When investigating all the possible microorganisms that live in very saline environments, literature on saline and hyper-saline lakes and seaswas researched, such as Mono Lake in California and the Black Sea in southeastern Europe. Sediments from these water sourceshave been collected and studied by other scholars. It was found that sulfate reducing bacteria (SRB) along with methanogenic and archaeal communities usually are present in the sediments (Leloup, Loy, et al. 2007, Foti, et al. 2007). In these saline or hyper-saline environments, microorganisms are forced to undertake relatively high energy demanding mechanisms so that the water inside the cell wall is not lost to the

surrounding environment (Stam, et al. 2010). In order to obtain that much energy from such an extreme environment, these microorganisms usually use carbon sources that have relatively high molecular mass as substrate and reduce them as much as they can, for example lactate, malate, and glycerol(Postgate 1984). Since sodium lactate is often used as an ideal carbon source for SRB, it was selected as the carbon source for the current research.

However, these ideal substrates are not always available in the natural environment. When the amount of nutrients available is scarce, microorganisms need to find alternative substrates and thus use different metabolic pathways to maintain their regular activities. Nitrate was found to be one possible alternative substrate that uses metal ions as electron acceptors, so-called autotrophic denitrification (Moura, et al. 1997). Nitrate is present in MIW due to explosives used for ore blasting. These growth conditions meet all the requirements if these microorganisms were to be used in treating the current mining wastewater. Therefore, experiments were designed in order to promote growth for these types of microorganisms (sulfate-reducers and denitrifiers). In particular, SRB were selected as the primary subject since the discovery of SRB raises many possibilities in wastewater treatment sector and many researches have been done on this subject. Therefore, more data would be available to be used in current study.

Many researchers have been studying the mechanisms of SRB and suggested a few possible pathways for sulfate reduction using lactate as the substrate (Postgate 1984, Stam, et al. 2010, Black 2010):

$$2 \text{ Lactate} + SO_4^{2-} = 2 \text{ acetate} + 2 CO_2 + 2 H_2O + S^{2-} \qquad \text{(Equation 2-1)}$$

$$\text{Lactate} + 3/2 \ SO_4^{2-} \rightarrow 3 \ HCO_3^- + 3/2 \ HS^- + 1/2 \ H^+ \qquad \text{(Equation 2-2)}$$

$$\text{Lactate} + 1/2 \ SO_4^{2-} \rightarrow \text{acetate} + HCO_3^- + 1/2 \ HS^- + 1/2 \ H^+ \qquad \text{(Equation 2-3)}$$

$$H_2S <-> HS^- + H^+ \qquad pKa = 7 \qquad \text{(Equation 2-4)}$$
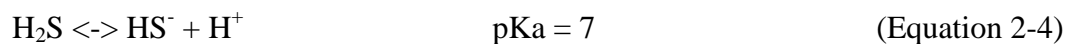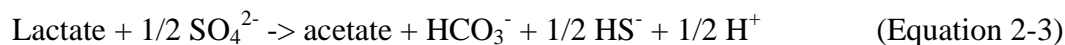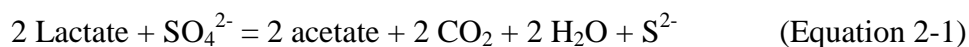
In the above equations, two possible end products that may pose questions to the current study are the sulfide ion (S^{2-}) and the bisulfide ion (HS^-). It is possible that the free sulfide ions and bisulfide ions will combine with the free hydrogen ions in the water and form hydrogen sulfide (H_2S). Hydrogen sulfide is very toxic to living organisms and may inhibit growth of SRB during the wastewater treatment process (O'Flaherty, et al. 1998). If industrial sized treatment is built, it may also be a potential fire hazard since the concentration of hydrogen sulfide gas will increase proportionally to the size of the treatment plant (Praxair Inc. 2014). As mentioned in Section 2.2.2, ZVI has been used in biological treatment before and significant increase in sulfate and nitrate reduction has been observed (Karri, Sierra-Alvarez and Field 2005, Shin and Cha 2008). It can be used in the current study to help precipitate the sulfide ions through green rust production. When iron is combined with sulfide ions, it forms blackishprecipitate (FeS), which is a good visual indicator of the presence and activity of SRB (Postgate 1984). This will also decrease the amount of sulfide ions that may become hydrogen sulfide gas and decrease the toxicity level of the treatment process. Studies also have shown that SRB grow better in the presence of metal ions (Ghazy, et al. 2011). Therefore, other forms of iron sources will also be investigated in the current research.

Suitable reaction conditions can also be selected in order to reduce the effects of hydrogen sulfide to SRB. As shown in Equation 2-4, when the pH value is below 7, more sulfur will be present in the solution as hydrogen sulfide gas. When the pH value is above 7, more sulfur will be present in the solution as bisulfide ions. It was also suggested that when the pH value is between 7 and 7.5, SRB are less sensitive to sulfide ions and the growth speed is the least affected by the sulfide ions (O'Flaherty, et al. 1998). This pH range also falls within the operating range of ZVI as mentioned before. It was found that the amount of carbon source fed into the treatment process also plays an important role. The carbon source can be represented as a chemical oxygen demand (COD) value based on the amount of oxygen required to completely oxidize the carbon source. The theoretical amount of COD required for SRB to reduce 1 g of sulfate is estimated to be 0.67 g (Rodriguez, et al. 2012). This number will also be used to calculate the amount of sodium lactate would be needed for the experiment. Usually mesophiles can be grown at 30 °C. Since most SRB can survive in that condition, this temperature will be selected for the experiments. Similar with other types of anaerobic bacteria, SRB does not require sunlight to grow. Since some SRB species are sensitive to light, during this study, all the experiments will be performed in dark in order to minimize competition of nutrients between the anaerobic bacteria and possible photosynthetic bacteria (Postgate 1984).

2.4 Quantitative Real-Time Polymerase Chain Reaction

One possible way to observe the performance of the bacteria used in the experiments implemented in this study is by measuring the changes in the concentrations of sulfate, nitrate and selenium over time compared to an abiotic control. In the abiotic controls, the

same experimental conditions are set up except without bacteria. To confirm that bacteria are involved in reduction of these chemical compounds, the amounts of the genes responsible for their reduction can be measured over time using quantitative real-time polymerase chain reaction (qPCR).

In order to perform qPCR, DNA needs to be extracted first from the sludge or biomasssamples collected throughout the experiment. This DNA contains the genetic code from the entire bacteria population. Those genes of interest relevant to the processes of biological sulfate-reduction, denitrification and selenate-reduction need to be selectively quantified from this mixture. In order to do this, the DNA sample is mixed together with a working solution containing a DNA replication enzyme, nucleotide building blocks and buffer, to which are added specific primers for the genes of interest. The specific genes are amplified during many cycles of DNA replication and their rate of amplification is quantified by recording a florescence signal. The more of a gene that is present initially, the faster its rate of amplification. Genes that are not present in the original sample are not replicated and do not generate a signal (Life Technologies 2013).

During one qPCR cycle, there are three main steps: denaturation, annealing and elongation. During the denaturation stage, the temperature of the solution is increased to the point that the double helix structure of DNA cannot be maintained. DNAbecomesunfolded and becomes single stranded. Then in the annealing stage, the primers specific for a particular gene attach to the locations on the DNA corresponding to the beginning and end of a fragment within that gene. Finally, during the elongation stage, DNA in between the forward and reverse primer is replicated by DNA polymerase

using the nucleotides included in the working solution. This forms a PCR product called an amplicon. For each amplicon made, a fluorescing signal is produced and recorded by the machine (Life Technologies 2013).

There are mainly two types of fluorescent dyethat can be used for qPCR. The first type is by including probes that are biologically engineered with a fluorescent dye on one end and a quencher dye on the other. If these two ends were relatively close to each other, the fluorescent intensity of the dyes would cancel each other out. However, if target DNAwere present, the DNA polymerase would cleave the probe causing an increase in the intensity of fluorescent (Livak, et al. 1995).

They second type of fluorescent dye used is called SYBR Green.This fluorescent dye can bind in between DNA bases during the annealing step of qPCR. When it is free in the solution, its fluorescent intensity is very low. However, if it is inserted between the DNA bases, it can emit very bright light if observed under the proper wavelength (Life Technologies 2013). For the current research, population of bacteria responsible for sulfate, nitrate and selenium reduction would need to be determined using qPCR. Since SYBR Green fluorescent dye can be used generically for different groups of primer sets, it was selected for the experiments in the current study.

The intensity of fluorescence is measured at the end of each cycle and a threshold is usually set for the intensity of fluorescence in the qPCR. This threshold marks that the change of the light intensity, and thus the concentration of DNA, is statistically significant. By recording the number of cycles required to pass this threshold, the original concentration of the DNA can be calculated.Since the cycles were repeated over and over

again, the light intensity of the fluorescent dye can also be correlated to the original

concentration of the interested DNA by comparing the results obtained with standard

solutions that have known numbersof DNA copies (Life Technologies 2014).

3   Materials and Methods

In this Chapter, the materials and procedures used in the experiments will be described in detail. Many procedures were modified versions of existing methods taken from handbooks or journal articles. References will be included at the beginning of each section for these procedures. In order to select the suitable anaerobic bacteria for the experiment, sediment samples from different mining sites were collected and a preliminary comparison among the samples was performed first. A hypothesized workflow chart of the mine wastewater treatment can be found in Appendix A.

3.1 Enrichment Culturing of SRB from the Mine Site Sediment Samples

The purpose of the initial enrichment culturing was to select for bacteria that can grow in a high-sulfate environment. This is because the mine wastewater R.O. brine contained very high concentration of sulfate. Also, SRB are known to promote the reduction of selenium, the most important contaminant to be removed from the brine.

3.1.1 Sediment Collection

Three sediment samples were collected from different mine site locations. The first sediment sample was collected from a coalmine located in the Elk River Valley. In the coalmine, there are piles of waste coal that are not needed immediately. As water flows through the piles, it can result in leaching of selenium and sulfate into the aqueous phase. It is suspected that some anaerobic bacteria grow within thesepiles and they are adapted

to the chemical environment of the waste coal seepage water. Around 300 g of coal from approximately20 cm into the bottom of one pile was collected as inoculum for one enrichment culture. For convenience in the future, cultures using this sediment sample as inoculum are labeled as 'T' in short.

The second sediment sample was collected from a natural marsh, called the Goddard Marsh, in the Elk River Valley impacted by run-off from large waste rock piles on the mine site. Goddard Marsh receives seepage directly from the mine waste rock piles. Despite the high concentrations of sulfate, selenium and nitrate in the seepage water, the water quality within the Marsh is very good.Since the biodiversity of microorganisms found in an organic-rich marsh is expected greater than in places lacking vegetation on the mine site, such as inoculum T, it is possible that some sulfate- and selenium-reducing bacteria already exist in the natural environment. If these are get selected for and become adapted to the brine mine water over several culturing passages, they may outperform the bacteria found within the mine site. Around 300 g of sediment was collected at approximately 10 cm below the surface. Cultures using this sediment as inoculum were label as 'G' in short.

The third sediment sample was collected from a pilot-scale biological treatment system at an Imperial Metals' mine site. Previous studies of the microorganisms in this system revealed that there were many types of sulfate-reducers present. Anaerobic bacteria from this site were already tolerant to a saline environment such as the tailings pond water that was being treated to remove sulfate, nitrate and selenium. Microbes growing in mine tailings ponds are a greatsource of inoculum for developing biological treatment

processes (Bordenave, et al. 2010). Samples from the Imperial Metals' mine treatment system were collected close to shore. One sample from each corner of the treatment pond was collected. However, based on study performed by a colleague, it was found that even though all the samples were collected from different locations at the settling pond, it did not make a huge difference in the biodiversity of the inoculum or the performance of the inoculum. For this thesis, a random sediment sample was selected as inoculum for the experiments. Since the mine site is located near the Mount Polley area, all the cultures using this sediment as inoculum were labeled as 'M' in short.

After all the sediment samples were collected, they were kept in coolers around 4 °C. Then they were transferred to the anaerobic hood for storage.

3.1.2 Growth Medium

Since sulfate had the highest concentration in the sample wastewater, the growth of SRB should be favored. Therefore, a growth medium suitable for SRB was selected for initial enrichment of microbes from the sediment samples. Based on a comparison study among the different types of growth medium, it was found that either Postgate B or Starkey media promotes the growth of SRB the best (Ghazy, et al. 2011). As recommended by Dr. Postgate, medium B serves as a 'general purpose medium' in which a wide diversity of SRB can grow. In the sediment samples collected for this study, the types of bacteria present were unknown. Using medium B will help to enrich for many types of natural species, which may consequently achieve higher rates of sulfate reduction and selenium removal from the mine R.O. wastewater. Most of the chemical ingredients used to make

the medium can also be stored as a stock solution (Postgate 1984). Therefore, medium B was selected for the enrichment culture.

The stock salt solution was made first in 10 times concentration. This will allow the addition of other essential nutrients, such as carbon sources, and adjustment of the pH value. The stock salt solution was autoclaved first before it was stored at room temperature. The ingredients of the 10 times concentrated salt solution can be found in the table below:

Table 3-1 Composition of 1L 10 times concentrated stock salt solution

Chemical	Weight (g)
KH_2PO_4	5.0
NH_4Cl	10.0
$CaSO_4 \cdot 2H_2O$	12.6
$MgSO_4$	9.77
$FeSO_4 \cdot 7H_2O$	5.0

During the growth of bacteria, it is possible that some metallic elements might be needed in small amounts. A trace element solution was also made and used in the growth medium in order to provide the most nutritious environment for the bacteria in the sediments. The composition of the trace element solution is listed in detail below:

Table 3-2 Composition of 1L trace element solution

Chemical	Volume/Weight	Unit
HCl (25%; 7.7 M)	10.0	ml
$FeCl_2 \cdot 4H_2O$	1.5	g
$ZnCl_2$	70.0	mg
$MnCl_2 \cdot 4H_2O$	100.0	mg
H_3BO_3	6.0	mg
$CoCl_2 \cdot 6H_2O$	190.0	mg
$CuCl_2 \cdot 2H_2O$	2.0	mg
$NiCl_2 \cdot 6H_2O$	24.0	mg
$Na_2MoO_4 \cdot 2H_2O$	36.0	mg
Distilled Water	990.0	ml

When the growth medium is being made, 100 mL 10 times concentrated stock salt

solution, 750 mL distilled water, 1 g yeast extract along with 1 mL trace element metal

solution were added to a 1 L volumetric flask first. After the pH value of the growth

medium was adjusted to around 7.5 using concentrated sodium hydroxide, nitrogen gas

was bubbled into the growth medium for 10 to 20 minutes in order to drive out any

oxygen dissolved in the medium during the mixing of the ingredients. Then 2.67 mL

sodium lactate was added to the growth medium and the pH value was checked again

before the medium was filled up to 1 L. If less than 1 L of growth medium were to be

made, all the ingredients listed above would need to be adjusted proportionally.

3.1.3 Inoculation

The enrichment cultures for each sediment sample were prepared in 300 mL bottles. Ten

percent inoculum was used for each enrichment culture, which is approximately 30 mL.

However, the sediment samples were mostly solids or rocks. When transferring the

sediment samples into the bottles, 30 g of soil sample was estimated instead and used as inoculum for all the enrichment cultures. Then the bottles were filled with growth medium to the brim to minimize the amount of air bubbles present in the culture. At last, the bottles were sealed with caps tightly.

Growth medium used for the inoculation was freshly prepared on the day of the inoculation. Inoculation was performed inside the anaerobic hood to minimize exposure of the anaerobic bacteria to air. The culture was then transferred to an incubatorand kept at 30 °C in the dark.Each culture was grown for approximately one month before being inoculated into fresh medium. Anaerobic bacteria are slow growing and need up to a month to adjust to the new environment (medium) and to increase in population.

3.1.4 Sample Collection for Analyses

In order to confirm that the growth medium was suitable and to estimate the performance of the anaerobic bacteria from the sediment samples in the cultures, supernatant was collected for analyses. Three sets of samples were collected throughout the one-month period: the first set of sample was collected on the day of inoculation, the second set of samples was collected one week after the inoculation and the last set of samples were collected at the end of the one-month period, which was before the next inoculation was performed. A 10 mL syringe was used to collect supernatant from the bottles and freshly made growth medium was used to fill the bottles back to the brim. It was important to keep the bottles filled to the brim so as to eliminate any headspace containing oxygen. The procedures for making the growth medium were the same with what was described in Section 3.1.2. Since hydrogen sulfide is one of the potential products in the culture,

samples were collected in the fume hood instead of the anaerobic hood for better ventilation. However, it was ensured that when the bottles were sealed again, minimum amount of air bubbles were present in the bottles. After sample collection, all the bottles were transferred back to the oven and preserved in dark.

3.1.5 Sample Preservation

The 10 mL supernatant sample was first filtered through 0.22 μm syringe filters and then stored in 2% w/v zinc acetate. The filter would eliminate any suspended solids such as microorganisms from the filtrate sample. Thus, when the sample was stored, the concentrations of the remaining nutrients and sulfate would remain constant since any microbial activity would be absent. Zinc acetate was used to precipitate any sulfide ions present in the supernatant (Vester and Ingvorsen 1998). Based on a rough calculation on the total amount of sulfate ions in the growth medium, 2 mL of zinc acetate was added per 5 mL of supernatant. If more supernatant were collected, proportionally more zinc acetate would be required. All the samples were kept at 4 °C until needed.

At the end of the one-month period, 0.5 mL of culture sludge was collected and preserved in glycerol at -80 °C. This was to make sure that if the culture needed to be grown again, there was inoculum readily available. Finally, sludge from the bottom the bottle was spun at 10,000 rpm for 15 minutes in 50 mL Falcon tubes. The supernatant was discarded and the sludge samples were stored at -20 °C for DNA extraction.

3.2 Acclimatization of Bacteria to the RO Brine

Next the enrichments from the Postgate B medium were adapted to the high salinity environment of the R.O. brine progressively. Since the R.O. brine before the SR process has higher salinity, it was used to acclimatize the anaerobic bacteria so that they have higher tolerance to harsh environments than what is needed. It is rather easier to adjust to environment with less salinity than with higher salinity.

3.2.1 Inoculum

To select for bacteria adapted to an environment with high sulfate concentration, three passages were performed at increasing sulfate concentrations. Each time, the concentration of sulfate was approximately doubled compared to the last passage. The inoculum used for the first passage of this experiment were taken directly from the enrichment cultures from the last experiment after they had been growing for approximately three weeks. Sludge from the bottom of the bottles were used as inoculum if it was possible since some species of SRB may be more prone to grow on the surface of particles within sediments (Postgate 1984). Then the inocula used for the subsequent passages were collected from thecurrent passage. All cultures were given approximately three weeks to adjust to the new environment and sludge from the bottom of the bottles was used if possible as the inoculum for the next passage.

3.2.2 Growth Medium

The same trace element solution was used in the growth medium for this part of the experiment as well. Since the concentration of sulfate doubles compared to the last passage, a different stock salt solution was made for the common part of the growth medium among the three passages. The detailed composition of the stock salt solution can be found in the table below:

Table 3-3 Composition of 1L 10 times concentrated stock salt solution of passages

Chemical	Weight (g)
KH_2PO_4	5.00
NH_4Cl	10.00
$CaCl_2 \bullet 2H_2O$	10.76
$MgCl_2 \bullet 6H_2O$	16.50
$FeCl_2 \bullet 4H_2O$	3.58

Similar proceduresas those used to make the growth medium for the first enrichment culture were followed for the acclimatization growth medium except a suitable amount of R.O.brine wastewater from the mine site was used to increase the sulfate concentration. These specific amounts of R.O.brine used in each passage are listed in Table 3-4:

Table 3-4 Volume of R.O. brine used in each passage

Passage Number	Volume (mL)
1	33.33
2	66.67
3	125.00

100 mL stock salt solution and the wastewater sample were added to a 1L volumetric flask first along with 1 g yeast extract, 1 mL trace element solution and 650 mL distilled water for the passages number one and two. After the pH value of the growth medium was adjusted to around 7.5, nitrogen gas was bubbled into the medium for 10 to 20 minutes to drive out any dissolved oxygen during mixing. Then 2.67 mL sodium lactate was added. However, for the third passage, since the concentration of sulfate is significantly higher than the reference enrichment culture, increased amount of nutrition was added proportionally. Instead of 1 g of yeast extract and 1 mL of trace element solution, 4 g of yeast extract and 4 mL of trace element solution were added. 10.74 g additional $FeCl_2 \Box 4H_2O$ was also added to help sulfide ions precipitate and reduce the potential toxicity level of hydrogen sulfide to the SRB. Theoretically, 0.67 g of COD would be required by SRB to reduce 1 g of sulfate. Based on this theoretical ratio, 4.83 mL of sodium lactate was added to the growth medium for the third passage instead of 2.67 mL. The growth medium was then filled up to 1 L after the pH has been checked again. If less than 1 L growth medium need to be made, all the ingredient values need to be adjusted proportionally.

3.2.3 Inoculation

Three hundred-milliliter bottles were used in the first two passages and 500 mL bottles were used in the third passage.Ten percent (by volume)inoculum was used for all the passages. Sludge from the bottom of the bottles was collected as inoculum if possible. All the inoculation procedures were carried out in the anaerobic hood to minimize exposure of bacteria to air and any growth medium used were freshly made on the day of the

inoculation. Bottles were filled to the brim with growth medium to eliminate possible air bubbles after the bottles were sealed. Then the bottles were carried over to an incubator set at 30 °C for approximately three weeksto one month before another inoculation was performed. The incubator door was covered with aluminum foil to prevent any light from penetrating into the oven, as some SRB species are sensitive to light (Postgate 1984). After passage number 3, the cultures were preserved in the same conditions with fresh growth medium until new sets of experiments were performed.Three hundred-milliliter bottles were used again when passage number 3 was repeated.

3.2.4 Sample Collection for Analyses

During each passage, samples were collected in order to check the performance of the culture. For passage number 1, since the growth medium was very similar with the enrichment culture, samples were collected only at the beginning and at the end of the passage. For passage number 2, samples were collected on the day of the inoculation, one week after the inoculation and at the end of the passage. Finally for passage number 3, samples were collected more often since the growth condition was a lot more different and a lot more harsher compared to passages number 1 and number 2. The bacteria were also given more time to get adjusted to the new environment and to reduce the concentrations of sulfate, nitrate and selenium. The same collection procedure as described in Section 3.1.4 was used to collect supernatant samples for the three passages.

3.2.5 Sample Preservation

The same procedures as described in Section 3.1.5 were used to preserve the samples collected from the three passages. In addition to preserving the supernatant of the cultures with zinc acetate, a few drops of environmental grade nitric acid was added as well because of the presence of selenium in the growth medium. The nitric acid prevents selenium from oxidizing when exposed to air. Similarly, the sludge samples located at the bottoms of the bottles were spun at 10,000 rpm for 15 minutes in 50 mL Falcon tubes. The supernatant samples from passages number 1 and number 2 were discarded. The supernatant samples from passage number 3 were filtered with 0.22 μm filters and stores with 2% w/v zinc acetate and environmental grade nitric acid. These samples were sent to ALS Environmental for detailed analyses on the compositions of the supernatant at the end of the passage number 3. Finally, 0.5 mL of supernatant was stored in 0.5 mL glycerol at -80 °C in case the cultures need to be grown again in the future. Supernatant from passage number 3 was only stored for the first time since all the following growth conditions were the same. It was assumed that the microbial communities for the three inoculums would not change very much in the following passages.

3.3 Sulfate Reduction, Selenium Removal and Denitrification of Mine R.O. Brine Wastewater Using the Adapted Cultures

In this experiment, one of the enrichment cultures prepared previously was used to examine to what extent sulfate, selenium and nitrate could be removed from an actual R.O. brine wastewater from the mine. In addition, certain amendments were tested to see if they improved removal rates. Due to high sulfate concentration in the brine, environmental samples were enriched with SRB since some SRB were also known to reduce selenium (Hockin and Gadd 2003).

3.3.1 Inoculum

For this part of the experiment, only one of the previously acclimated cultures was used for test on actual R.O. brine from the mine. Based on sulfate reduction analyses performed on the three acclimatization enrichments, it was found that inoculum T did not perform as well as the other two mine inocula. Therefore, only inoculum G and M were used for this part of the experiment. Passage number 3 was repeated and sludge samples collected after three weeks in order to make sure that the bacteria were fully adapted to the high salinityenvironment. This ensures the bacterial cultures obtained were at the optimum condition and also had a large enough population. Sludge samples were collected from the bottom of the bottles if possiblefor this experiment.

3.3.2 Growth Medium

In this experiment, R.O.brine wastewater after the SR process was used asthe growth medium. The goal was to investigate the minimum amount of additional nutrients that would be required to optimize the growth conditions for the bacterial communities responsible for reductions of sulfate, nitrate and selenium.The R.O. brine wastewater after the SR process isless saline than the brine used in the acclimation experiment since a large amount of sulfate had been removed as gypsum in the SR process (Table 2-1).Since the bacteria had been acclimatized to a very saline environment through several passages, it was hypothesized that adjusting to the new less saline environment would be quick. It was also anticipated that the microbes wouldperform better in terms of sulfate, nitrate and selenium reduction. Therefore, even if the nutrients become restricted, regular microbial activities can still be carried out. However, the microbial population may change due to competition for nutrients.

Since the R.O. process was still at the testing stage, the amount of wastewater sample received from the mine was not enough to use for the growth medium. Therefore, artificial brine was made based on the chemical analysis of the actual mine R.O. brine. This used as growth medium along with additional nutrients and trace elements, which were not present in the brine, but needed for optimal growth. Three different conditions were considered and three separate growth mediums were made. Since the concentration of sulfate in the post-SR R.O. brine wastewater was similar to the concentration of sulfate in Postgate medium B, it was better to make sure that concentrations of the other essential

Postgate medium B nutrients were at least the same in the brine growth medium. The common ingredients in all growth media are listed in Table 3-5:

Table 3-5 Common ingredients used for all three growth media

	Weight/Volume	Unit
$MgSO_4$	0.25	g/L
$CaSO4•2H_2O$	2.74	g/L
Na_2SeO_4	0.0045	g/L
NaCl	0.096	g/L
$CaCl_2•2H_2O$	0.23	g/L
$Ca(NO_3)_2•4H_2O$	1.26	g/L
KH_2PO_4	0.50	g/L
HNO_3	0.27	mL/L
Yeast Extract	1.00	g/L
Sodium Lactate	2.67	mL/L
Trace Element	1.00	mL/L

There was no ammonium salt in the artificial brine and it was not known if ammonium would be required for microbial growth or not. There was also no iron in the artificial brine. Iron is needed to remove sulfide so as to reduce sulfide toxicity. Thus two media compositions were compared. In Condition #1, both ammonium and iron were added, whereas in Condition #2, only iron was added (Table 3-6). Iron requirements are high in sulfate-reducing bioreactors due to the very high sulfate concentrations of mine wastewater. A convenient and inexpensive iron source is needed. To see if ZVI could be as effective as adding an additional treatment was carried out with ZVI added instead of iron (II) chloride salt.

Table 3-6 Differences among the three growth media

Condition #1	Concentration (g/L)
NH$_4$Cl	1.00
FeCl$_2$•4H$_2$O	3.58
Condition #2	
FeCl$_2$•4H$_2$O	3.58
Condition #3	
ZVI	18.64

By comparing test results obtained from condition number 1 with condition number 2, whether additional ammonia source needs to be added to the growth medium can be determined. By comparing test results obtained from condition number 2 and condition number 3, the optimum form of iron source in the growth medium can be determined. When the experiment was set up, samples collection and exposure of culture to air was taken into account. Since it is difficult to prevent oxygen from getting into the culture, it is not possible to take multiple samples from the same bottle for anaerobic cultures. Separate bottles were used for each sampling point so that the cultures can be grown without interference until the sample collection time. Triplicate bottles were also set up using the same inoculum for each condition at random time points. This is because growth medium has to be made on the same day of inoculation. Considering the size of the experiment, it is not feasible to set up triplicates for each sample collection time point.

All the weights and volumes used in the tables above were calculated based on the concentration of elements found in Table 2-1 or the concentrations used in the Postgate medium B. The concentration of ZVI was calculated based on the optimum reaction condition recommended in the literature (Karri, Sierra-Alvarez and Field 2005). The ZVI

used in this part of the experiment was generously provided by Connelly-GPM Inc. Detailed reports on the analyses of the wastewater samples and specifications of the ZVI can be found in Appendix C.

All the chemical compounds were added to 800 mL distilled water along with 1 g yeast extract and 1 mL trace element. Nitrogen gas was then bubbled into the growth medium after the pH was adjusted to around 7.5 with concentrated sodium hydroxide. Then sodium lactate was added to the growth medium and filled up to 1 L with distilled water. Exception was made for condition number 3 since ZVI is in the powder form. If ZVI was added into the growth medium along with other chemical compounds, it will settle at the bottom of the volumetric flask and it will not dissolve. This will make it difficult to pour out the ZVI and distribute it evenly among the bottles. Therefore, growth medium for condition number 3 was made without ZVI.

3.3.3 Inoculation

A total of fifty70 mL bottles were set up with the three different media (Table 3-6). Three-week acclimatized cultures from Goddard Marsh and Mount Polley were used as inocula (10% v/v) for 25 bottles each respectively. ZVI was also weighed and poured into the bottles separately according to the concentration described in Section 3.3.2. Growth media were freshly made on the day of the inoculation using the same procedures described in Section 3.1.2 and the bottles were kept around 30 °C in the dark. The experiment was run for 8 weeks in total and samples were collected regularly in order to observe the performance of the cultures under three different conditions.

3.3.4 Sample Collection for Analyses

From the previous culturing experiments, it was anticipated that growth in such a saline medium would be very slow. Except for the ZVI medium, individual bottles were sacrificed as samples every two weeks. Since reduction of sulfate, nitrate and selenium were possibly faster in the presence of ZVI, samples from that medium were collected initially every day for the first week of inoculation. Due to the size of the experiment, triplicate cultures for each condition and time point were not prepared. Otherwise, it would not be possible to perform all the chemical analyses quickly after sampling. Representative triplicate samples were collected for one time point for each of the medium to determine experimental error. In this way, each time point sample represents a different culture. While this may introduce variability into the experiment, the confounding factor of exposure to air due to repeated sampling from one bottle was removed. The following table shows a layout of the days where samples were collected. '3X' marks the collection of triplicate samples. The total number of data points listed in the table turns out to be 50 in total.

Table 3-7 Layout for sample collection days where colored areas marks the sacrifice of a 70 mL bottle

Time	Goddard Marsh			Mount Polley		
	Condition #1	Condition #2	Condition #3	Condition #1	Condition #2	Condition #3
Day 0						
Day 1						
Day 2						
Day 3			3X			3X
Day 4						
Day 5						
Day 6						
Day 7						
Week 2						
Week 4	3X			3X		
Week 6		3X			3X	
Week 8						

3.3.5 Sample Preservation

The same procedure described in Section 3.2.5 was used to preserve samples collected for this part of the experiment. However, some supernatant was taken after filtration to measure the concentrations of ammonia and nitrate in the cultures right away. The rest of the supernatant was preserved at 4 °C and the sludge samples were kept at -20 °C until needed.

3.4 Analytical Procedures

Throughout the experiments, several tests were performed at different stages of the experiment. The following sections will describe the procedures used for these tests performed.

3.4.1 Total Dissolved Solids Test

One possible way to monitor the removal of dissolved sulfate, nitrate and metals was by measuring the change in TDS over time. TDS measures the sum of all charged particles dissolved in the water. Cations and anions are the main contributors to the TDS. If there are microbial activities present, the TDS of the brine wastewater should decrease over time since bacteria consume all the chemicals as nutrients. It can also be correlated to the conductivity of the water since higher concentration of charged particles help conduct electricity better (Fondriest Environmental Inc. 2014). The TDS of the wastewater sample was included in the analysis reported by ALS Environmental. By performing a series of dilutions on the wastewater sample and measuring their conductivities

accordingly, a standard curve relating TDS and conductivity of the solution to the
concentration of brine can be constructed. Then the TDS of the cultures can be calculated
using this standard curve and monitored throughout the experiment. The conductivity
meter used for the experiment was WTW BtInolab pH/Cond 7200.

3.4.2 Ammonia Concentration Test

Ammonia concentration was estimated using a CHEMets kit only accurate to +/- 1 mg/L.
Samples were diluted by 100 times first since the kit only measures samples with less
than 10 ppm ammonia. After the sample was stabilized and reacted to the reagents inside
the vials, a color change can be observed after approximately 1 minute. Then the color of
the vial was compared to standards to determine the concentration of ammonia. This
concentration of ammonia is very subjective to change since the color for some standards
are very close to each other which was why it was difficult to obtain very precise results.
This test was used since ammonia must be measured immediately after sample removal
since it can volatilize and change quickly. The kit enables one to do this within a minute
or two.

3.4.3 Sulfate Concentration Test

Sulfate was measured using the barium sulfate turbidimetric method $4500\text{-}SO_4^{2-}$ from the
Standard Method for the Examination of Water and Wastewater (SMWW) handbook
(Clesceri, Greenberg and Eaton 1999). All the standard solutions were made fresh every
time. After a standard curve was obtained, all the samples were diluted in order to get the
diluted concentrations to be within the linear range of the standard curve. When the

concentrations of sulfate were calculated, the dilution factor and also the 2% w/v zinc acetate added into the samples for preservation were accounted for. Buffer solution A was used throughout the experiments. The spectrophotometer used for the sulfate concentration determination was Ultrospec 1000 UV/Visible spectrophotometer.

3.4.4 Nitrate Concentration Test

The procedure used to test the concentration of nitrate in the culture was a modified version of the method used in the CHEMets kit for nitrate. Since the kit measures nitrate concentration from 0 to 3 ppm, all the samples were diluted first so that the concentration of nitrate was roughly in this range. Based on the instructions included in the kit, 15 mL diluted sample was mixed with a package of zinc powder included in the kit. After mixing on the vortex for about 10 seconds, only the supernatant was taken and mixed with the acidifier solution also included in the kit. Instead of taking the ampoules in the kit, a color reagent was made according to the procedure included in Section 4500-NO_2^- B. 3b of the SMWW handbook. The proportion of color reagent added to the sample was also obtained based on Section 4500-NO_3^- E of the SMWW handbook (Clesceri, Greenberg and Eaton 1999). For 15 mL sample, 0.6 mL color reagent was used. A standard curve was also constructed using the same method of preparation. This modified procedure ensures that more accurate results can be obtained from the spectrophotometer instead of comparing colors by eye measurements. The spectrophotometer used for the nitrate concentration test was GENESYS[TM] 10S UV-Vis Spectrophotometer.

3.4.5 Selenium Concentration Test

The concentration of selenium cannot be detected with existing equipment in the lab. Therefore, samples were diluted by 10 times and sent to ALS Environmentalforinductively coupled plasma-mass spectrometry (ICP-MS) analysis. This was to reduce the interference of other ion particles to the test since selenium has very low concentration compared to other elements. Some samples were also analyzed in the civil engineering lab on campus. To compare the accuracy of the results, few replicate samples were selected and sent to ALS Environmental for confirmation.

3.5 Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qPCR) was used to quantify genes involved in sulfate-reduction, denitrification and selenium-reduction as a proxy for microbial activity. Genes for the biochemical pathways of interest, sulfate-, selenate-reduction and denitrification were quantified (Table 3-8). The gene copy numbers were normalized to the total bacteria as measured by the 16S gene. This represents the relative abundance of the functional groups in the total population, not the actual concentration of microbes. This was done to determine when the functionally important microorganism were most prevalent and to correlate treatment with bacterial growth.

3.5.1 DNA Extraction Protocols

At the end of each enrichment culture passage and during the acclimatization experiment, all of the sludge/sediment that were not used for inoculating the next culture was collected, centrifuged and stored at -20 °C. Two DNA extraction protocols were used.

The first protocol was developed to extraction DNA from sediment samples from early cultures that still may contain soil particles and contaminants. In this protocol, more robust mixing was implemented to release microbes from the soil particles and additional chemicals were used in order to remove contaminating compounds typically found in soil. Finally,DNA was purified by running it through a diethylaminoethyl sephacel column.

The other protocol used phenol/chloroform extraction to obtain DNA from sludge samples at the bottom of the culture bottles of later passages that were mostly biological debris or precipitated metal ions from microbial activities. The amounts of the bacteria would be greater at these times, so less harsh conditions could be used to lyse the cells and cleaner and better quality DNA obtained.

Detailed step-by-step procedures can be found in Appendix D. Two extractions from each sludge or sediment sample were performed in order to double-check the reproducibility of the extractions and to have more DNAs to work with for qPCR later. The nano-pure distilled water used to suspend DNA was collected from ELGA PureLab Option-Q. The suspended DNAs were preserved in -20 °C until prior to qPCR set-up.

3.5.2 Gel Electrophoresis

After the DNA obtained was suspended in nano-pure distilled water,its quality was checked using gel electrophoresis (GE). This was to ensure that the protocol developed in the lab can successfully extract DNA from the sludge or sediment samples and a single, clear band, indicating intact genomic DNA, was visible under UV light. To make the gel,1% w/v agarose was dissolved in TAE and 100 bp molecular weight ladder was used as a reference. The dye used for GE was SYBR safe DNA gel stain from Invitrogen (catalog number S33102) and the dye used for the DNA was diluted from 5 times concentrated nucleic acid sample loading buffer made by Bio-Rad (catalog number 170-8352). 2 µL of SYBR dye was used for a 50 mL gel. 6 µL of dye and around 20 µg of DNA were used for loading onto the gel. GE needs to be made hot to dissolve the agarose in TAE buffer. It was made sure that the gel has cooled down to approximately 65 °C before it was poured onto the plate. After the gel has completely cooled down, DNAs and the molecular rulers were loaded. It was run in TAE buffer solution for 30 minutes at 75 V. Then the picture was taken usingan Alpha InnotechMultiImage Light Cabinet.The size of the DNA obtained was not a concern since it will be checked using qPCR.

3.5.3 qPCR Set-up

3.5.3.1 Qubit

Before qPCR was performed, all the concentrations of DNA obtained from each culture were quantified through Qubit. Then the DNA would be diluted in order to obtain a concentration close to 1 ng/µL. This will simplify the qPCR procedure and comparison

between results since similar quantity of DNA would be used for the same qPCR test. When calculating the population of the bacteria, the concentration of DNA would also be needed.

Based on the number of samples being tested each time, one master mix solution was made according to Figure A-2found in Appendix A and then distributed to all the samples and standards. 1 part of dye and 199 part of buffer solution were used to make the master mix solution. Ten microliters of standards and 2 μL of DNA sample were used and the rest of the volume made up with the master mix solution. After mixing on a vortex, all the samples and standards were left siting for 2 minutes to allow enough time for the DNA and dye to react. Then readings were collected using Qubit 2.0 Fluorometer. Eppendorf tubes were lightly tapped to made sure that there were no air bubbles present before the samples were put into the fluorometer. Using these steps, the DNA concentrations were checked with broad range buffer solution first. Then after dilution, the concentrations were checked again with high sensitivity buffer solution using the same procedures described above.

3.5.3.2 qPCR Primers and Reaction Conditions

Since reduction of sulfate, nitrate and selenium were the focusof this research; the biochemical pathways responsible for these were of interest. As a result, total bacteria and primer sets for each functional group were used. *dsrA* and *dsrB* represent two regions of the dissimilatory sulfite reductase gene found in SRB. *nifH* is the nitrogen fixation gene that transforms nitrogen to ammonia. *nosZ* is the nitrous oxide reductase gene that involves in denitrification. *serA* is the catalytic subunit of the selenium reductase gene

and *srdB* is another selenate reductase gene found in bacteria able to respire on selenate. The detailed sequencesof the primers and the product sizesobtained from each primer set are listed in Table 3-8:

Table 3-8 Primer sequences used for qPCR

Primer Name	Primer Sequence	Product Size
Total Bacteria		
BACT1369F	CGG-TGA-ATA-CGT-TCY-CGG	272
BACT1492R	GGW-TAC-CTT-GTT-ACG-ACT-T	
dsrA		
dsr-1F+	ACS-CAC-TGG-AAG-CAC-GGC-GG	220
dsr-R	GTG-GMR-CCG-TGC-AKR-TTG-G	
dsrB		
2060 F	CAA-CAT-CGT-YCA-YAC-CCA-GGG	376
4R	GTG-TAG-CAG-TTA-CCG-CA	
nifH		
PolF	TGC-GAY-CCS-AAR-GCB-GAC-TC	342
PolR	ATS-GCC-ATC-ATY-TCR-CCG-GA	
nosZ		
nosZ-F	CGY-TGT-TCM-TCG-ACA-GCC-AG	434
nosZ1622R	CGS-ACC-TTS-TTG-CCS-TYG-CG	
serA		
serA_765_F	CAC-ACC-AAG-GAC-GGC-AAG-TTC	210
serA_975_R	CAA-TCT-CGG-CTT-TCA-GGC-GTT-C	
srdB		
srdB_838_F	TAC-CGT-CCG-GTT-ATC-GAG-GAA-GAG	162
srdB_1000_R	GCA-ATA-GCG-ACA-ACC-GAT-ACA-CTT	

Most of these primer sets were developed or recommended by other researchers. To determine the population of SRBs, primer sets for *dsrA* and *dsrB* were used (Leloup, Fossing, et al. 2009, Paisse, et al. 2013). To determine the population of nitrogen fixing and denitrifying bacteria, primer sets *nifH* and *nosZ* were used (Poly, Monrozier and Bally 2001, Throback, et al. 2004). Finally, the primer sets used to determine the

populations of selenium-reducing bacteria were *serA* and *srdB*. These primer sets were developed in house by colleagues in the lab. All the primers were prepared to be 5 mM in the qPCR reaction mixture.

These primer sets were used in conjunction withSsoFast™EvaGreen® supermix(catalog number 175-5201) and distilled water. A total volume of 15 µL was used for all the qPCR reactions, which comprised 7.5 µL SsoFast™EvaGreen® supermix. 0.5 µL distilled water, 1 µL forward primer, 1 µL reverse primer and 5 µL DNA or standard solution. Plasmid gene standards for total bacteria, *dsrA* and *dsrB* genes were made from pure cultures (*Desulfosporosinum orientis* and *Desulfomicrobium baculatum* purchased from DSMZ) and standards for all the other genes were amplified from mixed cultures previously grown in the lab. Reaction conditions for most primer sets were: 3 minutes at 95 °C for enzyme activation, 40 cycles of 10 seconds at 95 °C for denaturation and 30 seconds at 55 °C for both annealing and extension. The*dsrB*amplicon PCR conditions were: 40 cycles of 10 seconds at 95 °C for denaturation, 20 seconds at 55 °C for annealing and 20 seconds at 72 °C for extension were used as reaction conditions. The plates were set up with three replicates of standard dilutions and two replicates of each sample. The equipment used to run qPCRs was the CFX Connect Real-Time PCR Detection System from Bio-Rad.

3.6 Statistical Analyses Methods

3.6.1 Margin of Errors

As described above, not all experiments were performed in triplicates due to time constraints. Representative triplicate samples were set up and collected for each condition and inocula. These were used to determine experimental errors. It was not possible to perform triplicates for all conditions since the amount of workload would not feasible to be completed alone. The error bars in the graphs were calculated using standard deviation function (stdev.p) in excel. If significant amount of changes could not be surely determined based on the graph alone, then data were entered into program R for further analyses.

3.6.2 Analyses in R

There are errors associated with all experiments. Some can be minimized through repetition and some others cannot be eliminated. For this particular thesis, it is possible that when the cultures were inoculated at the beginning of each passage, the variety of bacteria in each bottle is slightly different from each other. Since it is hard to judge the population of microorganisms through eye measurement, only estimated can be made when inoculating the cultures. Small differences at the beginning of the experiment may cause significant differences at the end of the experiment when the population of the bacteria increases exponentially. Especially when the adapted cultures were used for sulfate-reduction, denitrification and selenium-reduction for the R.O. brine wastewater, all the cultures were inoculated in separated bottles. Even though triplicates were set up,

they may still not be very good representatives of the entire population. Besides the differences in inocula and treatment conditions, there may also be other factors that can affect the results obtained for the experiments, for example the combined influences of the inocula and the treatment conditions.

There are two analyses used in program R for this thesis. The first analytical method is called the analysis of covariance (ANCOVA) and the second analytical method is called the Tukey's test. In ANCOVA, it was assumed that all the data points are random and independent from each other since all the cultures were grown in separate bottles. The data were checked whether they were normally distributed first (Figure E-1, E-2 and E-3). With equal sample sizes, the slope between the covariate and the dependent variables are constant(Zaiointz 2014). The variables in the R.O. brine wastewater treatment experiment are the time duration of the experiment, treatment conditions and the inoculum used. The covariates are the interaction terms of these three variables since there could be more than one factor affecting the change of concentrations. Using program R, all these terms can be calculated and tabulated so that they can be easily compared. If the probability is greater than 0.05, the term is not very significant. Thus its effect can be omitted. If the probability is smaller than 0.05, the term has a significant effect on the overall results.

In the Tukey's test, two groups of data are compared with each other after ANCOVA to determine if there are any differences. Based on the types of inoculum and treatment condition used, the concentrations of sulfate, nitrate and selenium are compared. If the inocula and the treatment conditions have different effects on these concentrations, the

optimum parameters can be determined for industrial reactor design. The scale of difference can be determined as probability similar with described above. If the probability is less than 0.05, then the two groups are significantly different. The better treatment condition for each inoculum can thus be determined.

3.6.3 Analyses in Excel

As the population of different groups of bacteria changes, the concentration of sulfate, nitrate and selenium changes as well. The populations of bacteria based on the functional primers described in Table 3-8 will be calculated as percentage of total bacteria. These changes will be related to the changes in concentrations. If the slope is negative, then the concentration of sulfate, nitrate or selenium decreases as the specific population of bacteria increases. This trend is more desired. However, if positive slopes are observed, then the specific combination of inoculum and treatment conditions is not suitable for the reduction of sulfate, nitrate or selenium since their concentration increases as the population of bacteria increases.

4 Results

In this Chapter of the thesis, primary results obtained throughout the experiments will be presented. Results will be presented in chronological order of the experiments performed. The results obtained for the enrichment cultures using sediment samples collected from mines sites will be presented first. Then three of the passages used to acclimatize the bacteria to saline environment will be presented. Lastly, results related to the treatment of R.O. brine wastewater will be presented, including the changes in concentrations of sulfate, nitrate and selenium, TDS and qPCR results.

4.1 Enrichment of Bacteria Able to Grow in High Sulfate Medium

The purpose of this experiment was to enrich for SRB from three different mine sediment samples under optimum growth conditions to create inocula for subsequent experiments. Sulfate concentrations weremeasured in order to confirm that bacteria in the Postgate medium B were active (Figure 4-1). Based on the results obtained, the time suitable to perform the acclimatization experiment was determined.
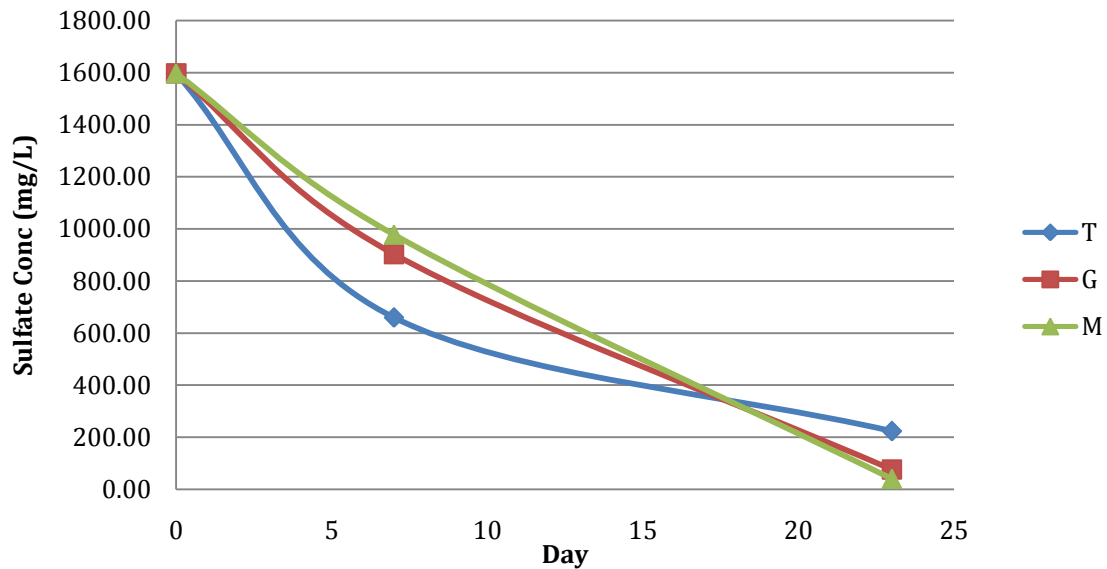
Figure 4-1 Change of sulfate concentration over time for the enrichment culture

4.2 Acclimatization of Bacteria to R.O. Brine from the Mine Site

In this experiment, inocula collected from the previous enrichment cultures were slowly trained to tolerate the very saline environment of the mine R.O. brine through a series of passages at increased concentrations of brine. The sulfate concentration increased from around 2000 mg/L in the first passage to almost 5000 mg/L in the final culture.

For passages 1 and 2, the medium conditions were very similar to those in the enrichments. Since the cultures performed well previously, it was predicted that they would adapt quickly and samples were not collected very often so as to not disturb the cultures. Similarly with the enrichment culture, the concentration of sulfate was tested in order to help determine whether a new passage needs to be performed. The change of sulfate concentration over time for passage 1 and passage 2 can be found in Figure 4-2.

61
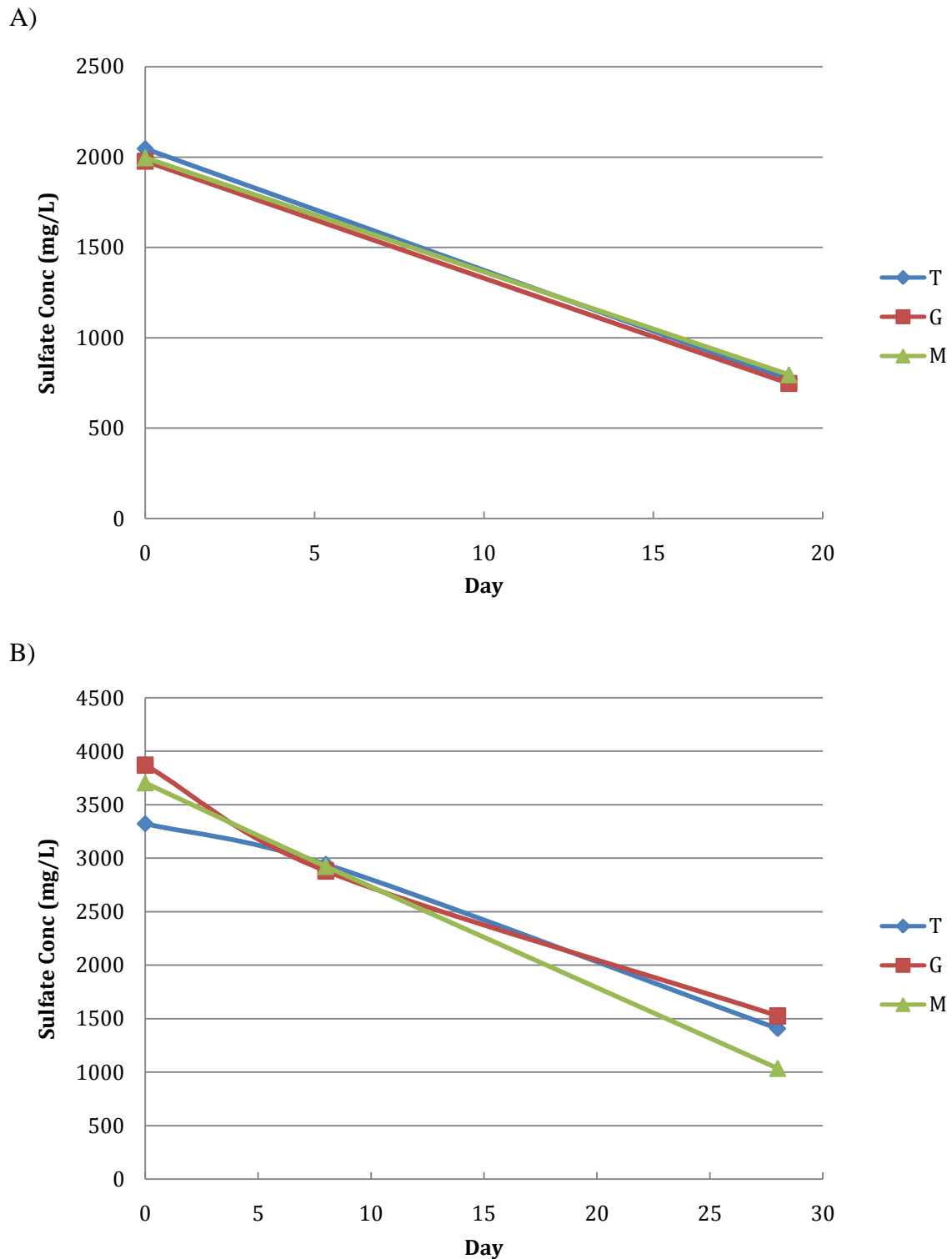
Figure 4-2 Change of sulfate concentration over timefor A) passage 1 and B) passage 2

(Errors calculated based on triplicate tests performed on the same sample)

As shown in Figure 4-2, all three inocula have performed well with respect to sulfate reductionin passage 1, approximately 60% sulfate was reduced on average over the three-week period and no significant differences were observed between the three inocula. In passage 2, approximately 64% sulfate was reduced on average over fourweeks.Inoculum M achievedmore sulfate reduction compared to the other two. Since sulfate concentrations were still high at the end of passages 1 and 2 (above the BC M.O.E. water quality guidelines), passage 3 was observed for a longer period of time to allow for more time for complete sulfate reduction.

The mediumfor passage 3 contained more brine, so its sulfate concentration was higher: around 5000 mg/L compared to 3700 mg/L in passage 2. Due to the higher sulfate concentration, the amounts of nutrients in the media were increased proportionally.Samples were collected more often (every 2 to 3 days) in order to monitor the performancesof the three different inocula. At each sampling time, the pH of the culture was measured using pH paper. Significant changes in pH were not observed for this passage. However, inoculum G has a relatively shorter lag phase compared to the other 2 inocula and the most amount of sulfate reduction was observed. Even though inoculum T and M performed similarly during the lag phase, more reduction was observed in inoculum M compared to inoculum T.
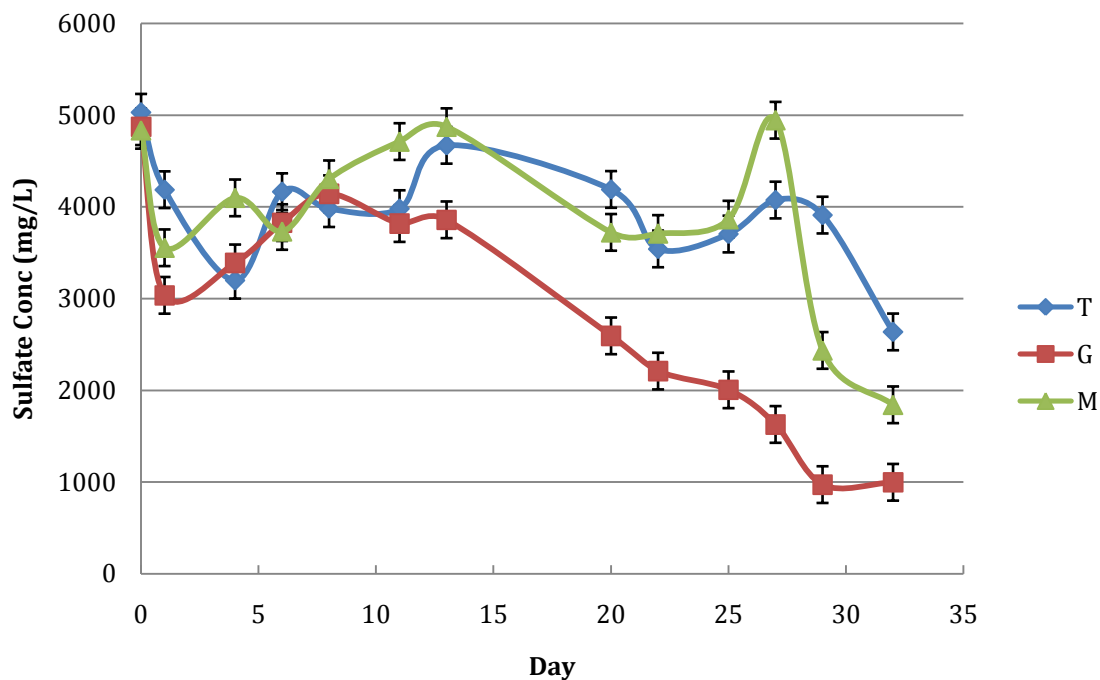
Figure 4-3 Change in sulfate concentration over time for passage 3

At the same time that culture samples were collected for sulfate concentration measurement, the conductivities of the cultures were also measured. The TDS of a solution can be linearly correlated to the conductivity of the solution (Fondriest Environmental Inc. 2014). Based on the standard curve constructed in Figure 4-4, the TDS of the culture was calculated andFigure 4-5 was obtained.
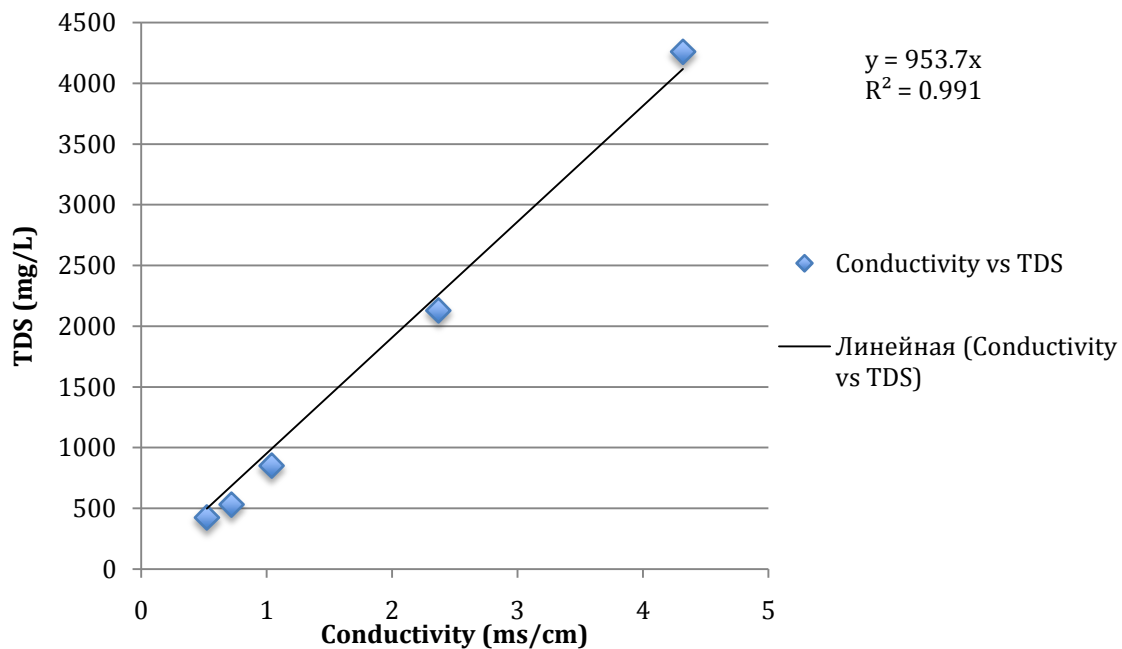
Figure 4-4 Standard curve correlating conductivity and TDS for the brine wastewater
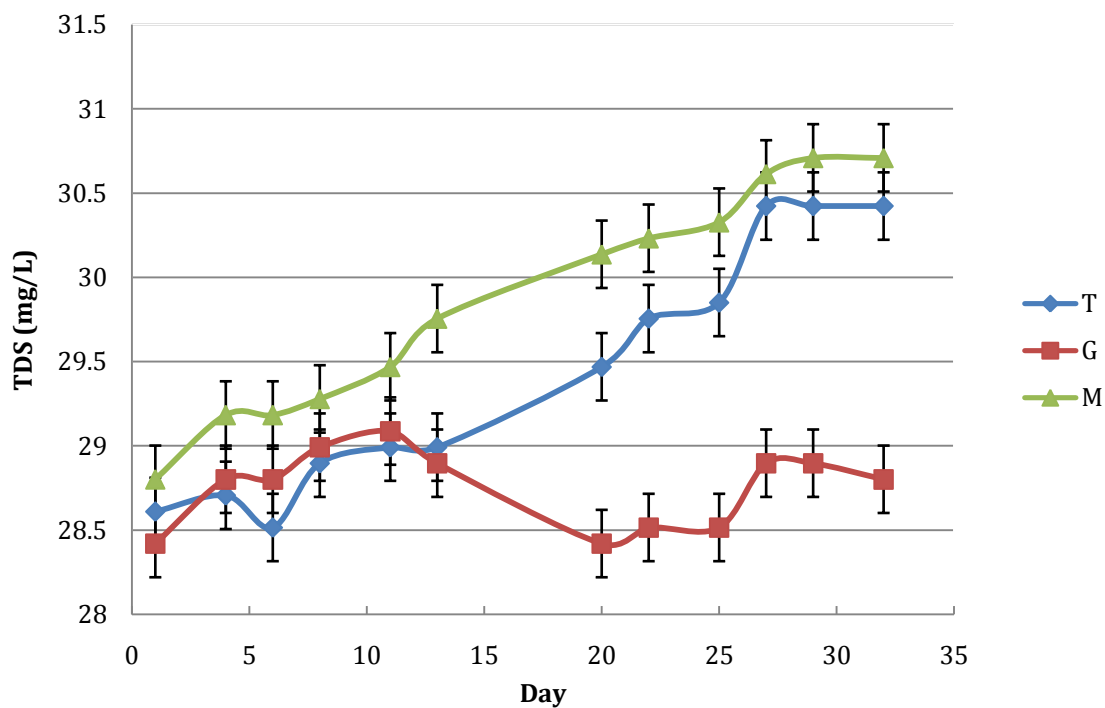
Figure 4-5 Change of TDS over time for passage 3

If the population of bacteria increases in the cultures, the TDS was expected to decrease over time as some elements in the brine get consumed as nutrients. However, from Figure 4-5, increase in TDS was observed. It is possible that some organic molecules affected the measurements for TDS (Atekwana, et al. 2004). After passage #3 was repeated 2 more times, it was concluded that inoculum T did not perform as well as inoculum G and M. Therefore, this inoculum was not further considered in the following experiments. Only inoculum G and M were studied.

4.3 Use of Acclimatized Enrichment Cultures for Sulfate, Nitrate and Selenium Removal from Mine R.O. Brine

A different brine wastewater was provided by the company for this experiment. An additional treatment step after R.O. removed sulfate by precipitation as gypsum. Chemical precipitation was deemed much faster than biological sulfate reduction and more feasible for the mine. The residual brine wastewater still contained contaminants above water quality guidelines and the objective of this experiment was to see if biological treatment could remove the sulfate, nitrate and selenium to below water quality guidelines. Selenium removal was specifically desirable due to the extreme toxicity of this element and its recalcitrance to removal using any chemical process. All the cultures in this experiment were observed for 8 weeks.

Results that will be presented in this sectioninclude the change of concentrations for sulfate, nitrate and selenium over time and qPCR results for species of bacteria related to these concentration changes. Errors included in the graphs werebased on the procedure

described in Section 3.6.1. After deleting any outliers, only the average values were used to construct graphs.

4.3.1 Change in Sulfate, Nitrate and Selenium Concentrations

The nitrate concentration of the cultures was measured right after the samples were collected since it is subject to change over time. Sulfate and selenium concentrations were measured after the 8-week period of the experiment. Sulfate concentration was measured in the lab using procedure described in Section 3.4.3. Part of the samples was sent to the civil engineering lab at UBC for selenium concentration measurements and the other part sent to ALS Environmental. The change of concentrations over time for inoculum G was presented first and then the inoculum M. All the concentrations are presented as percentage of the original concentration.

As shown in Figure 4-6, the concentration of sulfate did not change by a significant amount in 8 weeks. Nitrate concentrations continued decreasing for the entire 8-week period for all conditions. Finally, the concentration of selenium showed slowly decreasing trends for conditions #1 and #3. However, the change of selenium in condition #2 was not as obvious as the other two conditions. The error bars associated with the sulfate and nitrate measurements overlapped fro some of the treatments making it difficult to determine statistical significance visually. The concentration values were input into the program R so as to perform statistical analysis(Chapter 5).
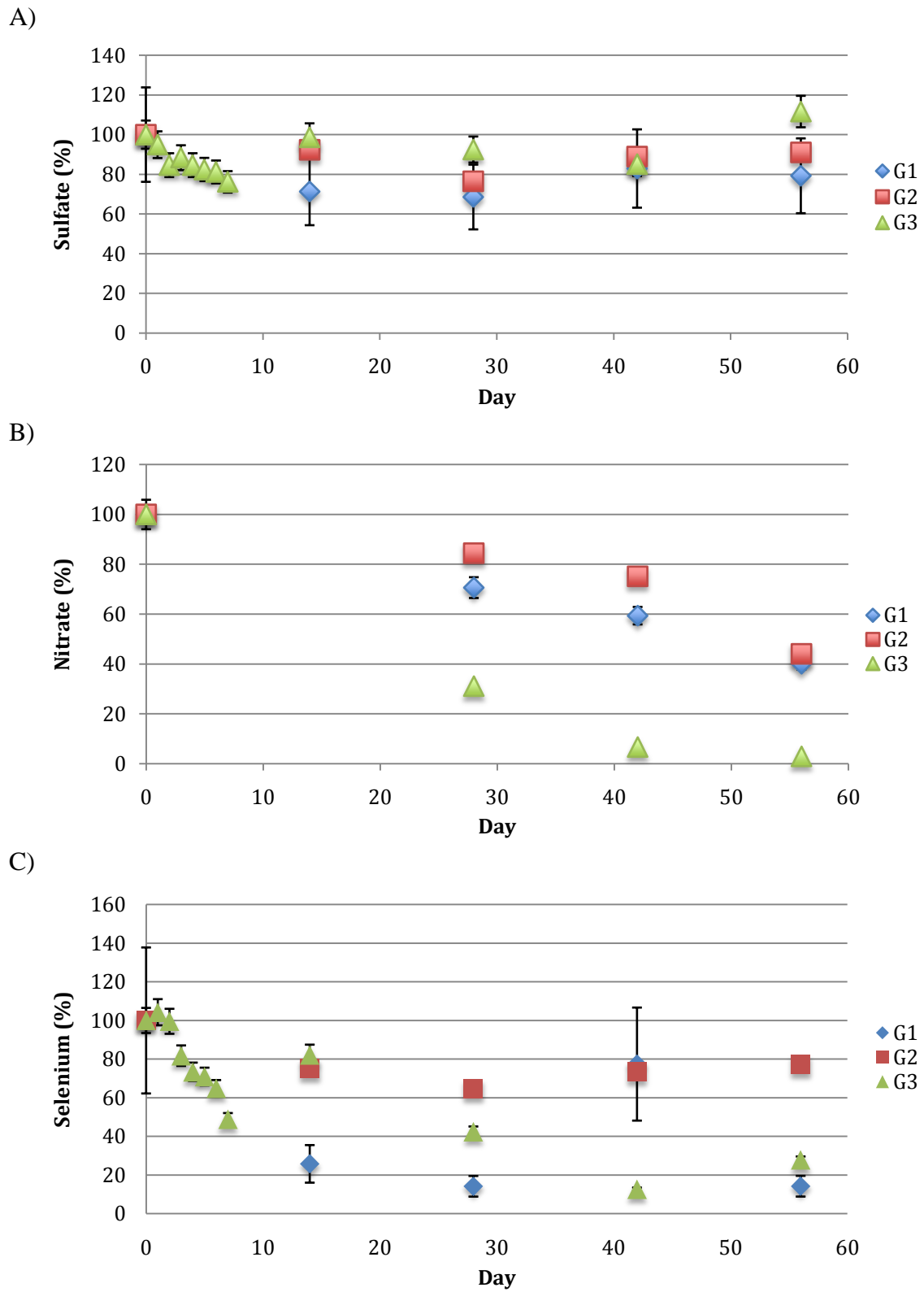
Figure 4-6 Change of A) sulfate, B) nitrate and C) selenium concentrations over time for

inoculum G (in percentage of the original concentration)
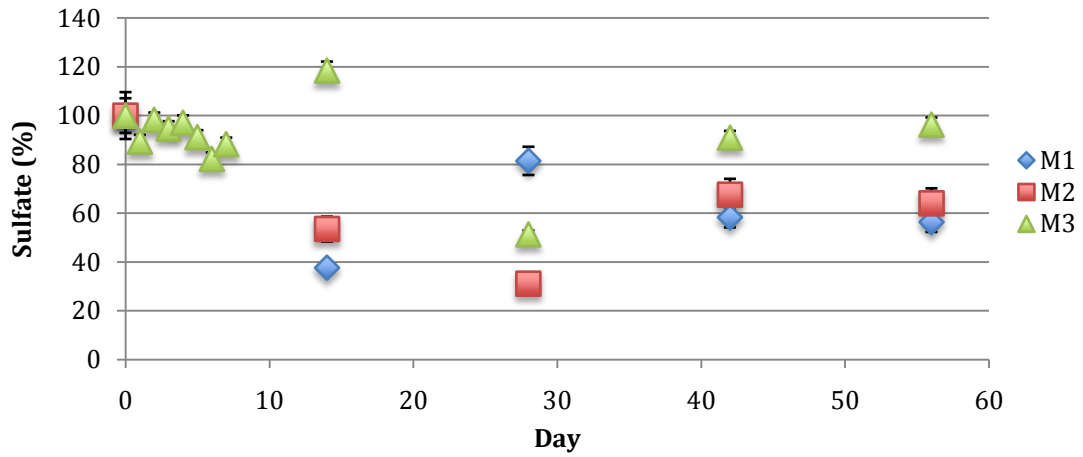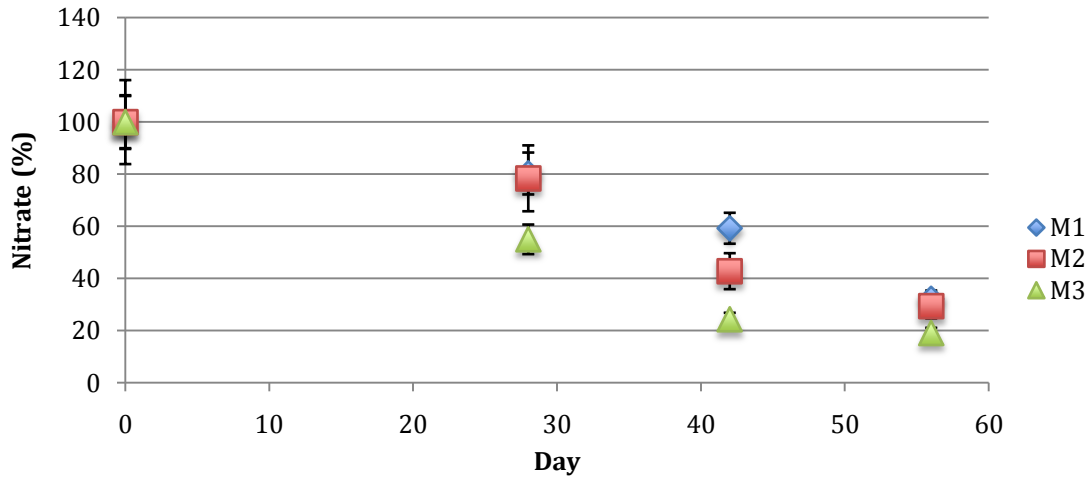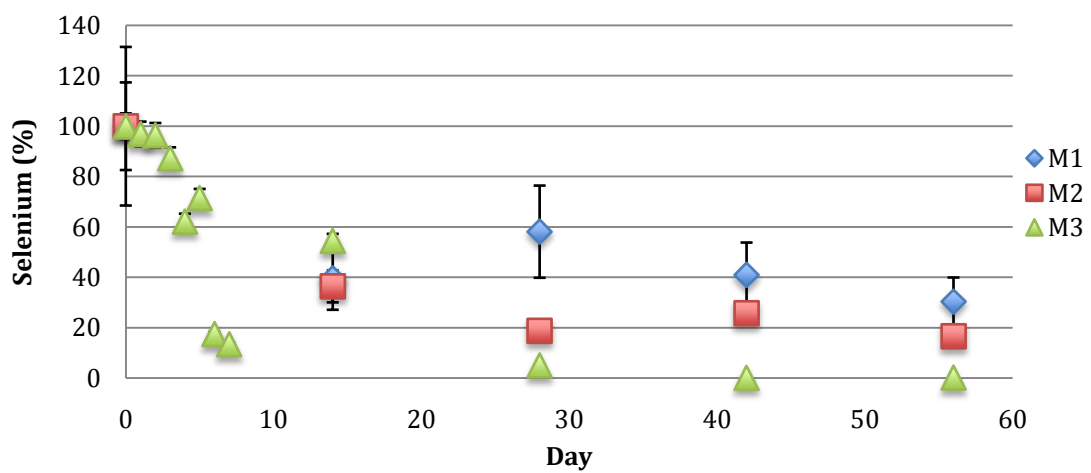
Figure 4-7 Change of A) sulfate, B) nitrate and C) selenium concentrations over time for

inoculum M (in percentage of the original concentration)

Similarly with the trends observed for inoculum G, the sulfate concentrations in the inoculum M cultures did not change significantly over the 8 weeks and a steady decreasing trend was observed for nitrate concentrations. However, only condition #3 for inoculum M showed significant amount of reduction in selenium concentration. The other two conditions showed similar results. These data were also transferred to program R for further analyses. See Chapter 5 for detailed description of analyses in R.

4.3.2 Change in the Population of Total Bacteria

In order to measure the functional microbial community structure of the cultures with respect to the reduction of sulfate, nitrate and selenium in the wastewater, a baseline was needed. This baseline was the amount of total bacteria in each DNA sample that was used for qPCR. These numbers do not reflect the actual amounts of bacteria cells in each of the cultures since all the DNA samples extracted were diluted to the same concentration of approximately 1 ng/μL using the Qubit procedures described in Section 3.5.3.1. Since the nature of qPCR is to make copies of the DNA in the sample, if there were very small differences at the beginning of the reaction between two samples, these differences will be exaggerated by multiple times at the end of the reactions. Thus, all the results obtained from the qPCR were normalized to the population of total bacteria and presented on a log to the base of 2 scale.

The primer set used in qPCR to measure total bacteria targets the 16S ribosomal ribonucleic acid (rRNA) gene in a cell. This primer set has been used by other researchers and it was proved to provide very good estimate of the total bacterial population present

in the culture. It was assumed that there are 3.6 16SrRNA genes in a cell (Suzuki, Taylor and DeLong 2000, Vigneron, et al. 2013).

4.3.3 Change in the Population of Sulfate Reducers(*dsrA* and *dsrB*Genes)

The functional gene that is related to sulfate reduction in SRB is *dsrAB* gene. For the qPCR experiment, two parts of the same gene were used to determine the population of sulfate reducers in the cultures: *dsrA* and *dsrB*. This method only measures the presence of the genes. To measure whether the genes were actively expressed or not, mRNA needs to be used instead. However, it is very difficult for mixed cultures from the environment. Based on Figure 4-8 and 4-9, differences in the number of sulfate reducers present in the cultures can be observed for the two inocula. The 3 different conditions associated with the inocula were noted as G1, G2 and G3 for inoculum G and M1, M2 and M3 for inoculum M. In inoculum M, the number of *dsrAB* gene tends to fluctuate more for condition #1 and #2. Whereas in condition #3, the number of *dsrAB* gene tends to be more stable and increased continuously. Similar trend can be observed for inoculum G where the population of sulfate reducers fluctuated over time under conditions #1 and #2 and stayed relatively stable under condition #3.

A)

B)
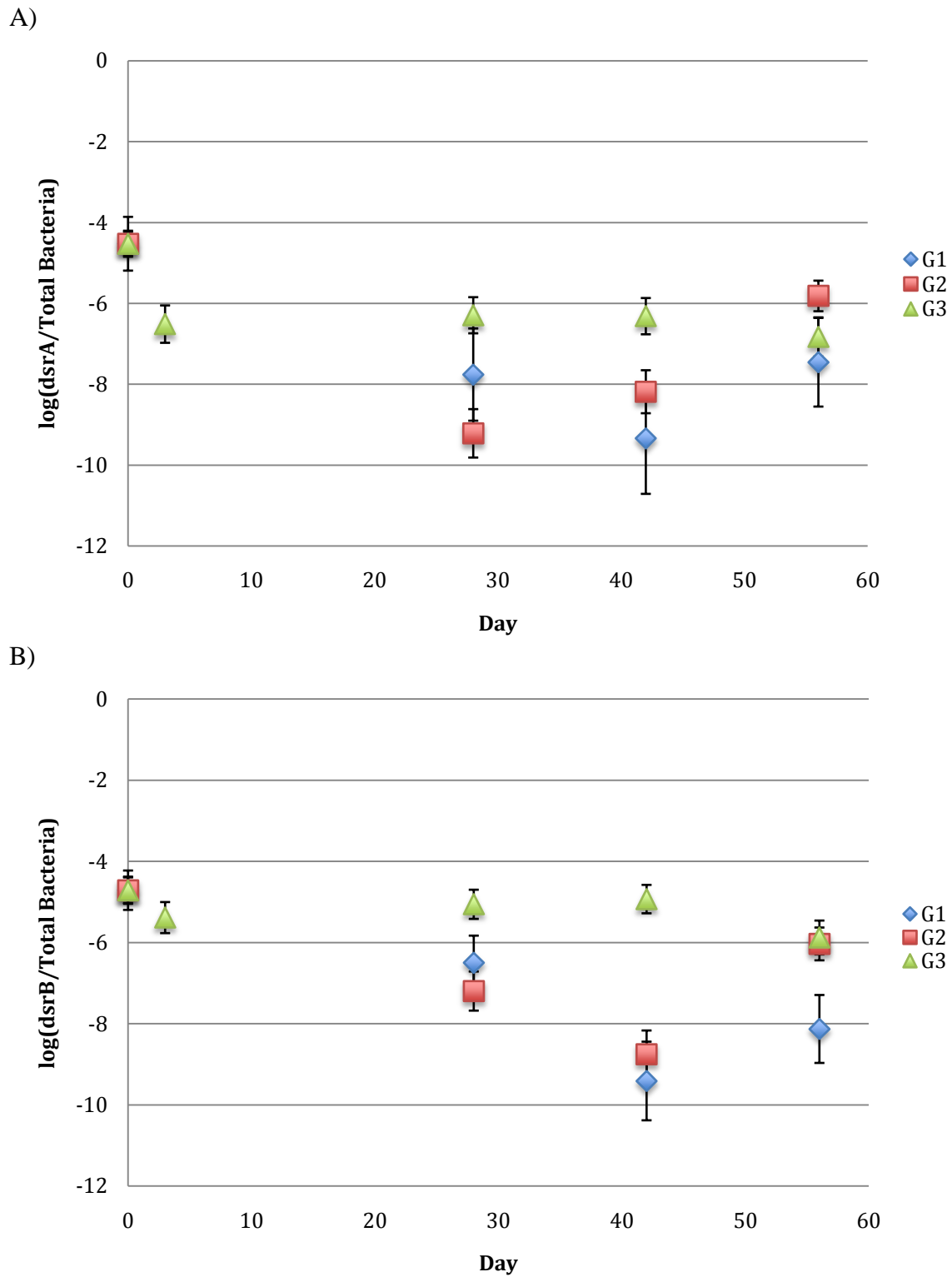
Figure 4-8 Number of A) *dsrA* and B) *dsrB* gene copies over time for inoculum G

A)

B)
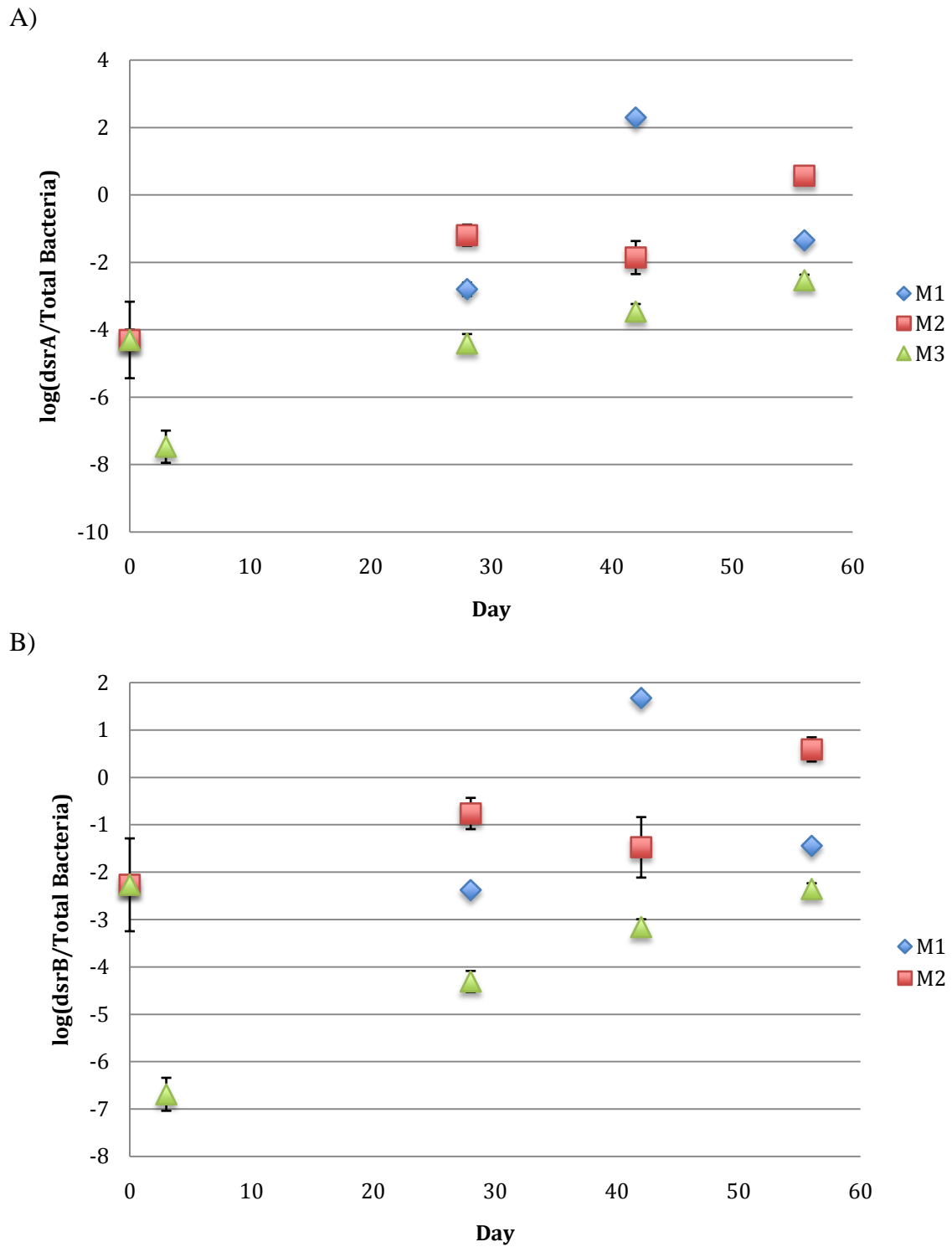
Figure 4-9 Number of A) *dsrA* and B) *dsrB* gene copies over time for inoculum M

4.3.4 Change in the Population of Denitrifying Bacteria(*nifH* and *nosZ*Genes)

Two primer sets were used to calculate the population sizes of nitrate reducing bacteria. The *nifH* gene was used to capture the population size of nitrogen-fixing bacteria and the *nosZ* gene was used to capture the population size of denitrifying bacteria (Poly, Monrozier and Bally 2001, Throback, et al. 2004). After the results were compiled, the Figure 4-10and 4-11 were obtained.

In inoculum G, the concentration of denitrifying bacteria stayed relatively the same throughout the 8-week period under all conditions. Even though the change of population in nitrogen-fixing bacteria is not as much as the denitrifying bacteria, an overall increase in population size can still be observed. On the other hand, similar trends were observed between nifH and nosZ genes in inoculum M. When the population size for nitrogen-fixing bacteria decreased, the population size for denitrifying bacteria decreased. Greater increase in the population of denitrifying bacteria was also observed compared to the changes of nitrogen-fixing bacteria. Overall, under condition #3, the proportion of both types of bacteria stayed very stable from week 4 to 8 compared to the other two conditions.

A)
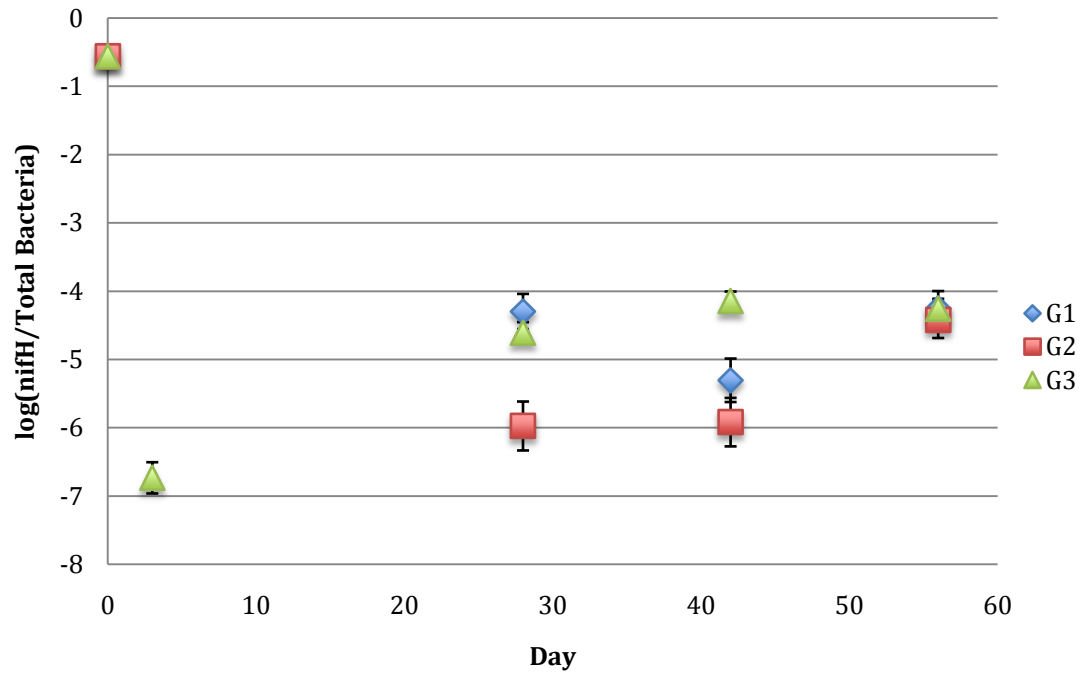
B)
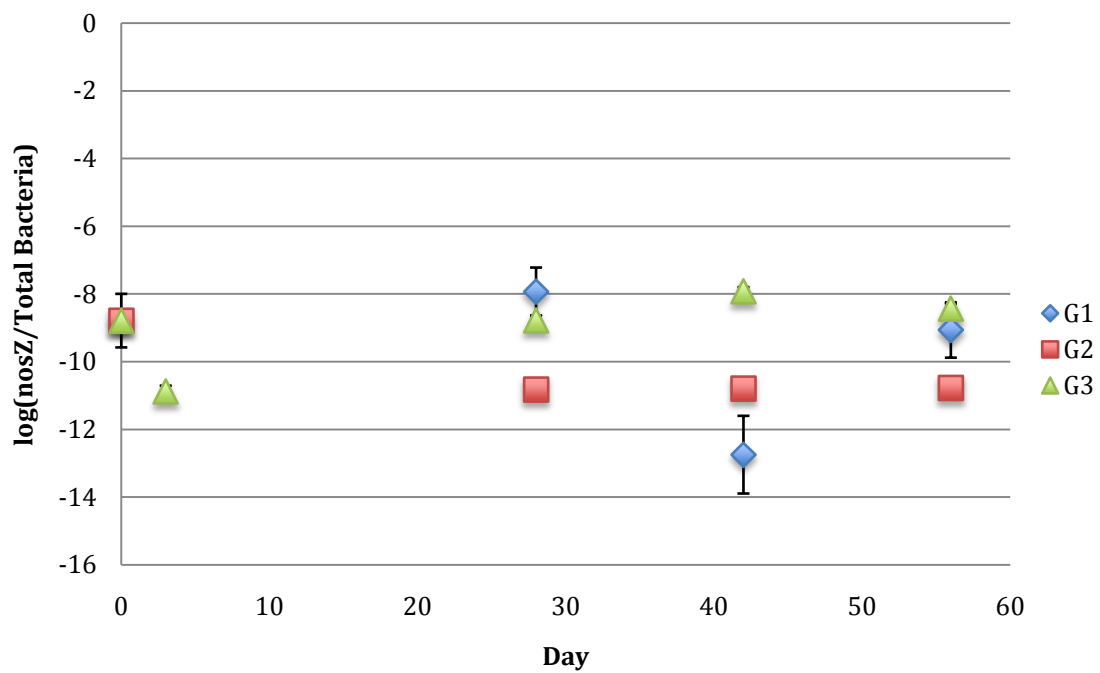
Figure 4-10 Number of A) *nifH* and B) *nosZ* gene copies over time for inoculum G

A)

B)
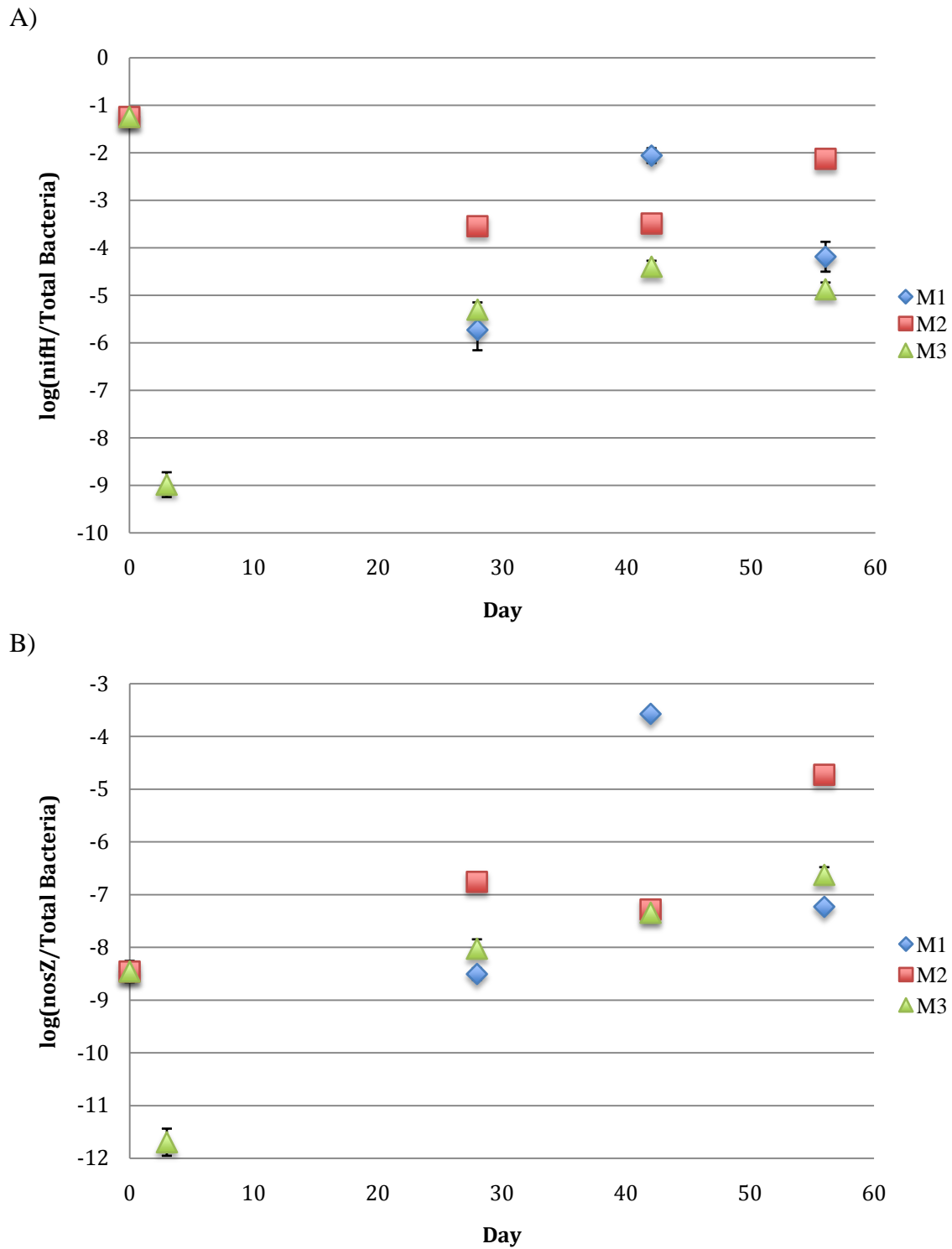
Figure 4-11 Number of A) *nifH* and B) *nosZ* gene copies over time for inoculum M

4.3.5 Change in the Population of Selenium Reducers(*serA* and *srdB*Genes)

Two primer sets were used to detect the population size of selenium-reducing bacteria in the culture: *serA* and *srdB*. These primer sets were developed in the lab using cultures grown in the lab as well as by another colleague. These primer sets have shown very good performance in detecting selenium-reducing bacteria in lab. Therefore, they were also used in the current study. After deleting all the outliers, Figure 4-12 and 4-13 were constructed as estimations of the population sizes.

Similar with other functional genes, it was assumed that there are only one serA or srdB gene in the selenium-reducing bacteria. The original population size for selenium reducing bacteria was zero. For inoculum G, gradual decrease of population for both types of genes can be observed under all three conditions. Whereas for inoculum M, some population increase can still be observed towards the end of the experiment. However, the numbers of *serA* and *srdB* genes under condition #3 were always higher than the other two conditions.
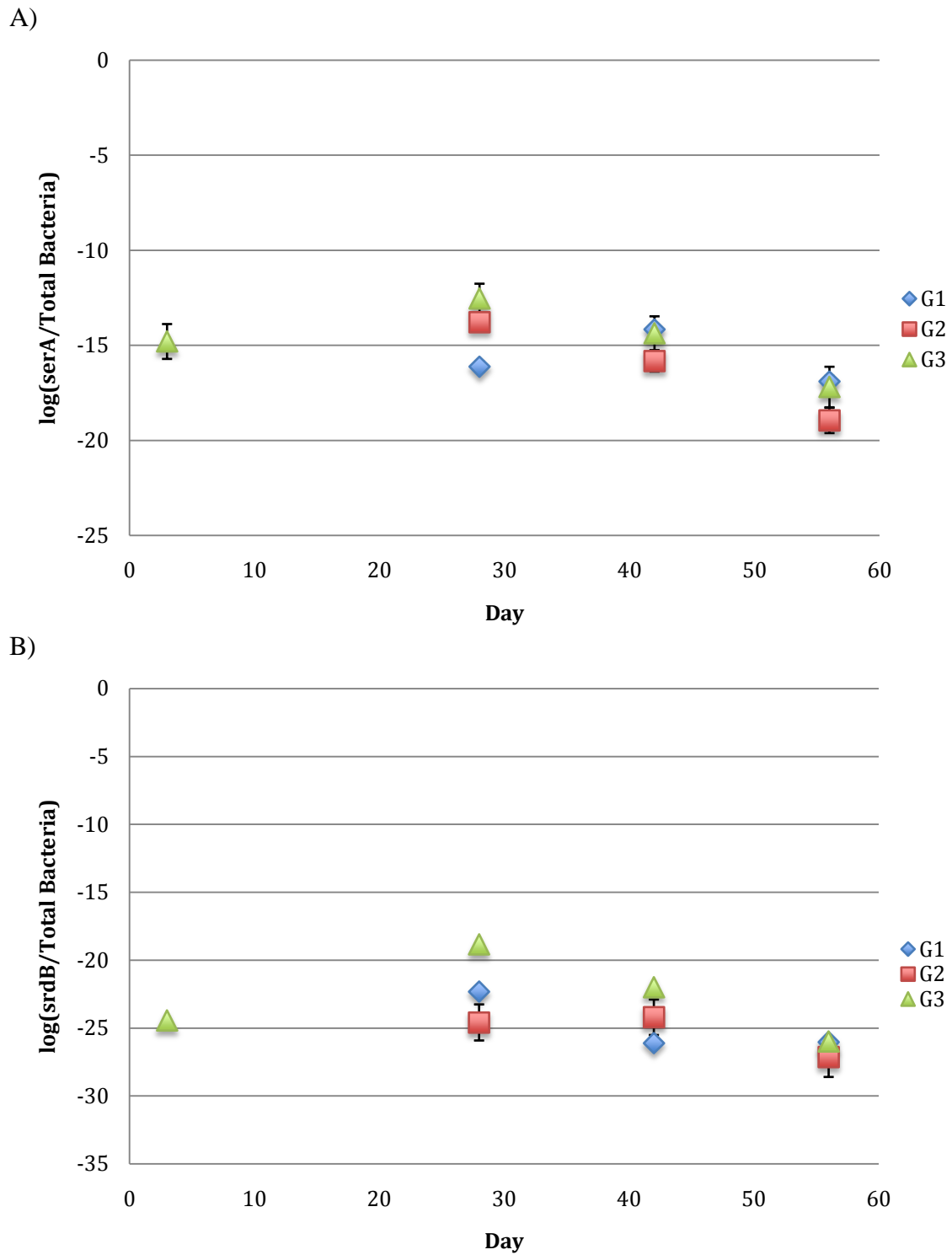
Figure 4-12 Number of A) *serA* and B) *srdB* gene copies over time for inoculum G

A)

B)
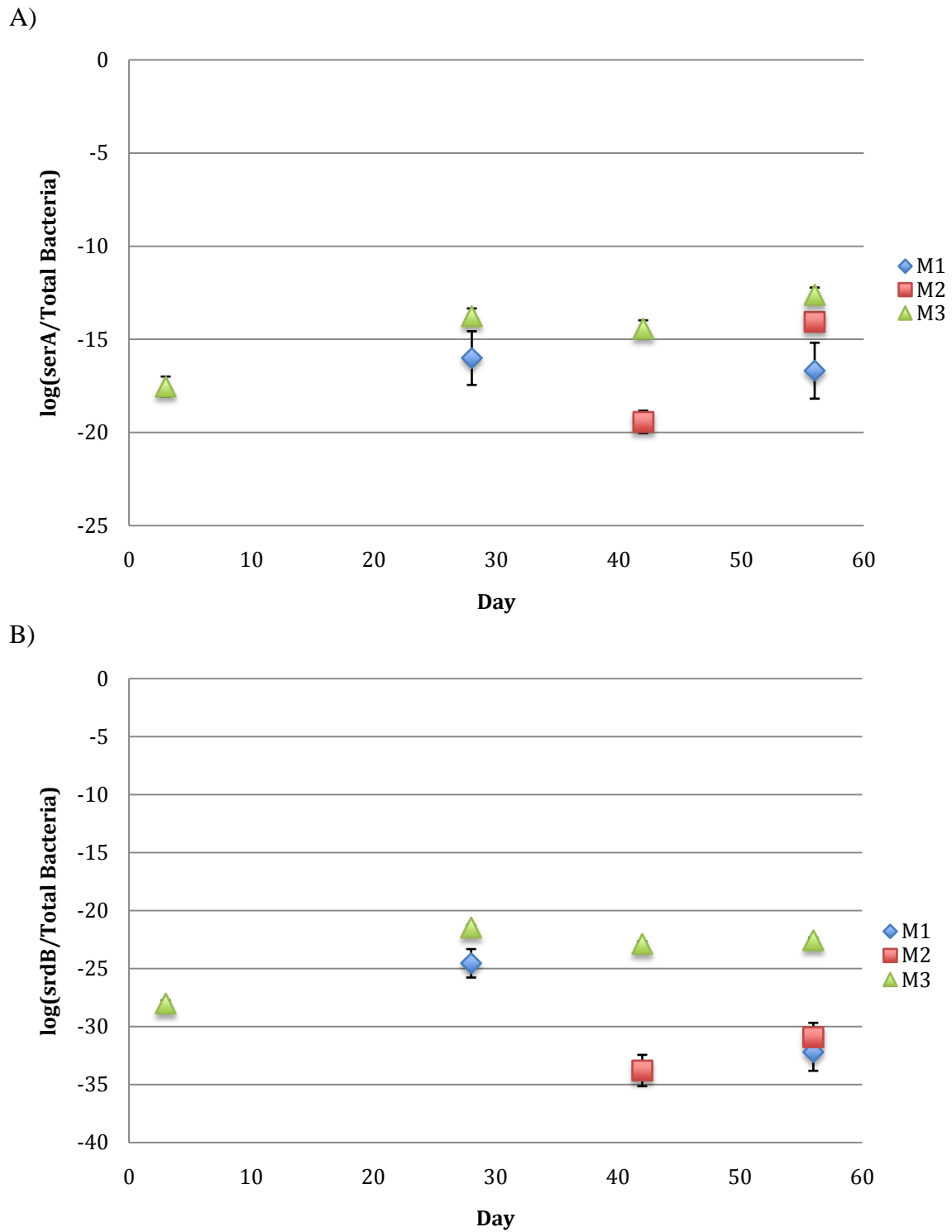
Figure 4-13 Number of A) *serA* and B) *srdB* gene copies over time for inoculum M

5 Discussion

In this Chapter, results presented in Chapter 4 are discussed in detail. Results related to the enrichment cultures using sediments collected on site will be discussed first. Then results of the passages used to acclimatize the enrichment inocula will be discussed. Finally, results of the treatment of R.O. brine wastewater using the acclimatized inocula will be discussed. At the end of this chapter, a suitable inoculum would be recommended for bioreactor designs.

5.1 Enrichment of Bacteria Able to Grow in High Sulfate Medium

On average, greater than 90% of the initial sulfate concentration was reduced in all enrichment cultures over approximately three weeks. At the end of the experiment, the sulfate concentration in inoculum T was higher than that in the other two cultures, which were similar to each other. Since triplicate measurements were not performed, the errors associated with the tests were not known. However, from Figure 4-1, it was clearly shown that the sulfate concentration on day 23 has decreased by a significant amount compared to day 0. This indicates that the SRB are active in all three cultures and they are ready to be used in the acclimatization experiment.

5.2 Acclimatization of Bacteria to R.O. Brine from the Mine Site

The change of sulfate concentration over time was analyzed first. As shown in Figure 4-3, the sulfate concentrations appeared to decrease very quickly in the first few days. Then it increased a little bit before it was constantly reduced over time. These trends are not statistically significant due to the overlapping error bars. It was difficult to get accurate sulfate concentration measurements for these very saline solutions. Fluctuations in bacterial cultures are not unexpected. Since the original inocula were not pure cultures, there were many different types of bacteria present able to perform many tasks. Usually microorganisms cooperate with each other so that they can survive together in very harsh environments. Using SRB as an example, the sulfide ions produced from their metabolism can be used by other types of sulfur-oxidizing bacteria (SOB) to convert it back to sulfate (Postgate 1984). Sulfur-oxidizing bacteria usually need some oxygen, albeit in low concentrations, which may have been present initially.

One possible scenario might have been when the enrichment inoculum was introduced into a new environment; the group of bacteria that favored that environment thrived first. This might explain the sudden drop in sulfate concentration. As time passes, the environment becomes favorable for SOB due to the production of sulfide. As their population grows, the concentration of sulfate might slowly increase as SOB oxidize sulfide back to sulfate. At some point, SOB are no longer favored, perhaps due to absence of any oxygen and SRB are more active, further reduction in sulfate can be observed. With the production of sulfide, conditions become more reducing and more favorable for

SRB population, an overall decrease in sulfate concentration can be observed. The amount of sulfate reduction achieved was around 50% by inoculum T, 80% by inoculum G and 60% by inoculum M.

The change of TDS over time for passage 3 was considered next. By comparing Figure 4-3 with Figure 4-5, the concentration of sulfate is negatively related to the TDS of the solution. As TDS measures all the charged particles present in the solution, theoretically, it should decrease over time as the population of microorganisms increases since some particles are consumed as nutrients. However, opposite to the trend observed in sulfate concentration, TDS constantly increased slowly over time for inoculum T and M. This unexpected trend observed may be related to the changes of microbial population over time. The conductivity meter used in the experiment measures the amount of particles passes through the space between two sensing pillars and translate it into conductivity. The increase in bacterial population might be higher than the decrease in charged particles and resultedhigher conductivity measurements overall. Other researchers have also observed similar effects when hydrocarbons are present in water sources (Atekwana, et al. 2004).

For inoculum G, a drop in TDS was observed two weeks after the inoculation. This may because a lot of gas was being produced for this specific culture. When the bottle was opened for sample collection, gas bubbles constantly rose from the bottom of the culture that made it very difficult to collect a stable conductivity reading. Only estimated values were used. However, this showed that the species of bacteria present in this particular inoculum was very different from the other two inoculums based on the type of end

product being produced. The amount of gas observed also indicated that there were a large amount of bacterial activities happening.

5.3 Use of Acclimatized Enrichment Cultures for Sulfate, Nitrate and Selenium Removal from Mine R.O. Brine

In this section of the discussion chapter, results related to the sulfate, nitrate and selenium reduction of R.O. brine from the mine would be discussed. The qPCR results using primers listed in Table 3-8 will also be discussed and related to the amount of sulfate, nitrate and selenium reduced in the R.O. brine. The estimated total bacteria population will be discussed first since many results in the qPCR experiment use this result as a baseline for comparison. Then the calculated amount of sulfate reduction will be discussed. Finally, the functional genes related to this reduction are correlated to the amount of sulfate reduced in the culture. The same discussion order will be used for nitrate and selenium reduction of the R.O. brine.

5.3.1 Change in the Population of Total Bacteria

For inoculum G, the total population of bacteria increased steadily over the 8-week period in all conditions (Figure 4-8). However, the speed at which the population grows slowed down towards the end of the experiment since the amount of nutrients became limiting. The population on the third day of the experiment for condition #3 was higher than the population in week 4. This may be caused by the adjustments microbial communities made to a new environment.

On the other hand, total bacteria already increased to a peak population on the third day of experiment in condition #3 for inoculum M. The total population in the samples collected afterwards slowly decreased over time. Similar trend can be observed in the other two conditions,but the largest amount of total bacteria was observed in week 4 instead. Comparing to results obtained in inoculum G, this inoculum showed that it could adjust to a new environment faster and consume the nutrients in the culture faster. It may also have a shorter life cycle compared to inoculum G since bacteria already started dying after 4 weeks.

5.3.2 Change in Sulfate Concentrations and Related Bacteria Populations

Among the three different media designed for the experiment, in conditions #1 and #2, the concentration of sulfate can only be reduced through microbial activities. Whereas in condition #3, it is also possible for sulfate to be adsorbed to the surface of ZVI and slowly released back into the solution over time or precipitated by green rust (Sandy and DiSante June, 2010). Therefore, the sulfate reduction speed for condition #3 is theoretically faster than other two conditions.

As shown in Figure 4-6, the overall concentrations of sulfate did not decrease significantly for all three conditions in inoculum G. Even though decreasing trend was observed for condition #3 in the first week of experiment, concentration of sulfate started to fluctuate over time after week 2. The sulfate concentration change obtained for inoculum M was similar with inoculum G (Figure 4-7). Reduction in sulfate concentration was observed for the first 2 weeks of the experiment under condition #1

and only for the first week under condition #3. The most amount of sulfate reduction was observed under condition #2 since the concentration kept reducing for 4 weeks before it started to fluctuate. Since SRB was not the only group of bacteria present in the inoculum, there might be other bacteria that can affect the sulfate concentration of the culture. The change of sulfate concentration over time was also observed from another angle by looking at the population change of SRB using *dsrAB* genes. As described in Table 3-8, it was engineered into two primers as *dsrA* and *dsrB* genes. Assuming that there is only one *dsrAB* gene present in one SRB cell (Paisse, et al. 2013), the number of the genes present in the sludge sample can be used to represent the total number of SRB cells.

As shown in Figure 4-8, the population proportion of SRB decreased compared to the original inoculation. This may be caused by a harsher environment compared to the previous growth condition. However, the concentration of sulfate still decreased. This was the case during the first week of the experiment. Then the population of SRB fluctuated with the sulfate concentration. When the sulfate concentration increased, the population of SRB decreased.

Based on the rough population count performed by qPCR, SRB favored condition #3 the most since a larger population was observed constantly under this condition. However, towards the end of the experiment, SRB population in condition #2 increased dramatically even though the overall sulfate concentration did not change very much. As for condition #1, SRB population was very similar with the other two conditions in week 4, but more sulfate reduction was observed.

Results obtained for inoculum M (Figure 4-9) were then compared with inoculum G. It showed that the SRB communities between the two were very different. In condition #1 of inoculum M, even though the concentration of sulfate continued decreasing from week 4 to week 8, the population of SRB fluctuated indicating further adjustments in the microbial communities. In condition #2, the population of SRB increased as the concentration of sulfate decreased. Finally in condition #3, even though the population proportion of SRB decreased by a large amount on the third day, it kept increasing over the 8 weeks and sulfate concentration kept decreasing for the first 2 weeks of the experiment. However, the overall sulfate concentration observed later on fluctuated with time regarding the increasing population size of SRB. The largest amount of sulfate reduction was achieved on week 4 under condition #2 where more *dsrAB* gene copies were observed compared to other conditions.

5.3.3 Change in Nitrate Concentrations and Related Bacteria Populations

The second parameter investigated in the current study was the change of nitrate concentration over time. Nitrate is usually reduced into nitrite first before it is further reduced into either nitrogen gas or ammonia, depending on the pathway taken by the bacteria (Moura, et al. 1997). Since nitrite is chemically unstable, samples were taken and analyzed for nitrogen content right away. The concentration of nitrite was measured to be very small throughout the experiment and the concentration of ammonia increased over time. Since the method to test the concentration of ammonia was not precise, the results were not shown. Results obtained for the first two weeks of the experiment were also not

shown because a different person was performing measurements and the results were not compatible with the rest of the data.

The change of nitrate concentration in inoculum G is discussed first (Figure 4-6). The nitrate concentration kept decreasing throughout the experiment for all three conditions. The largest amount of reduction can be observed under condition #3 where the concentration of nitrate was always lower than the other two conditions at the same time points. The final nitrate concentration was close to zero for condition #3 whereas approximately 60% of nitrate reduction was achieved in the other two conditions at the end of the experiment. Similar with the trend observed for inoculum G, continuous nitrate reduction was observed throughout the experiment for all three conditions. Nitrate reduction for inoculum M was the larges under condition #3 as well. However, the differences among the three conditions are not as obvious as inoculum G. Approximately 70% of nitrate reduction was achieved under condition #1 and #2, whereas approximately 80% of nitrate reduction was achieved under condition #3.

When the samples were prepared for measurements, cloudy particles were present after the samples were mixed with the zinc powder. Unstable readings from the spectrophotometer were obtained even though it was prepared from the same culture. Some other times, the color of the sample was clearly higher than the other sample judging by eye, but the opposite results were obtained using the spectrophotometer. Since the growth medium used for the experiment was very saline and had a lot of different kinds of particles present in it, it is possible that other particles are interfering with the nitrate test.

When the qPCR results were calculated, it was assumed that only one *nifH* gene or *nosZ* gene can be found in each cell. After the nitrogen gas is produced by the denitrifying bacteria, the nitrogen-fixing bacteria use it to produce ammonia. Since theses two primer sets represented two different groups of bacteria that work in sequence, when the population size of bacteria is increasing for one species, the population size of the other should also be increasing. In inoculum G, this correlation can be observed in all three conditions by comparing Figure 4-10 with Figure 4-11. After the inoculum was first introduced to a new environment, the population size of denitrifying bacteria, represented by the proportion of *nosZ*gene in respect to the total bacteria population, decreased compared to the inoculum as it is trying to adapt to a new and harsher environment. Meanwhile, the nitrogen-fixing bacteria, represented by the proportion of *nifH*gene in respect to the total bacteria population, experience the same effect and decreased in population. As the denitrifying bacteria grow in population, more nutrients are available for nitrogen-fixing bacteria. Then both types of bacteria start to increase in population together after 4 weeks of the inoculation since by that time, they have fully adjusted to the new environment. However, the population size found in condition #3 was always higher than the population sizes found in the other two conditions. This was also reflected in the more nitrate reduction observed in condition #3 by the end of the experiment where the concentration of nitrate almost approached 0. For the other two conditions, around 24% nitrate reduction was achieved for condition #1 and 38% for condition #2.

On the third day of the experiment, similar correlation between nitrogen-fixing bacteria and denitrifying bacteria can also be observed in inoculum M. However, the population of denitrifying bacteria showed more increase in population sizes for all three conditions.

This is shown by the higher proportion of *nosZ* genes compared to the proportion in the inoculum.The growth speed under condition #3 was steadier compared to the other 2 conditions. The population sizes for nitrogen-fixing bacteria fluctuated a lot in contrast. Under condition #1, the population size continued decreased until after week 4. Large amount of growth was observed in week 6, then the proportion of *nifH* gene decreased. Under condition #2, the population size of *nifH* genestayed almost the same between week 4 and 6, then it reached maximum in week 8. Finally under condition #3, the most amount of *nifH* gene was observed in week 6.

5.3.4 Change in Selenium Concentrations and Related Bacteria Populations

The selenium concentrations were tested by sending diluted samples to ALS Environmental. Since the concentration of selenium in the culture was very low and the growth medium was very saline, samples were diluted in order to reduce the interference caused by other particles present in the solution. The growth medium designed for the experiment also had precipitation when it was prepared. This made it difficult to distribute the growth medium evenly among all the bottles, especially when the growth medium was prepared in large volumetric flasks. Therefore, the concentration of selenium at the beginning of the experiment under different conditions was not the same with each other.

For inoculum G, continuous reduction in selenium could be observed in general for all conditions (Figure 4-7). The most amount of reduction can be observed under condition #3 on week 6. Then the concentration of selenium increased again on week 8. However,

the concentrations of selenium under condition #1 and #2 were higher than before on week 6. This might be resulted from unevenly distributed selenium at the beginning of the experiment. Or the selenium-reducing bacteria are less active in the specific bottles taken for sample collection since all the cultures were in separate bottles.

For inoculum M, better selenium reduction can be observed in general since a continuous decreasing trend can be observed under all conditions (Figure 4-7). However, the speed at which the reduction happens is not the same for the three different conditions. Some fluctuation was also observed under condition #2 after week 4 of the experiment. Overall, around 70%, 85% and 99% selenium was reduced under condition #1, #2 and #3 respectively.

Since the concentration of selenium was very low in the culture, the population of the selenium-reducing bacteria was also very low compared to the overall population of bacteria in the culture. This has made qPCR analyses a lot harder as the results became more scattered.Selenium-reducing bacteria was not detected in the inocula using qPCR because the population size was very small. Based on Figure 4-12, most of the population sizes increased to a maximum on week 4 in inoculum G. Then bacteria started to die off as the competition for nutrients become more severe. Both the *serA* gene and the *srdB* gene have the same trend throughout the experiment. The most amount of reduction was observed under condition #1 where 86% of selenium was reduced at the end of the experiment. On the other hand, only 23% selenium was reduced under condition #2 and 72% under condition #3.

The qPCR results obtained for inoculum M are more scattered (Figure 4-13). Generally, the population of selenium-reducing bacteria is higher under condition #3 than the other two conditions indicating better growth of bacteria. A continuous increase can be observed under condition #2 for both types of gene. Even though continuous decreasing in selenium concentration was observed, *serA* gene population decreased on week 6 and increased again at the end of the experiment for both conditions #1 and #2. The same trend was observed for *srdB* gene.Under conditions #1 and #2, the population proportion dropped on week 6 and increased again towards the end of the experiment. On the other hand, the maximum population proportion was observed on week 4 under condition #3. Then the population of *srdB* gene decreased.

5.4 Analysis of Concentration Changes Over Time using R

The Table 5-1 was generated for analyses in program R where 0 represents inoculum G and 1 represents inoculum M under the inoculum column. Under the treatment column, 0 represents condition #1, 1 represents condition #2 and 2 represents condition #3. Data collected in the first week of the experiment was not included in the table for condition #3 in order to have equal number of data points for all conditions and inocula.

Table 5-1 Concentration table for program R

Time	Inoculum	Treatment	Nitrate (mg/L)	Sulfate (mg/L)	Selenium (mg/L)
0	0	0	235.000	1998.260	0.881
14	0	0	94.346	1424.960	0.227
28	0	0	156.220	350.220	0.077
28	0	0	115.312	1044.470	0.172
28	0	0	175.908	1695.330	0.718
42	0	0	139.601	1656.930	0.682
56	0	0	94.346	1583.750	0.125
0	0	1	235.000	1872.330	1.029
14	0	1	50.369	1729.840	0.771
28	0	1	198.408	1431.590	0.664
42	0	1	122.215	1690.070	0.741
42	0	1	129.118	1723.210	0.747
42	0	1	156.988	1584.030	0.775
56	0	1	103.550	1704.630	0.794
0	0	2	235.000	1762.980	1.336
14	0	2	156.732	1739.780	1.097
28	0	2	73.124	1630.420	0.566
42	0	2	15.852	1501.180	0.169
56	0	2	6.903	1968.070	0.371
0	1	0	235.000	1716.580	0.909
14	1	0	62.386	646.204	0.360
28	1	0	169.772	1496.970	0.696
28	1	0	207.612	1298.620	0.527
28	1	0	116.334	669.450	0.363
42	1	0	139.346	1000.790	0.372
56	1	0	75.681	966.990	0.276
0	1	1	235.000	1481.300	1.087
14	1	1	73.636	792.010	0.395
28	1	1	184.345	460.630	0.204
42	1	1	85.908	1097.160	0.328
42	1	1	97.770	905.001	0.231
42	1	1	118.380	1400.890	0.572
56	1	1	69.289	948.390	0.180
0	1	2	235.000	1491.240	5.035
14	1	2	30.682	1766.290	2.742
28	1	2	129.374	765.500	0.253
42	1	2	57.272	1355.370	0.005
56	1	2	45.000	1434.990	0.010

It was assumed that the data points collected in this experiment was normally distributed as well as the variance was homogeneous among the different groups of data points. First, a linear model was fit to the data with interaction terms between time and inoculum, time and treatment and inoculum and treatment. If the probability of a term is less than 0.05, then this term poses a significant effect on the linear model developed. Otherwise, this term can be omitted and no significant changes would be observed in the linear model developed. Detailed program code and results obtained in R are listed and tabulated in Appendix E.

5.4.1 Analysis Procedures in R

Based on the results obtained, it was found that most of the interaction terms were not significant when considering the change of concentrations for sulfate and nitrate. However, the probability of the interaction terms for inoculum M under condition #3 on week 2 were very close to 0.05 for sulfate concentration change. If more data points were collected, whether these terms are truly significant can be determined. With that data available at the moment, these specifications for better sulfate reduction can be noted.

The probability of the interaction term under condition #3 on week 6 for nitrate reduction was significantly lower than 0.05. This indicated that a significantly amount of nitrate was reduced on the 6th week of the experiment whether it was for inoculum G or inoculum M. This specification of time period for nitrate reduction can be very useful for bioreactor design.

The linear model obtained for selenium reduction was more complex since more significant interaction terms were observed. The overall probability of coefficient obtained for inoculum M and conditions #3 were lower than 0.05. This indicated that more selenium reduction could be achieved through these two specifications. Any other parameters that interacted with these two specifications also obtained coefficients lower than 0.05. Specifically, when time passes week 4, any interaction terms related to either inoculum M or condition #3 were statistically significant.

Based on the observations made above, the linear models developed were updated by deleting the less significant terms. All the interaction terms for sulfate, nitrate and selenium reduction were omitted at this step since most of the interaction terms for sulfate and nitrate reduction were insignificant. All the interaction terms were significant for selenium reduction mostly because they were interacting with very significant parameters. Therefore, they were not further considered in the analyses performed next.

The Tukey's test was then performed using the updated versions of the linear models. In this test, pair-wise comparisons of the concentrations of sulfate, nitrate or selenium will be performed. Based on this test, the best inoculum and condition can be selected for each type of reduction desired. When two different parameters were compared, concentrations in the first parameter will be subtracted by the concentrations in the second parameter. If the concentrations in the first parameter were higher, then the results obtained would be positive. Otherwise, a negative number would be obtained. The parameter with the lower concentrations achieved more reduction in the same period of time. Therefore, it is

considered to be a better specification. The confidence interval was also considered for each Tukey's test performed to help select the better specifications.

5.4.2 Performance ofSulfate, Nitrate and Selenium Reduction in R.O. Brine Wastewater

For sulfate reduction, inoculum M performed better in general compared to inoculum G. The 95% confidence interval for this comparison was also always negative when inoculum M was subtracted by inoculum G. For treatment types, the least amount of difference was observed between condition #1 and #2 with condition #1 being lower in concentration. The 95% confidence interval for this comparison was rather symmetric about zero indicating that the two treatment conditions didn't make very big differences.

For nitrate reduction, similar conclusion can be made for inoculum M. The 95% confidence interval was more negative indicating that inoculum M was more likely to perform better than inoculum G. The results obtained from the Tukey's test for nitrate concentration indicated that the three conditions considered in the experiment had a lot of different influences on the amount of nitrate being reduced in 8-week period. This can be perceived from the large difference observed in the Tukey's tests and also a probability that is less than 0.05. It was found that conditions #3 performed the best in terms of nitrate reduction. The 95% confidence interval also indicated that condition #3 is more likely to perform better than the other conditions.

Finally for selenium reduction, inoculum G was observed to be more likely to perform better than inoculum M. The 95% confidence interval was more positive when inoculum

M was subtracted by inoculum G. However, the difference was rather small.Condition #1 was considered to perform better than other conditions for selenium reduction based on the 95% confidence interval. Again, similar with the Tukey's test performed for inoculum, the difference was rather small in values. Since the concentration of selenium was originally small, a small change can be very significant percentage-wise.

6 Conclusions and Future Work

Based on the analysis results obtained from program R, different specifications can be made if different treatment objectives need to be met. Since sulfur and selenium have similar chemical properties, if more sulfate and selenium need to be reduced in the wastewater, it is recommended to use inoculum M with condition #1. Even though the Tukey's test indicated that inoculum G was better in reducing selenium, there are still chances that inoculum M would perform better if more data were collected. If sulfate and nitrate are more of a concern in the wastewater, it is recommended to use inoculum M with condition #2. Even though condition #1 performed better in sulfate reduction, the performance of condition #2 is still comparative to condition #1.

The challenge would be to choose a proper condition in order to treat sulfate, nitrate and selenium all at the same time. Either the concentration of sulfate or the concentration of nitrate has to be compromised since different conditions favor the reduction of these two contaminants. Inoculum M can be determined for sure to be the better choice of inoculum. To make a moderate choice in the type of treatment, condition #2 would have to be selected for treating all three ingredients at the same time. Even though reduction in all three concentrations would be compromised, the overall results obtained from this combination can be acceptable. However, if more data points can be collected more often, the results can be potentially improved further.

One potential factor that may have affected the amount of reduction achieved can be the limitation in carbon sources. The growth medium designed using the post-SR R.O. wastewater sample was harsher than the enrichment culture since more magnesium and

calcium would need to be reduced for the same amount of carbon sources added. The competition for nutrient was more severe. Therefore, if more sodium lactate was added to the growth medium, more reduction may be achieved in all conditions.

Another way to gain more insights on the species of bacteria present in the culture and their reduction pathways is to perform more qPCR with more varieties of primers. Since the biodiversity of inoculum G was more than inoculum M, different sets of primers may be able to obtain products with proper sizes for inoculum G. Otherwise, DNA samples where more reduction of sulfate, nitrate or selenium was achieved can be sent for sequencing.

Bibliography

Atekwana, Eliot A., Estella A. Atekwana, Rebecca S. Rowe, D. Dale Werkema Jr., and Franklyn D. Legall. "The Relationship of Total Dissolved Solids Measurements to Bulk Electrical Conductivity in an Aquifer Contaminated with Hydrocarbon." *Journal of Applied Geophysics* (Elsevier) 56 (2004): 281-294.

Backer, Lorraine C. , Emilio Esteban, Carol H. Rubin, Stephanie Kieszak, and Michael A. McGeehin. "Assessing Acute Diarrhea from Sulfate in Drinking Water." *American Water Works Association* (American Water Works Association) 93, no. 9 (September 2001): 76-84.

Bai, He, Yong Kang, Hongen Quan, Yang Han, and Ying Feng. "Treatment of Copper Wastewater by Sulfate Reducing Bacteria in the Presence of Zero Valent Iron." *International Journal of Mineral Processing* (Elsevier) 112-113 (2012): 71-76.

Bartels, Craig, Rich Franks, and Keith Andes. "Operational Performance and Optimization of RO Wastewater Treatment Plants." *Singapore International Water Week*, June 2010: 1-12.

Black, Sarah Morgan. *pKa Chart.* 2010. http://cactus.dixie.edu/smblack/chem2310/summary_pages/pKa_chart.pdf (accessed March 2015).

Bordenave, Sylvain, Victoria Kostenko, Mark Dutkoski, Aleksandr Grigoryan, Robert J. Martinuzzi, and Gerrit Voordouw. "Relation Between the Activity of Anaerobic Microbial Populations in Oil Sands Tailings Ponds and the Sedimentation of Tailings." *Chemosphere* (Elsevier) 81, no. 5 (October 2010): 663-668.

Clesceri, Lenore S. , Arnold E. Greenberg, and Andrew D. Eaton. *Standard Methods for the Examination of Water and Wastewater.* American Public Health Association, 1999.

de Jonge, Victor N. , M. Elliott, and E. Orive. "Causes, Historical Development, Effects and Future Challenges of a Common Evironmental Problem: Eutrophication." *Hydrobiologia* (Springer) 475-476, no. 1 (May 2002): 1-19.

Feng, Yinghong, Yaobin Zhang, Xie Quan, and Suo Chen. "Enhanced Anaerobic Digestion of Waste Activated Sludge Digestion by the Addition of Zero Valent Iron." *Water Research* 52 (2014): 242-250.

Fondriest Environmental Inc. *Conductivity, Salinity & Total Dissolved Solids.* March 2014. http://www.fondriest.com/environmental-measurements/parameters/water-quality/conductivity-salinity-tds/ (accessed Januray 2015).

Foti, Mirjam, et al. "Diversity, Activity and Abundance of Sulfate-Reducing Bacteria in Saline and Hypersaline Soda Lakes." *Applied and Environmental Microbiology* 73, no. 7 (April 2007): 2093-2100.

Ghazy, E. A. , M. G. Mahmoud, M. S. Asker, M. N. Mahmoud, M. M. Abo ELsoud, and M. E. Abdel Sami. "Cultivation and Detection of Sulfate Reducing Bacteria (SRB) in Sea Water." *Journal of American Science* 7, no. 2 (2011): 604-608.

Harrison, John. *Earth Cycles The Nitrogen Cycle: Of Microbes and Men.* 2003. http://www.visionlearning.com/en/library/Earth-Science/6/The-Nitrogen-Cycle/98 (accessed March 2015).

Hayrynen, Katri, Jorg Langwaldt, Eva Pongracz, Virpi Vaisanen, Mika Manttari, and Riitta L. Keiski. "Separation of Nutrients from Mine Water by Reverse Osmosis for Subsequent Biological Treatment." *Minerals Engineering* (Elsevier) 21 (2008): 2-9.

Hockin, Simon L., and Geoffrey M. Gadd. "Linked Redox Precipitation of Sulfur and Selenium under Anaerobic Conditions by Sulfate-Reducing Bacterial Biofilms." *Applied and Environmental Microbiology* (American Society for Microbiology) 69, no. 12 (December 2003): 7063-7072.

Hoekstra, Gordon. *Mount Polley Mine Tailings Spill Nearly 70 per cent Bigger Than First Extimated.* September 3, 2014. http://www.vancouversun.com/Mount+Polley+mine+tailings+spill+nearly+cent+bigger+than+first+estimated/10172302/story.html (accessed January 20, 2015).

Karri, Srilakshmi, Reyes Sierra-Alvarez, and Jim A. Field. "Zero Valent Iron as an Electron-Donor for Methanogenesis and Sulfate Reduction in Anaerobic Sludge." *Wiley Periodicals, Inc.*, June 16, 2005: 810-819.

KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nitrogen metabolism - Reference pathway.* January 9, 2014. http://www.genome.jp/kegg-bin/show_pathway?map=map00910&show_description=show (accessed March 2015).

—. *Sulfur metabolism - Reference pathway.* June 3, 2014. http://www.genome.jp/kegg-bin/show_pathway?map=map00920&show_description=show (accessed March 2015).

Krafft, Torsten, Ashley Bowen, Friedbert Theis, and Joan M. Macy. "Cloning and Sequencing of the Genes Encoding the Periplasmic-Cytochrome B-Containing Selenate Reductase of Thauera selenatis." *DNA Sequence* 10, no. 6 (2000): 365-377.

Kuroda, Masashi, et al. "Molecular Cloning and Characterization of the srdBCA Operon, Encoding the Respiratory Selenate Reductase Complex, from the Selenate-Reducing Bacterium Bacillus selenatarsenatis SF-1." *Journal of Bacteriology* 193, no. 9 (May 2011): 2141-2148.

Leloup, Julie, Alexander Loy, Nina J. Knab, Christian Borowski, Michael Wagner, and Bo Barker Jorgensen. "Diversity and Abundance of Sulfate-Reducing Microorganisms in the Sulfate and Methane Zones of a Marine Sediment, Black Sea." *Environmental Microbiology* (Society for Applied Microbiology and Blackwell Publishing Ltd) 9, no. 1 (2007): 131-142.

Leloup, Julie, Henrik Fossing, Katharina Kohls, Lars Holmkvist, Christian Borowski, and Bo Barker Jorgensen. "Sulfate-Reducing Bacteria in Marine Sediment (Aarhus Bay,

Denmark): Abundance and Diversity Related to Geochemical Zonation." *Environmental Microbiology* (Society for Applied Microbiology and Blackwell Publishing Ltd) 11, no. 5 (2009): 1278-1291.

Lemly, A. Dennis. *Aquatic Hazard of Selenium Pollution from Mountaintop Removal Coal Mining.* North Carolina: Wake Forest University, April, 2009.

Lemly, A. Dennis. "Environmental Implications of Excessive Selenium: A Review." *Biomedical and Environmental Sciences* 10 (1997): 415-425.

Li, Winton. *Design of a Bioreactor for Reducing Sulphate in Cattle Drinking Water.* Thesis, Chemical and Biological Engineering, University of British Columbia, Vancouver, BC: University of British Columbia, 2009.

Life Technologies. *Essentials of Real-Time PCR.* 2013. https://www.lifetechnologies.com/ca/en/home/life-science/pcr/real-time-pcr/qpcr-education/essentials-of-real-time-pcr.html (accessed January 2015).

—. "Real-Time PCR Handbook." *qPCR Education.* 2014. http://www.lifetechnologies.com/ca/en/home/life-science/pcr/real-time-pcr/qpcr-education/real-time-pcr-handbook.html?cid=rtpcrhandbook-rtpcr (accessed January 2015).

Livak, Kenneth J. , Susan J. A. Flood , Jeffrey Marmaro, William Giusti, and Karin Deetz. "Oligonucleotides with Fluorescent Dyes at Opposite Ends Provide a Quenched Probe System Useful for Detecting PCR Product and Nucleic Acid Hybridization." *Genome Research* (Cold Spring Harbor Laboratory Press) 4 (1995): 357-362.

Ljung, Karin, Fiona Maley, Angus Cook, and Philip Weinstein. "Acid Sulfate Soils and Human Health - A Millennium Ecosystem Assessment." *Encironment International* (Elsevier) 35 (2009): 1234-1242.

Lorax Environmental. "Treatment of Sulphate in Mine Effluents." *The International Network for Acid Prevention.* October 2003.

http://www.inap.com.au/public_downloads/Research_Projects/Treatment_of_Sulphate_in_Mine_Effluents_-_Lorax_Report.pdf (accessed March 2015).

Malaeb, Lilian, and George M. Ayoub. "Reverse Osmosis Technology for Water Treatment: State of the Art Review." *Desalination* (Elsevier) 267 (2011): 1-8.

Matheson Tri-Gas Inc. "Hydrogen Selenide." *Material Safety Data Sheet.* December 9, 2014. http://www.chemadvisor.com/Matheson/database/msds/00244337000800003.PDF (accessed March 2015).

Meays, Cindy, and Rick Nordin. *Ambient Water Quality Guidelines For Sulphate.* Technical Appendix, Water Protection & Sustainability Branch, BC Ministry of Environment, BC Ministry of Environment, 2013.

Ministry of Environment, BC. *Standards for End of the Pipe Water Based Discharges from the Pacific Place Site.* November 27, 1991. http://www.env.gov.bc.ca/epd/remediation/standards_criteria/standards/end_of_pipe.htm (accessed January 2015).

Moura, Isabel, Sergey Bursakov, Cristina Costa, and Jose J.G. Moura. "Nitrate and Nitrite Utilization in Suflate-Reducing Bacteria." *Anaerobe* (Academic Press) 3 (1997): 279-290.

Nordin, R.N., and L.W. Pommen. *Water Quality Criteria for Nitrogen (Nitrate, Nitrite, and Ammonia).* Technical Appendix, Water Quality Unit, Ministry of Environment and Parks Province of British Columbia, Victoria, BC: BC MOE, 1986.

O'Flaherty, Vincent, Therese Mahony, Ronan O'Kennedy, and Emer Colleran. "Effect of pH on Growth Kinetics and Sulphide Toxicity Thresholds of a range of Methanogenic, Syntrophic and Sulphate-Reducing Bacteria." *Process Biochemistry* (Elsevier Science Ltd.) 33, no. 5 (1998): 555-569.

Paisse, Sandrine, et al. "Sulfate-Reducing Bacteria Inhabiting Natural Corrosion Deposits from Marine Steel Structures." *Applied Microbiology and Biotechnology* (Springer) 97, no. 16 (August 2013): 7493-7504.

Poly, Franck, Lucile Jocteur Monrozier, and Rene Bally. "Improement in the RFLP Procedure for Studying the Diversity of nifH Genes in Communities of Nitrogen Fixers in Soil." *Research in Microbiology* 152 (2001): 95-103.

Postgate, J. R. . *The Sulphate-Reducing Bacteria.* London: Cambridge University Press, 1984.

Praxair Inc. "Safety Data Sheets." *Praxair Inc.* June 2014. http://www.praxair.com/~/media/North%20America/US/Documents/SDS/Hydrogen%20Sulfide%20H2S%20Safety%20Data%20Sheet%20SDS%20P4611.pdf (accessed January 2015).

Rodriguez, R. P. , G. H. D. Oliveira, I. M. Raimundi, and M. Zaiat. "Assessment of a UASB Reactor for the Removal of Sulfate from Acid Mine Water." *International Biodeterioration and Biodegradation* 74 (2012): 48-53.

Sandy, Tom, and Cindy DiSante. *Review of Available Technologies for the Removal of Selenium from Water.* CH2MHILL, June, 2010.

Seghezzo, Lucas, Grietje Zeeman, Jules B. van Lier, H. V. M. Hamelers, and Gatze Lettinga. "A Review: The Anaerobic Treatment of Sewage in UASB and EGSB Reactors." *Bioresource Technology* 65 (1998): 175-190.

Shao, E, J Wei, A Yo, and R Levy. "Application of Ultrafiltration and Reverse Osmosis for Mine Waste Water Reuse." *Water in Mining Conference*, September 2009: 15-17.

Shin, Kyung-Hee, and Daniel K. Cha. "Microbial Reduction of Nitrate in the Presence of Nanoscale Zero-Valent Iron." *Chemosphere* 72 (2008): 257-262.

Sima, Jan, Katerina Diakova, Lenka Pavelcova, and Michal Havelka. "Redox Properties of a Constructed Wetland: Theoretical and Practical Aspects." *Chemistry and Biodiversity* 6 (2009): 341-355.

Smith, V. H. , G. D. Tilman, and J. C. Nekola. "Eutrophication: Impackts of Excess Nutrient Inputs on Freshwater, Marine and Terrestrial Ecosystems." *Environmental Pollution* 10 (1999): 179-196.

Stam, Marjolijn C. , Paul R.D. Mason, Céline Pallud, and Philippe Van Cappellen. "Sulfate Reducing Activity and Sulfur Isotope Fractionation by Natural Microbial Communities in Sediments of a Hypersaline Soda Lake (Mono Lake, California)." *Chemical Geology* (Elsevier) 278 (2010): 23-30.

Suzuki, Marcelino, Lance T. Taylor, and Edward F. DeLong. "Quantitative Analysis of Small-Subunit rRNA Genes in Mixed Microbial Populations via 5'-Nuclease Assays." *Applied and Environmental Microbiology* 66, no. 11 (November 2000): 4605-4614.

The United States Environmental Protection Agency. *Health Effects from Exposure to Sulfate in Drinking Water Workshop.* Office of Water, Atlanta, Georgia: The United States Environmental Protection Agency, September, 1998.

The World Health Organization. "Chemical Hazards in Drinking-Water: Nitrate and Nitrite." *Water Sanitation Health.* July 2011. http://www.who.int/water_sanitation_health/dwq/chemicals/nitratenitrite_background.pdf ?ua=1 (accessed January 2015).

—. "Chemical Hazards in Drinking-Water: Selenium." *Water Sanitation Health.* October 2011. http://www.who.int/water_sanitation_health/dwq/chemicals/selenium.pdf?ua=1 (accessed January 2015).

—. "Chemical Hazards in Drinking-Water-Sulfate." *Water Sanitation Health.* March 2004. http://www.who.int/water_sanitation_health/dwq/chemicals/sulfate.pdf (accessed January 2015).

Thermo Scientific. *Basic Principles of qPCR.* 2013.
http://www.thermoscientificbio.com/applications/pcr-and-qpcr/introduction-to-qpcr/
(accessed January 2015).

Throback, Ingela Noredal, Karin Enwall, Asa Jarvis, and Sara Hallin. "Reassessing PCR
Primers Targeting nirS, nirK and nosZ Genes for Community Surveys of Denitrifying
Bacteria with DGGE." *FEMS Microbiology Ecology* 49 (2004): 401-417.

U.S. Environmental Protection Agency. *Design and Evaluation of Tailings Dams.*
Technical Report, Office of Solid Waste, Special Waste Branch, Washington, DC: U.S.
Environmental Protection Agency, August, 1994.

United States Environmental Protection Agency. "Zero-valent Iron Nanoparticles."
Nanotechnology. February 16, 2012.
http://www.epa.gov/rpdweb00/docs/cleanup/nanotechnology/chapter-2-zero-valent.pdf
(accessed January 2015).

Van Den Brand, T. P. H., K. Roest, D. Brdjanovic, G. H. Chen, and M. C. M. Van
Loosdrecht. "Temperature Effect on Acetate and Propionate Consumption by Sulfate-
Reducing Bacteria in Saline Wastewater." *Applied Microbiology and Biotechnology*
(Springer) 98 (2014): 4245-4255.

Vester, Flemming, and Kjeld Ingvorsen. "Improved Most-Probable-Number Method to
Detect Sulfate-Reducing Bacteria with Natural Media and a Radiotracer." *Applied and
Environmental Microbiology* (American Society for Microbiology) 64, no. 5 (May 1998):
1700-1707.

Vigneron, Adrien, et al. "Archaeal and Anaerobic Methane Oxidizer Communities in the
Sonora Margin Cold Seeps, Guaymas Basin (Gulf of California)." *The ISME Journal*
(International Society for Microbial Ecology) 7 (2013): 1595-1608.

Yi, Zheng-ji, Bin Lian, Ying-qun Yang, and Jian-ling Zou. "Treatment of Simulated
Wastewater from in situ Leaching Uranium Mining by Zerovalent Iron and Sulfate

Reducing Bacteria." *Transactions of Nonferrous Metals Society of China* (Science Press) 19 (2009): 840-844.

Zaiointz, Charles. *Analysis of Covariance (ANCOVA).* 2014. http://www.real-statistics.com/analysis-of-covariance-ancova/ (accessed April 2015).

Zhang, Yiqiang, Juanfang Wang, Chris Amrhein, and William T. Frankenberger, Jr. . "Removal of Selenate from Water by Zerovalent Iron." *Journal of Environmental Quality*, March 2005: 487-495.

Zinck, Janice, and Wesley Griffith. *Review of Mine Drainage Treatment and Sludge Management Operations.* MEND Report, Natural Resources Canada, 2013.

Zinck, Janice, and Wesley Griffith. *Review of Mine Drainage Treatment and Sludge Management Operations.* MEND Report, Natural Resources Canada, March, 2013.

Appendices

A. Workflow Diagrams
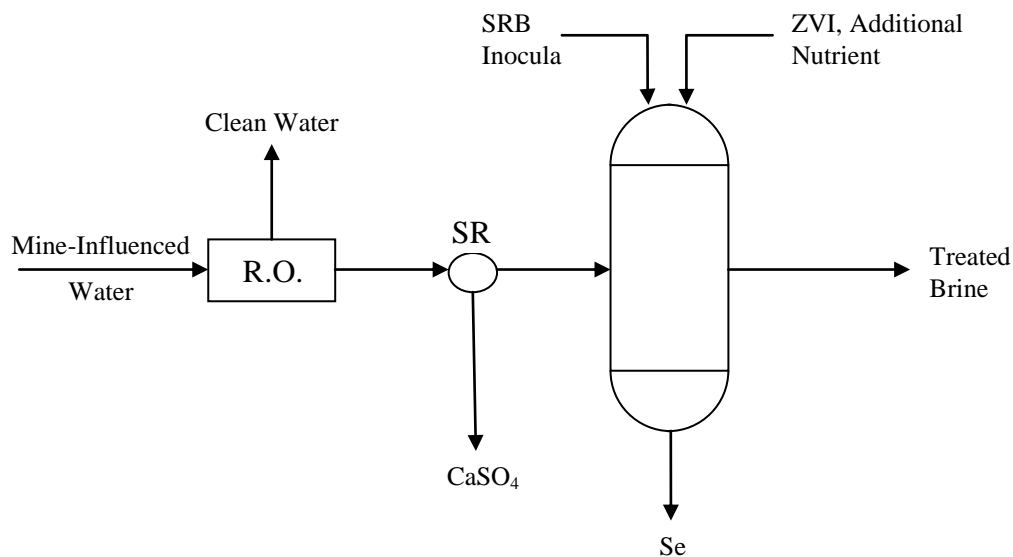
Figure A-1 Workflow diagram for the R.O. brine wastewater treatment process
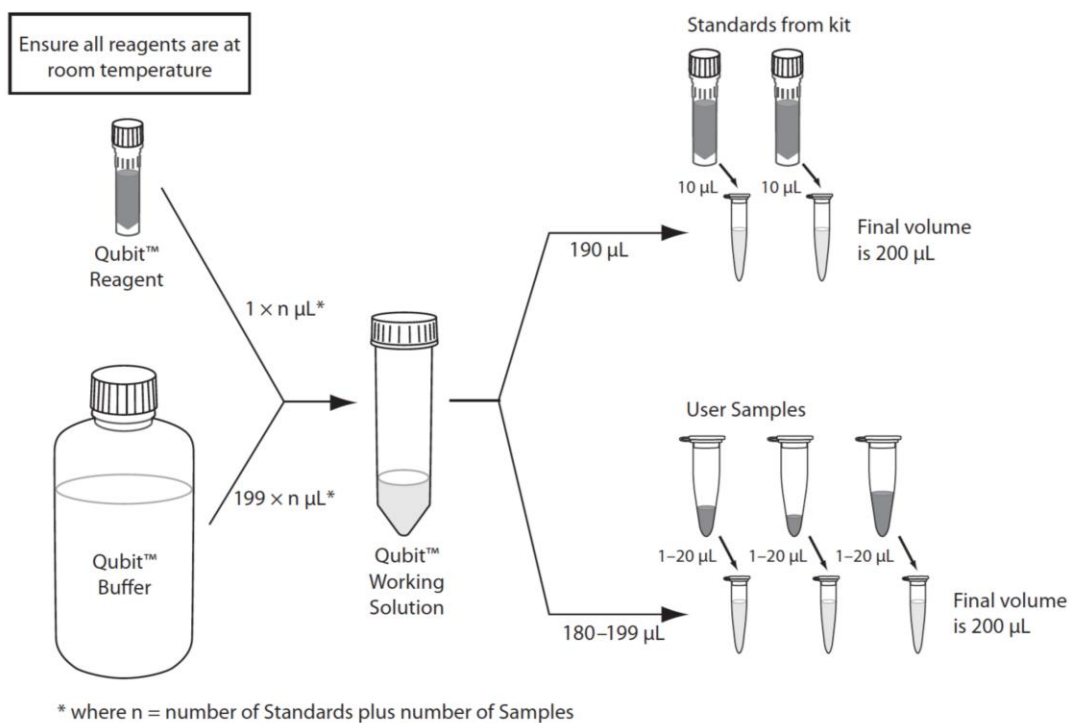
Figure A-2 Manufacturer's procedure for Qubit
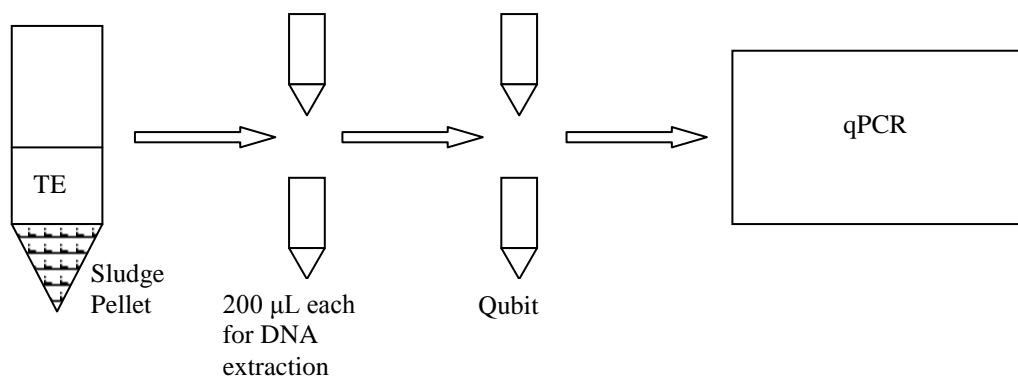
Figure A-3 Workflow diagram for qPCR

B. Analysis Reports

Table B-1 Analysis report of pre-SR R.O. brine wastewater from ALS Environmental

Name	Unit (mg/L)
Alkalinity, Total (CaCO$_3$)	1510
Ammonia, Total (as N)	-
Bromide (Br)	<5.0
Chloride (Cl)	96
Fluoride (F)	<2.0
Nitrate (as N)	56.9
Nitrite (as N)	<0.10
Sulphate (SO$_4$)	60300
Aluminum (Al)-Dissolved	<1
Antimony (Sb)-Dissolved	<1
Arsenic (As)-Dissolved	<1
Cadmium (Cd)-Dissolved	<1
Calcium (Ca)-Dissolved	76
Cobalt (Co)-Dissolved	<1
Copper (Cu)-Dissolved	<1
Iron (Fe)-Dissolved	<1
Lead (Pb)-Dissolved	<1
Magnesium (Mg)-Dissolved	55
Manganese (Mn)-Dissolved	<1
Molybdenum (Mo)-Dissolved	<1
Nickel (Ni)-Dissolved	<1
Potassium (K)-Dissolved	10
Selenium (Se)-Dissolved	7
Silicon (Si)-Dissolved	2
Sodium (Na)-Dissolved	35490
Zinc (Zn)-Dissolved	<1

Table B-2 Analysis report of post-SR R.O. brine wastewater from ALS Environmental

Name	Unit (mg/L)
Bromide (Br)	<1.0
Chloride (Cl)	113
Fluoride (F)	<0.40
Nitrate (as N)	235
Nitrite (as N)	<0.020
Sulfate (SO_4)	1730
Aluminum (Al)-Dissolved	<0.20
Antimony (Sb)-Dissolved	<0.20
Arsenic (As)-Dissolved	<0.20
Barium (Ba)-Dissolved	0.011
Beryllium (Be)-Dissolved	<0.0050
Bismuth (Bi)-Dissolved	<0.20
Boron (B)-Dissolved	<0.10
Cadmium (Cd)-Dissolved	<0.010
Calcium (Ca)-Dissolved	913
Chromium (Cr)-Dissolved	0.041
Cobalt (Co)-Dissolved	<0.010
Copper (Cu)-Dissolved	<0.010
Iron (Fe)-Dissolved	<0.030
Lead (Pb)-Dissolved	<0.050
Lithium (Li)-Dissolved	0.312
Magnesium (Mg)-Dissolved	51.1
Manganese (Mn)-Dissolved	<0.0050
Molybdenum (Mo)-Dissolved	<0.030
Nickel (Ni)-Dissolved	<0.050
Phosphorus (P)-Dissolved	<0.30
Potassium (K)-Dissolved	21.9
Selenium (Se)-Dissolved	1.87
Silicon (Si)-Dissolved	0.207
Silver (Ag)-Dissolved	<0.010
Sodium (Na)-Dissolved	38.8
Strontium (Sr)-Dissolved	0.631
Thallium (Tl)-Dissolved	<0.20
Tin (Sn)-Dissolved	<0.030
Titanium (Ti)-Dissolved	0.032
Vanadium (V)-Dissolved	<0.030
Zinc (Zn)-Dissolved	<0.0050

Table B-3 Analysis report of supernatants for passage #2 from ALS Environmental

Name	T (mg/L)	G (mg/L)	M (mg/L)
Bromide (Br)	<5.0	<5.0	<5.0
Chloride (Cl)	1710	1750	1730
Fluoride (F)	<0.030	<0.030	<0.030
Nitrate (as N)	852	924	805
Nitrite (as N)	<0.10	<0.10	<0.10
Sulfate (SO_4)	3380	2740	3350
Aluminum (Al)-Dissolved	<1.0	<1.0	<1.0
Antimony (Sb)-Dissolved	<1.0	<1.0	<1.0
Arsenic (As)-Dissolved	<1.0	<1.0	<1.0
Barium (Ba)-Dissolved	<0.050	<0.050	<0.050
Beryllium (Be)-Dissolved	<0.025	<0.025	<0.025
Bismuth (Bi)-Dissolved	<1.0	<1.0	<1.0
Boron (B)-Dissolved	<0.50	<0.50	<0.50
Cadmium (Cd)-Dissolved	<0.050	<0.050	<0.050
Calcium (Ca)-Dissolved	124	117	126
Chromium (Cr)-Dissolved	<0.050	<0.050	<0.050
Cobalt (Co)-Dissolved	<0.050	<0.050	<0.050
Copper (Cu)-Dissolved	<0.050	<0.050	<0.050
Iron (Fe)-Dissolved	<0.15	<0.15	<0.15
Lead (Pb)-Dissolved	<0.25	<0.25	<0.25
Lithium (Li)-Dissolved	<0.050	<0.050	<0.050
Magnesium (Mg)-Dissolved	157	153	155
Manganese (Mn)-Dissolved	<0.025	<0.025	0.025
Molybdenum (Mo)-Dissolved	<0.15	<0.15	<0.15
Nickel (Ni)-Dissolved	<0.25	<0.25	<0.25
Phosphorus (P)-Dissolved	31.8	20.3	27.1
Potassium (K)-Dissolved	166	162	165
Selenium (Se)-Dissolved	<1.0	<1.0	<1.0
Silicon (Si)-Dissolved	1.1	1.22	1.13
Silver (Ag)-Dissolved	<0.050	<0.050	<0.050
Sodium (Na)-Dissolved	2560	2600	2560
Strontium (Sr)-Dissolved	0.034	0.032	0.037
Thallium (Tl)-Dissolved	<1.0	<1.0	<1.0
Tin (Sn)-Dissolved	<0.15	<0.15	<0.15
Titanium (Ti)-Dissolved	<0.050	<0.050	<0.050
Vanadium (V)-Dissolved	<0.15	<0.15	<0.15
Zinc (Zn)-Dissolved	820	452	766

Table B-4 Analysis report of supernatants for passage #3 from ALS Environmental

Name	T (mg/L)	G (mg/L)	M (mg/L)
Bromide (Br)	<5.0	<5.0	<5.0
Chloride (Cl)	4730	4830	4770
Fluoride (F)	<0.030	0.031	<0.030
Nitrate (as N)	251	255	252
Nitrite (as N)	<0.10	<0.10	<0.10
Sulfate (SO_4)	7460	2700	4200
Aluminum (Al)-Dissolved	<2.0	<2.0	<2.0
Antimony (Sb)-Dissolved	<2.0	<2.0	<2.0
Arsenic (As)-Dissolved	<2.0	<2.0	<2.0
Barium (Ba)-Dissolved	<0.10	<0.10	<0.10
Beryllium (Be)-Dissolved	<0.050	<0.050	<0.050
Bismuth (Bi)-Dissolved	<2.0	<2.0	<2.0
Boron (B)-Dissolved	<1.0	<1.0	<1.0
Cadmium (Cd)-Dissolved	0.12	<0.10	0.1
Calcium (Ca)-Dissolved	234	178	212
Chromium (Cr)-Dissolved	<0.10	<0.10	<0.10
Cobalt (Co)-Dissolved	<0.10	<0.10	<0.10
Copper (Cu)-Dissolved	<0.10	<0.10	<0.10
Iron (Fe)-Dissolved	877	<0.30	3.46
Lead (Pb)-Dissolved	<0.50	<0.50	<0.50
Lithium (Li)-Dissolved	<0.10	<0.10	<0.10
Magnesium (Mg)-Dissolved	172	151	159
Manganese (Mn)-Dissolved	0.192	0.065	0.132
Molybdenum (Mo)-Dissolved	<0.30	<0.30	<0.30
Nickel (Ni)-Dissolved	<0.50	<0.50	<0.50
Phosphorus (P)-Dissolved	<3.0	<3.0	<3.0
Potassium (K)-Dissolved	288	244	254
Selenium (Se)-Dissolved	<2.0	<2.0	<2.0
Silicon (Si)-Dissolved	0.96	1.3	0.71
Silver (Ag)-Dissolved	<0.10	<0.10	<0.10
Sodium (Na)-Dissolved	5870	5410	5520
Strontium (Sr)-Dissolved	0.087	0.063	0.072
Thallium (Tl)-Dissolved	<2.0	<2.0	<2.0
Tin (Sn)-Dissolved	<0.30	<0.30	<0.30
Titanium (Ti)-Dissolved	<0.10	<0.10	<0.10
Vanadium (V)-Dissolved	<0.30	<0.30	<0.30
Zinc (Zn)-Dissolved	1010	365	876

Table B-5 Calibration curve for selenium concentration from UBC civil lab

Selenium (μg/L)	Average Reading	Reading 1	Reading 2	Reading 3
0	21.906	9.844	31.305	24.571
100	54.783	53.231	63.488	47.630
200	128.671	153.694	125.778	106.541
400	289.223	308.324	303.539	255.807
800	373.334	523.411	322.261	274.330
1000	587.795	1079.559	411.453	272.374
2000	1204.706	1232.629	1229.159	1152.329
5000	2877.644	2842.318	2869.047	2921.567
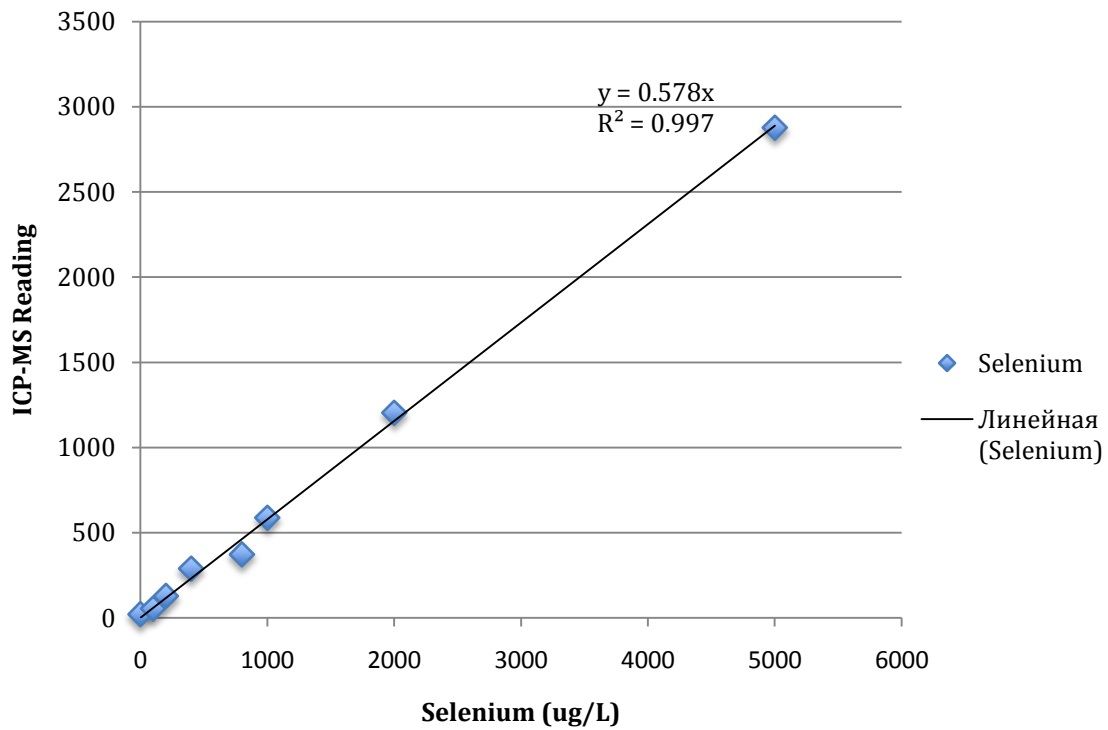
Figure B-1 Calibration curve for selenium concentration from UBC civil lab

Table B-6 Analysis report of selenium readings from UBC civil lab - part 1

Date	Code	Average	Reading 1	Reading 2	Reading 3
07-07-14	G1	517.341	503.472	514.572	533.980
07-07-14	G2	603.943	625.126	596.564	590.139
07-07-14	G3	783.169	791.945	772.237	785.323
07-07-14	M1	533.955	529.070	542.243	530.554
07-07-14	M2	637.886	623.161	642.994	647.502
07-07-14	M3	2900.948	2867.765	2944.987	2890.093
08-07-14	G3	816.331	804.577	837.424	806.993
08-07-14	M3	2812.936	2810.615	2814.049	2814.143
09-07-14	G3	779.765	757.587	791.199	790.510
09-07-14	M3	2798.017	2796.101	2798.147	2799.803
10-07-14	G3R1	608.441	585.320	608.482	631.521
10-07-14	G3R2	624.339	626.200	609.015	637.802
10-07-14	G3R3	689.897	698.833	685.991	684.867
10-07-14	M3R1	2703.166	2726.352	2709.188	2673.958
10-07-14	M3R2	2449.428	2421.465	2458.390	2468.430
10-07-14	M3R3	2458.686	2447.792	2438.511	2489.754
11-07-14	G3	575.575	560.880	588.056	577.790
11-07-14	M3	1818.699	1794.746	1818.996	1842.355
12-07-14	G3	556.411	550.799	569.468	548.966
12-07-14	M3	2087.647	2062.694	2081.741	2118.505
13-07-14	G3	509.270	510.789	503.086	513.935
13-07-14	M3	516.043	491.105	513.635	543.390
14-07-14	G3	383.496	371.929	381.016	397.543
14-07-14	M3	396.640	398.177	392.311	399.432

Table B-7 Analysis report of selenium readings from UBC civil lab - part 2

Date	Code	Average	Reading 1	Reading 2	Reading 3
21-07-14	G1	132.775	128.949	128.501	140.876
21-07-14	G2	453.014	436.961	453.106	468.974
21-07-14	G3	643.854	629.047	652.747	649.769
21-07-14	M1	211.128	202.996	218.266	212.123
21-07-14	M2	232.009	212.178	230.250	253.600
21-07-14	M3	1598.004	1605.751	1594.807	1593.453
04-08-14	G1R1	44.598	41.973	46.896	44.925
04-08-14	G1R2	100.266	100.597	101.136	99.065
04-08-14	G1R3	421.779	425.307	414.082	425.949
04-08-14	G2	390.103	379.138	393.051	398.120
04-08-14	G3	332.341	346.498	329.295	321.229
04-08-14	M1R1	408.663	398.094	423.320	404.574
04-08-14	M1R2	309.619	293.602	308.198	327.056
04-08-14	M1R3	212.858	214.530	202.752	221.293
04-08-14	M2	119.206	120.442	131.169	106.006
04-08-14	M3	148.109	137.173	144.532	162.622

Table B-8 Analysis report of selenium concentrations from UBC civil lab - part 1

Date	Code	Average mg/L	Concentration 1 mg/L	Concentration 2 mg/L	Concentration 3 mg/L
07-07-14	G1	0.881	0.857	0.876	0.909
07-07-14	G2	1.029	1.065	1.016	1.005
07-07-14	G3	1.336	1.351	1.317	1.339
07-07-14	M1	0.909	0.901	0.923	0.903
07-07-14	M2	1.087	1.062	1.096	1.103
07-07-14	M3	5.035	4.976	5.114	5.016
08-07-14	G3	1.392	1.372	1.429	1.376
08-07-14	M3	4.879	4.874	4.881	4.881
09-07-14	G3	1.330	1.292	1.349	1.348
09-07-14	M3	4.852	4.849	4.852	4.855
10-07-14	G3R1	1.037	0.997	1.037	1.076
10-07-14	G3R2	1.064	1.067	1.038	1.087
10-07-14	G3R3	1.176	1.191	1.169	1.167
10-07-14	M3R1	4.684	4.725	4.694	4.632
10-07-14	M3R2	4.234	4.185	4.250	4.268
10-07-14	M3R3	4.251	4.232	4.215	4.306
11-07-14	G3	0.980	0.955	1.002	0.984
11-07-14	M3	3.127	3.085	3.128	3.168
12-07-14	G3	0.948	0.938	0.970	0.935
12-07-14	M3	3.598	3.554	3.587	3.652
13-07-14	G3	0.867	0.870	0.857	0.875
13-07-14	M3	0.879	0.836	0.875	0.925
14-07-14	G3	0.653	0.633	0.649	0.677
14-07-14	M3	0.675	0.678	0.668	0.680

Table B-9 Analysis report of selenium concentrations from UBC civil lab - part 2

Date	Code	Average	Concentration 1	Concentration 2	Concentration 3
		mg/L	mg/L	mg/L	mg/L
21-07-14	G1	0.227	0.220	0.220	0.241
21-07-14	G2	0.771	0.744	0.771	0.798
21-07-14	G3	1.097	1.072	1.112	1.107
21-07-14	M1	0.360	0.346	0.372	0.361
21-07-14	M2	0.395	0.362	0.392	0.432
21-07-14	M3	2.742	2.756	2.737	2.735
04-08-14	G1R1	0.077	0.073	0.081	0.078
04-08-14	G1R2	0.172	0.172	0.173	0.170
04-08-14	G1R3	0.718	0.724	0.705	0.725
04-08-14	G2	0.664	0.645	0.669	0.678
04-08-14	G3	0.566	0.590	0.561	0.547
04-08-14	M1R1	0.696	0.678	0.721	0.689
04-08-14	M1R2	0.527	0.500	0.525	0.557
04-08-14	M1R3	0.363	0.366	0.346	0.377
04-08-14	M2	0.204	0.206	0.224	0.181
04-08-14	M3	0.253	0.234	0.247	0.277

Table B-10 Analysis report of 10 times diluted samples from ALS Environmental

Date	Code	Selenium Concentration (mg/L)
21-07-14	G1	0.0234
21-07-14	G2	0.0804
21-07-14	G3	0.104
21-07-14	M1	0.0318
21-07-14	M2	0.0319
21-07-14	M3	0.269
04-08-14	G1R1	0.00671
04-08-14	G1R2	0.0117
04-08-14	G1R3	0.0677
04-08-14	M1R1	0.0687
04-08-14	M1R2	0.0533
04-08-14	M1R3	0.0348
18-08-14	G1	0.0682
18-08-14	G2R1	0.0741
18-08-14	G2R2	0.0747
18-08-14	G2R3	0.0775
18-08-14	G3	0.0169
18-08-14	M1	0.0372
18-08-14	M2R1	0.0328
18-08-14	M2R2	0.0231
18-08-14	M2R3	0.0572
18-08-14	M3	<0.00050
01-09-14	G1	0.0125
01-09-14	G2	0.0794
01-09-14	G3	0.0371
01-09-14	M1	0.0276
01-09-14	M2	0.0180
01-09-14	M3	0.0010

C. Specification of ZVI

CONNELLY – GPM, INC.

ESTABLISHED 1875

3154 SOUTH CALIFORNIA AVENUE   CHICAGO, ILLINOIS 60608-5176

PHONE: (773) 247-7231 • www.ConnellyGPM.com • FAX: (773) 247-7239

April 17, 2014

SCREEN SPECIFICATION
CC-1349

U.S. SCREEN
 NUMBER (Opening Size)

20	(0.850 mm)	100% PASSING
40	(0.420 mm)	98 - 100% PASSING
60	(0.250 mm)	80 - 100
100	(0.150 mm)	40 - 75
200	(0.075 mm)	10 - 40

MATERIAL WEIGHS APPROXIMATELY 195 - 215 POUNDS PER CUBIC FOOT

TYPICAL ANALYSIS OF IRON AGGREGATE

Metallic Iron/Iron Oxide	87-93%
Total Carbon	2.85-3.23
Manganese	0.14-0.60
Sulphur	0.067-0.107
Phosphorous	0.000-0.132
Silicon	1.0-1.85
Nickel	0.05-0.21
Chromium	0.03-0.23
Vanadium	ND
Molybdenum	0.08-0.15
Titanium	0.004-0.1
Copper	0.11-0.20
Aluminum	0-0.005
Cobalt	ND
Magnesium	0.01
Boron	0.01
Zinc	0.01
Zirconium	0.01

GALEN B. DIXON
Technical Director

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D. DNA Extraction Protocols

D.1 Reagents Used for DNA Extractions

1. 1 M Tris-HCl

   - Weigh 12.11 g of tris base

   - Add 70 mL of distilled water

   - Put in around 10 mLhydrochloric acid (HCl)

   - Adjust the pH to 7.4

   - Fill the solution to 100 mL with distilled water

2. 100 mM Ethylenediaminetetraacetic Acid (EDTA)

   - Weigh 2.9224 g of EDTA

   - Pour around 50 mL distilled water and adjust the pH to 8.0

   - Fill the solution to 100 mL with distilled water

3. DNA Extraction Buffer

   - Pour 25 mL of 1 M Tris-HCl into a 300 mL volumetric flask

   - Weigh 7.306 g of EDTA

   - Weigh 4.099 g of monosodium phosphate (NaH_2PO_4)

   - Weigh 1.461 g of NaCl

   - Weigh 2.5 g of cetyltrimethylammonium bromide (CTAB)

   - Mix everything together with 100 mL distilled water and adjust the pH to 8.0

   - Fill the solution to 250 mL with distilled water

4. TE Buffer

   - Pour 2.5 mL of 1 M Tris-HCl into a 300 mL volumetric flask

   - Pour 2.5 mL of 100 mM EDTA into the same volumetric flask

   - Add 100 mL distilled water and adjust the pH to 8.0

   - Fill the solution to 250 mL with distilled water

5. 0.3 M NaCl in TE

   - Weigh 4.383 g sodium chloride (NaCl)

   - Mix with 150 mL TE buffer

   - Adjust the pH to 8.0

   - Fill the solution to 250 mL with TE buffer

6. 30% Polyethylene Glycol 6000 in 1.6 M NaCl

   - Weigh 75 g of polyethylene glycol 6000

   - Weigh 23.376 g of NaCl

   - Mix with distilled water to obtain a final volume of 250 mL

7. 0.5 M Calcium Chloride ($CaCl_2$) in TE Buffer

   - Weigh 18.376 g of $CaCl_2$

   - Add 150 mL of TE buffer

   - Adjust the pH to 8.0

   - Fill the solution to 250 mL with TE buffer

8. 0.3 M NaCl

   - Weigh 4.383 g of NaCl

   - Mix with distilled water to obtain a final volume of 250 mL

D.2DNA Extraction Protocol for Soil Samples

1. Mix 3-6g of soil with $1 - 2$ mL of distilled water (diH$_2$O) in a 50 mL falcon tube and pretreat for 10 min with 0.5- 1 mLof 50 mg/mL lysozyme at 25 °C.

2. After pretreating add 13.5 mL of DNA extraction buffer and 100 µL of 10 mg/mL proteinase Kand shake horizontally at 225 rpm for 30 min at 37°C.

3. After the shaking treatment, add 1.5 mL of 20% sodium dodecyl sulfate (SDS), and incubate the samples in a 65°C water bath for 2hour with gentle end-over-end inversions every 15 to 20 min.

4. Collect the supernatants after centrifugation at 4000 rpm for 10 min at room temperature and transfer to a clean 50 mL centrifuge tube.

5. (Optional Step) Extract the soil pellets two more times by adding 4.5 mL of the extraction buffer and 0.5 mL of 20% SDS, mixing using the vortex for 10 s, incubating at 65°C for 10 min, and centrifuging as before. Alternatively the soil pellet can be stored at -20 °C if insufficient DNA was obtained.

6. If doing multiple extractions, combine supernatants and mix with an equal volume of 24:1 vol/vol chloroformisoamyl alcohol for 30min.

7. Recover the aqueous phase by centrifugation ensuring not to disturb the interface.

8. Precipitate the DNA by incubating at room temperature for 1hour in 2 volumes of 30% polyethylene glycol 6000 in 1.6M NaCl.

9. Centrifuge at 4000 rpm for 30 min.

10. Wash the DNA pellet with 5 mL fresh 70% ethanol and centrifuge for 5 min.

11. (Stop Point) DNA can be stored as a pellet overnight before proceeds to the next step.

12. Re-suspend diethylaminoethyl sephacel and add 1 mL to spin columns.

13. Equilibrate the sephacel by passing 4 mL of 0.3M NaCl in TE.

14. Re-suspend DNA in 1 mL of 0.3M NaCl and add to the sephacel column.

15. Wash the column with 4 mL of 0.3 M NaCl in TE.

16. Elute the DNA with 4 mL of 0.5 M CaCl in TE.

17. Precipitate with 0.6 volume of isopropanol at room temperature for 1hour.

18. Pellet the nucleic acids by centrifuging at 4000rpm for 30 min.

19. Wash pellets with 70% fresh ethanol and centrifuge at 4000 rpm for 7 min.

20. Re-suspend the DNA pellets in sterile water.

D.3 Phenol-Chloroform DNA Extraction Protocol

1. Wash the pellet with TE buffer.

2. Suspend the pellet in 500 µL TE buffer.

3. Add 10 µL 150 mg/mL lysozyme and incubate at 56 °C for 1 hour.

4. Add 75 µL of 10% SDS and 5 µL of 20 mg/mL proteinase K.

5. Incubate at 56 °C for 1 hour.

6. Add 5 µL of RNase A.

7. Incubate at 37 °C for 30 min.

8. Add 400 µL of tri-saturated phenol (pH 8.0).

9. Centrifuge at 10000 rpm for 10 min.

10. Take the supernatant and add 200 µL of tris-saturated phenol and 200 µL of chloroform:isoamyl alcohol (24:1).

11. Centrifuge at 10000 rpm for 10 min.

12. (Optional Step) Again take the supernatant and repeat the above step.

13. Take the supernatant and add 400 μL of chloroform:isoamyl alcohol (24:1).

14. Centrifuge at 10000 rpm for 10 min.

15. (Optional Step) Repeat step 13 and 14.

16. Take the supernatant and add 0.1 volume of 3 M sodium acetate and 2 volume of absolute ethanol chilled to -20 °C.

17. Mix gently and incubate at -20 °C overnight.

18. Centrifuge at 15000 rpm for 30 min at 0 °C.

19. Decant the supernatant and wash the pellet with 600 μL of 70% ethanol by centrifugation at 10000 rpm for 15 min.

20. Decant the supernatant and dry the pellet at room temperature.

21. Suspend the pellet in nano-pure water.

E. R Command Lines, Graphs and Tables

E.1 R Command Lines

Set the workplace and read the data

setwd("~/Dropbox/UBC/Masters/Selenium Treatment/Thesis/ANCOVA")

Data <- read.csv("Table for ANCOVA.csv")

Make Time, Inoculum and Treatment a factor

Data$Time<- factor(Data$Time)

Data$Inoculum<- factor(Data$Inoculum)

Data$Treatment<- factor(Data$Treatment)

Check homogeneity of the data

boxplot(Data$Sulfate ~ Data$Treatment)

boxplot(Data$Nitrate ~ Data$Treatment)

boxplot(Data$Selenium ~ Data$Treatment)

Load all the libraries/packages needed for analysis

install.packages("car")

library(car)

install.packages("multcomp")

library(multcomp)

Fit data into linear models

m1<- lm(Sulfate~(Time+Inoculum+Treatment)^2, data=Data)

m2<- lm(Nitrate~(Time+Inoculum+Treatment)^2, data=Data)

m3<- lm(Selenium~(Time+Inoculum+Treatment)^2, data=Data)

```
# Display results

summary(m1)

summary(m2)

summary(m3)

# Adjust the models for the Tukey test temporarily omitting the interaction terms

m1a<- update(m1,~Time+Inoculum+Treatment)

m2a<- update(m2,~Time+Inoculum+Treatment)

m3a<- update(m3,~Time+Inoculum+Treatment)

# Perform the Tukey test on the types of treatment and inoculum based on the models
developed

test1T<- glht(m1a,linfct=mcp(Treatment="Tukey"))

test1I<- glht(m1a,linfct=mcp(Inoculum="Tukey"))

test2T<- glht(m2a,linfct=mcp(Treatment="Tukey"))

test2I<- glht(m2a,linfct=mcp(Inoculum="Tukey"))

test3T<- glht(m3a,linfct=mcp(Treatment="Tukey"))

test3I<- glht(m3a,linfct=mcp(Inoculum="Tukey"))

# Display the Tukey test results

summary(test1T)

summary(test1I)

summary(test2T)

summary(test2I)

summary(test3T)

summary(test3I)
```

Calculate the confidence interval for the treatment and the inoculum
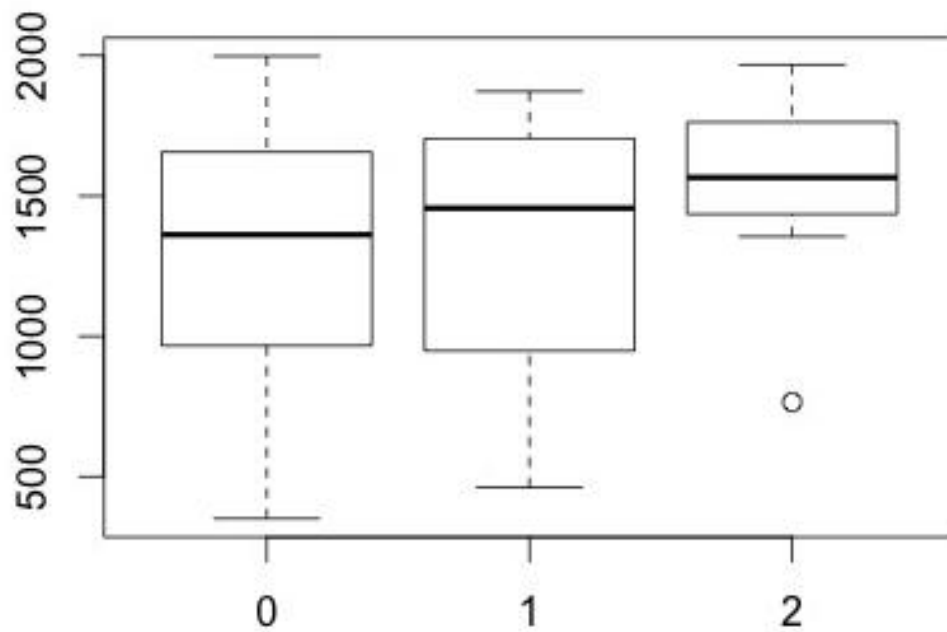
confint(test1T)

confint(test1I)

confint(test2T)
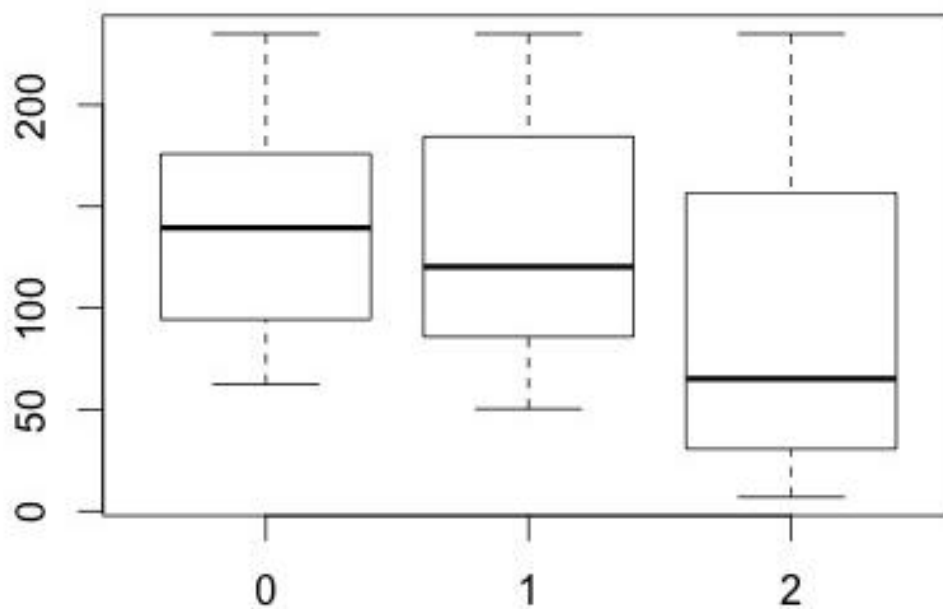
confint(test2I)

confint(test3T)

confint(test3I)

E.2 Graphs Obtained in R

Figure E-1 Average sulfate concentration for 3 different treatments

Figure E-2 Average nitrate concentration for 3 different treatments
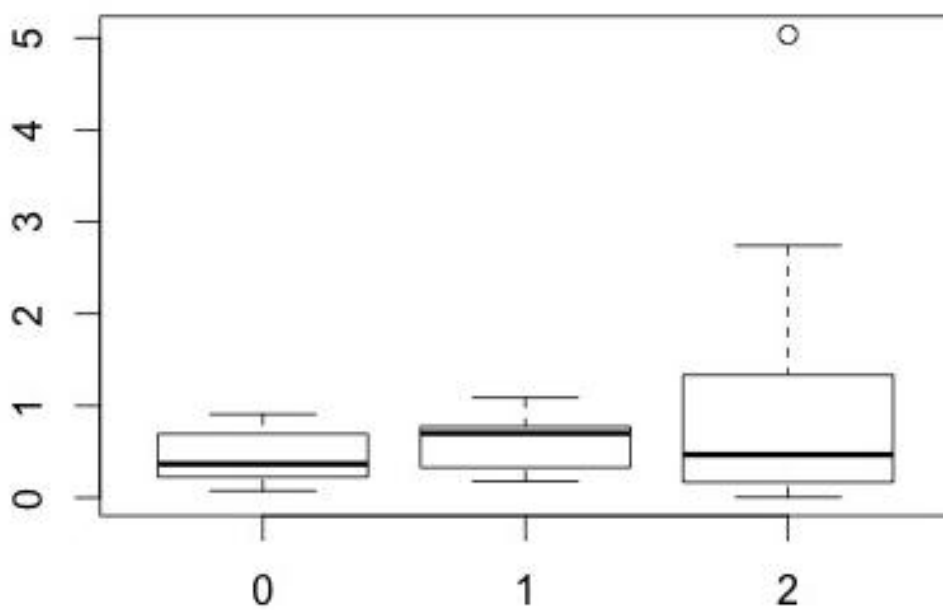
Figure E-3 Average selenium concentration for 3 different treatments

E.3 Tables Obtained in R

Table E-1 Summary of coefficients obtained in model 1 for sulfate

	Estimate	Std. Error	t Value	Pr(>|t|)	
(Intercept)	1944.325	313.610	6.200	1.27E-05	***
Time14	-697.567	426.054	-1.637	0.121	
Time28	-748.031	358.162	-2.089	0.053	.
Time42	-492.916	416.726	-1.183	0.254	
Time56	-421.778	426.054	-0.990	0.337	
Inoculum1	-173.810	348.027	-0.499	0.624	
Treatment1	6.319	397.617	0.016	0.988	
Treatment2	-205.724	400.674	-0.513	0.615	
Time14:Inoculum1	-248.542	426.054	-0.583	0.568	
Time28:Inoculum1	-33.758	387.396	-0.087	0.932	
Time42:Inoculum1	-71.288	387.396	-0.184	0.856	
Time56:Inoculum1	-320.543	426.054	-0.752	0.463	
Time14:Treatment1	405.948	521.807	0.778	0.448	
Time28:Treatment1	34.205	476.343	0.072	0.944	
Time42:Treatment1	251.805	476.343	0.529	0.604	
Time56:Treatment1	231.745	521.807	0.444	0.663	
Time14:Treatment2	947.763	521.807	1.816	0.088	.
Time28:Treatment2	335.760	476.343	0.705	0.491	
Time42:Treatment2	329.725	521.807	0.632	0.536	
Time56:Treatment2	656.470	521.807	1.258	0.226	
Inoculum1:Treatment1	-373.849	296.367	-1.261	0.225	
Inoculum1:Treatment2	-49.172	312.398	-0.157	0.877	

Table E-2 Summary of coefficients obtained in model 2 for nitrate

	Estimate	Std. Error	t Value	Pr(>|t|)	
(Intercept)	234.909	28.082	8.365	3.090E-07	***
Time14	-134.177	38.151	-3.517	0.003	**
Time28	-86.950	32.072	-2.711	0.015	*
Time42	-91.087	37.316	-2.441	0.027	*
Time56	-147.515	38.151	-3.867	0.001	**
Inoculum1	0.182	31.164	0.006	0.995	
Treatment1	5.266	35.605	0.148	0.884	
Treatment2	-4.992	35.878	-0.139	0.891	
Time14:Inoculum1	-44.914	38.151	-1.177	0.256	
Time28:Inoculum1	17.620	34.689	0.508	0.618	
Time42:Inoculum1	-8.880	34.689	-0.256	0.801	
Time56:Inoculum1	-4.943	38.151	-0.130	0.899	
Time14:Treatment1	-16.364	46.725	-0.350	0.731	
Time28:Treatment1	34.517	42.654	0.809	0.430	
Time42:Treatment1	-21.077	42.654	-0.494	0.628	
Time56:Treatment1	1.406	46.725	0.030	0.976	
Time14:Treatment2	15.341	46.725	0.328	0.747	
Time28:Treatment2	-55.611	42.654	-1.304	0.211	
Time42:Treatment2	-102.912	46.725	-2.202	0.043	*
Time56:Treatment2	-59.062	46.725	-1.264	0.224	
Inoculum1:Treatment1	-10.531	26.538	-0.397	0.697	
Inoculum1:Treatment2	9.985	27.974	0.357	0.726	

Table E-3 Summary of coefficients obtained in model 3 for selenium

	Estimate	Std. Error	t Value	Pr(>|t|)	
(Intercept)	0.318	0.447	0.712	0.487	
Time14	-0.204	0.608	-0.336	0.741	
Time28	0.155	0.511	0.304	0.765	
Time42	0.319	0.594	0.537	0.599	
Time56	0.074	0.608	0.121	0.905	
Inoculum1	1.153	0.496	2.324	0.034	*
Treatment1	0.370	0.567	0.652	0.524	
Treatment2	1.921	0.571	3.363	0.004	**
Time14:Inoculum1	-0.794	0.608	-1.308	0.210	
Time28:Inoculum1	-1.249	0.552	-2.261	0.038	*
Time42:Inoculum1	-1.374	0.552	-2.488	0.024	*
Time56:Inoculum1	-1.536	0.608	-2.529	0.022	*
Time14:Treatment1	0.127	0.744	0.170	0.867	
Time28:Treatment1	-0.155	0.679	-0.227	0.823	
Time42:Treatment1	-0.124	0.679	-0.183	0.857	
Time56:Treatment1	0.124	0.744	0.166	0.870	
Time14:Treatment2	-0.665	0.744	-0.893	0.385	
Time28:Treatment2	-2.307	0.679	-3.396	0.004	**
Time42:Treatment2	-2.731	0.744	-3.670	0.002	**
Time56:Treatment2	-2.301	0.744	-3.092	0.007	**
Inoculum1:Treatment1	-0.414	0.423	-0.979	0.342	
Inoculum1:Treatment2	0.739	0.445	1.658	0.117	

Table E-4 Summary of the Tukey's test for sulfate linear model - treatment

	Estimate	Std. Error	t Value	Pr(>|t|)
1 - 0 == 0	3.466	130.884	0.026	1.000
2 - 0 == 0	203.778	137.964	1.477	0.316
2 - 1 == 0	200.312	137.964	1.452	0.328

Table E-5 Summary of the Tukey's test for sulfate linear model - inoculum

	Estimate	Std. Error	t Value	Pr(>|t|)	
1 - 0 == 0	-442	105.7	-4.18	0.000232	***

Table E-6 Summary of the Tukey's test for nitrate linear model - treatment

	Estimate	Std. Error	t Value	Pr(>|t|)	
1 - 0 == 0	2.314	14.127	0.164	0.9853	
2 - 0 == 0	-39.186	14.891	-2.632	0.0345	*
2 - 1 == 0	-41.501	14.891	-2.787	0.0241	*

Table E-7 Summary of the Tukey's test for nitrate linear model - inoculum

	Estimate	Std. Error	t Value	Pr(>|t|)
1 - 0 == 0	-6.642	11.413	-0.582	0.565

Table E-8 Summary of the Tukey's test for selenium linear model - treatment

	Estimate	Std. Error	t Value	Pr(>|t|)
1 - 0 == 0	0.160	0.302	0.530	0.857
2 - 0 == 0	0.627	0.318	1.971	0.137
2 - 1 == 0	0.467	0.318	1.469	0.320

Table E-9 Summary of the Tukey's test for selenium linear model - inoculum

	Estimate	Std. Error	t Value	Pr(>|t|)
1 - 0 == 0	0.137	0.244	0.562	0.578

Table E-10 Summary of the 95% confidence interval for sulfate linear model - treatment

	Estimate	Lower	Upper
1 - 0 == 0	3.467	-319.066	325.999
2 - 0 == 0	203.778	-136.201	543.757
2 - 1 == 0	200.312	-139.667	540.290

Table E-11 Summary of the 95% confidence interval for sulfate linear model - inoculum

	Estimate	Lower	Upper
1 - 0 == 0	-441.983	-657.924	-226.042

Table E-12 Summary of the 95% confidence interval for nitrate linear model - treatment

	Estimate	Lower	Upper
1 - 0 == 0	2.314	-32.504	37.133
2 - 0 == 0	-39.186	-75.888	-2.484
2 - 1 == 0	-41.501	-78.203	-4.799

Table E-13 Summary of the 95% confidence interval for nitrate linear model - inoculum

	Estimate	Lower	Upper
1 - 0 == 0	-6.642	-29.950	16.665

Table E-14 Summary of the 95% confidence interval for selenium linear model -

treatment

	Estimate	Lower	Upper
1 - 0 == 0	0.160	-0.584	0.903
2 - 0 == 0	0.627	-0.157	1.410
2 - 1 == 0	0.467	-0.317	1.251

Table E-15 Summary of the 95% confidence interval for selenium linear model -

inoculum

	Estimate	Lower	Upper
1 - 0 == 0	0.137	-0.361	0.635