IDENTIFICATION AND SURVIVAL OF ISOLATES COLLECTED FROM THE MARS ROVER, CURIOSITY, UNDER SIMULATED MARTIAN CONDITIONS AND IDENTIFICATION OF A SELENATE REDUCTION PATHWAY IN A NOVEL DECHLOROMONAS ISOLATE

A Dissertation

Presented in Partial Fulfillment of the Requirements for the

Degree of Doctorate of Philosophy

with a

Major in Environmental Science

in the

College of Graduate Studies

University of Idaho

by

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May 2014

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Authorization to Submit Dissertation

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Abstract

The work presented herein is a compilation of two separate research projects. The first project analyzed almost 400 isolates collected from the Mars rover, Curiosity, prior to launch. For the first time in 40 years of Mars spaceflight, we are beginning to understand in-depth the types of culturable microorganisms present on spacecraft and their possible impact on efforts to both detect life and preserve any existing ecosystems on planetary bodies. The research presented here shows that the microorganisms remaining on the spacecraft, after numerous attempts to eradicate them, are very resistant to extreme environmental conditions and are some of the hardiest microorganisms. Most can survive extended periods of desiccation, grow at low temperatures, grow in medium containing elevated salt or pH, and can grow anaerobically utilizing sources known to be available on Mars, such as sulfate, perchlorate, and iron, to conserve energy. Further studies will provide additional insight as to whether these organisms can truly inhabit other worlds, ways to mitigate their initial contamination of spacecraft, and provide additional knowledge as to the limits of life as we know it.

The second study focused on the characterization of a novel dissimilatory selenate reducing organism belonging to the *Dechloromonas* genus. Several selenate reducing microorganisms have been identified previously but only one microorganism, *Thauera selenatis*, had been studied in-depth when this study commenced. With a lot of luck, and then an opportunity to perform full-genome sequencing on this *Dechloromonas* isolate, it was discovered that the selenate reductase pathway is very closely related to the one found in Thauera. There is still much work that needs to be done to have a better understanding of the Se reduction enzymes and their regulatory controls in microorganisms. However, this work is increasing the ability to understand selenium cycling in nature and could lead to the development of bioremediation strategies to be applied in selenium contaminated environments.

Acknowledgements

I wish to thank my committee members for all of the support, patience, and guidance they have provided me throughout the years. Dr. Strawn has shown an enormous amount of patience and support thoughout this process for which I am extremely grateful and I can not thank him enough. Dr. Childers has not only been a mentor for the technical aspects of my work, but has also mentored me during the difficult times when I needed someone to help me re-center and focus. She has put a lot of effort into reviewing this dissertation and I wouldn't have been able to finish this work without her. Dr. Crawford gave me the initial opportunity to begin this journey and has been a wonderful mentor throughout my master's and doctoral work. I have always greatly appreciated his kindness and support. He gave students the room to discover themselves and their capabilities while always being there as a mentor and avid supporter. I also wish to thank Dr. Cosens for providing tremendous input into the legal aspects of selenium regulation. She has always been concerned with making sure that I am on the right track to achieve my educational and professional goals. I greatly appreciate her support.

None of this work would have been possible without Dr. J. Nick Benardini at Jet Propulsion Laboratory. None of this MSL work could have been done without him providing me with the isolates, and technical support. This collaboration has provided me with multiple funding opportunities and research support. I am beyond thankful that he came to me when JPL wanted to embark on a different journey with the archive collection. There are not enough words to express my gratitude.

Dr. Paszczynski has been absolutly wonderful for letting me work in his laboratory and do what I needed to do to keep this going. I can not thank hm enough. I also wish to thank my co-workers in Dr. Paszczynski's lab, and the former EBI, who have provided me with both friendhsip and technical support. There have been numerous students and recent graduates who have directly contributed to this project: David Anderl, Mike Schrader, Emmaleen Wear, Jamilee Lords, Kyle Petersen, and Connor Chapek. Your tremendous contributions are not forgotten and this work couldn't have been completed without you.

Dedication

This dissertation is dedicated to my family, all of whom have stood by me and patiently waited for me to complete this journey, while providing me with unconditional support throughout this process. I couldn't have done this without each and every single one of you and my gratitude cannot be expressed enough.

I also wish to specifically dedicate this to my son, Connor Rohde. Your arrival into my life made it even more imperative that I finish my degree, so that I may give you the very best in life and all that you deserve. I hope that this will one day pave the way for you to have every opportunity at success. Remember that if you stick with it, you can accomplish your dreams.

Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction to Dissertation

This dissertation is the result of four years of work in two different, but not completely unrelated, areas of study. The first area of study was looking at microbial selenate reduction in a *Dechloromonas* bacterium isolated from seleniferous shales collected in southeastern Idaho. The second area of study was to identify and characterize microbial isolates collected from the NASA Mars Science Laboratory prior to launch. This introduction will explain the significance of each of these studies from the science to the larger impact at home and in space.

Microbial Selenate Reduction Studies

In August 2008 I began work in Dr. Susan Childers' lab studying bacteria that respire selenium. Selenium (Se) is a naturally occurring element in the environment and is often associated with shales, coal mining wastes, agricultural drainage water, and oil refining wastewater (Barceloux 1999, Lenz 2009). High concentrations of selenium in soils is usually the result of weathering and leaching of bedrock, and leaching of selenium from these soils can result in contamination of nearby groundwater and in the accumulation of toxic levels of selenium (Häsänen 1997).When shales are exposed to air during the mining of oil, coal, and phosphate, soluble forms of Se are released into surface water and enter watersheds posing a health threat to aquatic species. Additionally, consumption of oil shale or burning of coal for energy production results in the generation of flue gas and fly ash, which can contain selenium and can lead to the entry of toxic forms of Se into watersheds (Stolz 1999). The Se present in a watershed is absorbed by plants such as legumes, grasses, shrubs and forbs. When livestock or wildlife, such as deer and elk, feed on these plants, they ingest large amount of Se leading to selenosis (selenium toxicity) and livestock often have to be euthanized, resulting in economic losses to ranchers. Fish and waterfowl are also at risk for increased Se uptake through the ingestion of contaminated water or aquatic plants. Se also presents a health risk to humans who consume plants that have accumulated large amounts of Se or from animals that have fed off of plants containing high levels of Se.

Many treatment options are being considered for the attenuation and remediation of excess environmental selenium in impacted areas. The parameters at different sites can vary in the total concentrations of Se, the species of Se present, biological and physio-chemical differences, and interference by other anions, which can make it difficult to develop a one-size fits all approach (Lenz 2009). Additionally, many of the processes currently in use are expensive and often result in the accumulation of selenium wastes that still need disposal.

Microorganisms that can reduce selenate to Se(0), an insoluble form of selenium, have been identified, and these organisms may be able to play a major role in affecting the fate of selenium species in the environment . Even though microbial reduction of selenium has been studied, and preliminary results show that use of microorganisms could result in a cost effective method for altering the fate of selenium in the environment, little is known about the diversity of microorganisms in the environment that show Se reducing capabilities.

Many microorganisms have evolved pathways to utilize the reduction of selenium as a means of conserving energy. However, only one Se reducing microorganism, *Thauera selenatis*, has been well studied. There are a number of unanswered questions as to what regulates Se reduction by this organism and the role of certain genes within the Se reduction operon (Schröder 1997). The pathways used by various microorganisms to reduce selenium, the regulatory elements involved in these pathways, and the optimal conditions for Se reduction by microbes in the natural environment need further study in order to optimize Se reduction by microbes in the field. Our studies have indicated that there may be different metabolic pathways used by Se reducing microorganisms that reduce selenium to conserve energy versus those that reduce selenium to lessen the toxicity. The project, described in more detail in Chapters 5-7, was undertaken to provide a better understanding of microbial Se reduction processes. The information is useful towards the development of a strategy for Se remediation that encompasses all Se reduction metabolic processes likely to occur *in situ*. The objective of this research was to identify and characterize the microbial selenate reduction mechanisms within novel Se respiring organisms. Our efforts will further our understanding of Se cycling in the environment, and should lead to the development of

methods to remove toxic forms of Se from the environment or prevent their release into the environment.

Planetary Protection Studies

In the Fall of 2009 I had the opportunity to identify and characterize microorganisms isolated from pre-launch spacecraft. I worked with Dr. James N. Benardini, in the Planetary Protection division at Jet Propulsion Laboratory (JPL) in Pasadena, CA. The pre-launch isolates were collected from spacecraft residing in JPL spacecraft assembly facilities. JPL personnel had isolated and archived a number of microorganisms collected from various spacecraft over the decades and were uncertain what should be done with the microorganisms given that little information about the microbes had been collected before they were stored. It was unclear exactly what was in the archive or the relevance of the archive to JPL, NASA and the scientific community. A plan was initiated to begin identification and characterization of the microorganisms in the archive beginning with the isolates collected from the Mars Scientific Laboratory (MSL) rover, Curiosity. A research proposal submitted in response to a call for research initiation grant proposals through the Idaho Space Grant Consortium was funded in March 2010, and work began on the MSL isolates in the summer of 2010.

There is ongoing discussion regarding the potential for contamination of Mars by microorganisms transported from Earth on spacecraft destined for the Mars surface. Furthermore, the unresolved issue of forward contamination of Mars or other solar bodies by Earth microorganisms carried on spacecraft is of concern in our search for extraterrestrial life. Despite the precautions taken to decrease the risk of microbial contamination of other worlds by the arrival of future spacecraft, microbes have been isolated from various clean room facilities used during mission preparation (LaDuc 2004; Kempf 2005; LaDuc 2007). For example, JPL has collected approximately 350 microbial isolates from the MSL spacecraft that launched in 2011 and arrived on Mars on August 5, 2012 at 10:31 pm. The 350 microbes isolated from the MSL, none of which had been identified or characterized as to their potential to survive and/or grow on Mars, managed to survive the ‘cleaning’ of the

spacecraft through various testing phases in preparation for launch. Since the microbes persisted on the spacecraft through the preparatory phases, there is a risk that some of the microbes could contaminate Mars, assuming they can survive and grow on the Martian surface.

One of the primary concerns hindering the assessment of forward contamination of Mars is that there is not enough experimental information accumulated about the survival of Earth microorganisms in a Martian environment. Several *Bacillus* species isolated from clean room facilities have been used to study survival after exposure to Martian environmental conditions because *Bacillus* readily form spores when stressed, and spores are known to be resistant to a multitude of stresses (Setlow 2001; Nicholson 2002; Setlow 2006). Few studies have tested whether non-spore forming microorganisms can withstand Martian environmental conditions. Furthermore, studies characterizing the ability of potential Mars contaminating microbes to utilize available Mars electron acceptors such as iron, sulfur, or perchlorate, are lacking. Characterizing the 'hardiness' of a diverse array of microbes with potential to exploit Mars carbon and energy resources is of utmost importance for determining if forward contamination of Mars is a real possibility. Identification of microorganisms resistant to irradiation, desiccation, and oxidizing environments that can utilize potential Mars energy sources would lead to a better understanding of microorganisms which could live and persist on Mars or present a threat to planetary protection.

The goal was to identify the MSL isolates and to characterize isolates for their potential to grow and survive on Mars. Chapters 3-5 of this dissertation give more detailed information on the project, the results of my studies, and questions that remain to be answered. The results of these studies will provide NASA with critical information for developing protocols to ensure the protection of planetary bodies from contamination with Earth microbes, as well as shed light on the types of microorganisms most likely to survive and/or inhabit Mars.

Conclusion

Although the two research projects discussed above are quite different from one another and will be discussed separately in the dissertation, they are not mutually exclusive. Both studies examine the physiology and biochemistry of microorganisms and their ability to conserve energy by utilizing different electron acceptors and carbon sources. Studies on the MSL isolates will yield information of the organisms' ability to thrive under limited nutrient conditions. Having information on the metabolic pathways used for selenium reduction will advance the total knowledge on the ability of organisms to acquire various reductive pathways and utilize the pathways to their benefit to conserve energy and survive under adverse environmental conditions on Earth. Both studies will allow us to gain information which could be used for bioremememdiation or to understand how organisms may survive and utilize energy sources on Mars.

The information gathered from isolates collected from the MSL imparts a better understanding of the diversity of organisms that persist on spacecraft after repeated bioburden reduction methods are administered. It is unclear how microorganisms resist the bioburden reduction procedures and the mechanisms employed by the microorganisms to survive such treatments. It is also unclear how the human-controlled environment (i.e. a cleanroom atmosphere) affects the evolution of microorganisms found in such environments. My research aims to elucidate the resistance mechanisms employed by these microorganisms, and to identify a wide range of microorganisms with resistance capabilities to extreme conditions such as high salt and pH, dessication and irradiation. Moreover, my research will provide vital information to the scientific community regarding microbial resistance mechanisms, information which can be applied to improving clean room technologies, enhancing water treatment and bioterrorism countermeasures, and can be utilized by multiple government agencies and private companies.

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Chapter 2: Planetary Protection and the Search for Life on Mars and Other Planetary Bodies

Abstract

Planetary protection is governed by the Outer Space Treaty, under the supervision of COSPAR, and includes the practice of protecting planetary bodies from contamination by Earth life. COSPAR ensures that compliance by space faring nations is being met and the voting body sets planetary protection policies. In the US, the NASA Office of Planetary Protection is responsible for complying with COSPAR planetary protection policy and sets NASA adherence and procedural policies, which include in part, the allowable bioburden on spacecraft which is dependent on the category of each mission.

Mars is considered a likely place to look for extraterrestrial life, given its proximity to Earth, the presence of carbon and other essential elements, and the presence of water in some form. Although studies are constantly expanding our knowledge about life in extreme environments, it is still unclear whether organisms from Earth, traveling on Mars-bound spacecraft, can survive and grow in a Martian atmosphere where there is intense radiation, high oxidation potential and extreme desiccation. Knowing if microorganisms from Earth can survive in conditions simulating those on the Martian surface is paramount to addressing the issue of whether microorganisms from Earth, traveling on spacecraft, could potentially pose a risk to future challenging planetary protection missions such as life detection missions.

Extensive research has shown that the potential for forward contamination is very real and precaution must be taken if we are to protect our ability to accurately search for present or past life on Mars or other planetary bodies such as Europa. The current bioburden limits set by COSPAR and NASA focus only on the number of spore forming units on the entire spacecraft and do not address the presence of other microorganisms such as fungi and archaea, many of which are known to survive in some of the most inhospitable environments on Earth. This understanding of diverse life on Earth implies that 1) even though the surface of Mars is very inhospitable to life, it is possible that there may be indigenous

microorganisms on Mars especially in the subsurface where they are more protected, and 2) organisms from Earth, traveling on spacecraft, may have the ability to survive the trip to Mars and thrive on the red planet if introduced to an environment that provides suitable conditions for growth.

Ultimately, a better understanding of the diversity of life on Earth is needed to determine if microbes from Earth pose a true forward contamination risk, or if there is any real possibility that life may exist on Mars. Until such research has been conducted, and culminates in the conclusion that forward contamination is not possible, or that life cannot exist on Mars, we must take precautions to protect the red planet from possible contamination by us. This is of paramount importance both for preserving our ability to conduct accurate research determining if Earth life can survive on Mars, and for potentially preserving any extant life on Mars.

1. Introduction

The search for extraterrestrial life is bolstered by our long-standing quest to determine if we are alone in the Universe. Mars and Europa are two likely candidates to target in the search for extraterrestrial life, since both have carbon, potential energy sources, and water in some form (Malin 2000; Malin 2003; McKay 2004; Roth 2014). The current focus to search for life on Mars is supported by the fact that although Mars is quite cold and dry, current conditions are thought to be analogous to conditions on early Earth when single-celled life was gaining a foothold (Rasmussen 2009). Furthermore, because there is a diversity of microorganisms known to thrive in the most inhospitable habitats on Earth, it is not unreasonable to think that microorganisms could live on Mars. Thus there exists a real possibility that microorganisms from Earth transported to Mars on spacecraft may be able to live in the harsh Mars environment.

While continuing Mars explorations confirm that all of the basic necessities for microbial life are present, it remains unclear whether microorganisms that are metabolically capable of living on Mars can actually survive in the Mars environment. The Mars surface presents a

very inhospitable habitat for life because of the intense radiation, highly oxidizing conditions, concentrated evaporative salts, and extremely low water activity. Determining if microbes can survive those surface conditions is vital to discussions regarding the potential contamination of Mars by microorganisms transported on spacecraft launched from Earth. Microorganisms have been isolated from various clean room facilities used during mission preparations, despite the precautions that were taken to reduce the microbial load (LaDuc 2004; Kempf 2005; LaDuc 2007b).

One of the primary issues hindering assessment of the likelihood of the forward contamination of Mars is that there is not enough experimental information accumulated about the survival of Earth microorganisms in the Mars environment. Several studies have reported on the survival of spore-forming *Bacillus* species isolated from clean room facilities after exposure of microbes to specific Mars-like environmental conditions (Setlow 2001; Nicholson 2002; Setlow 2006), but recent culture independent analyses show that spore formers are not the only types of microorganisms present in assembly and clean room facilities (LaDuc 2007; Moissl 2008). Other studies have focused on testing survival of extremophilic microbes that have the metabolic capabilities to inhabit Mars (La Duc 2007; Morozova 2007; Nicholson 2012) though those microorganisms have not been detected on spacecraft. Few studies have been done using non-extremophilic, non-spore forming microbes to address survival of cells exposed to Mars-like environmental conditions (Berry 2010; La Duc 2007b; Osman 2008), and only a handful of studies have used isolates collected directly from Mars-bound spacecraft hardware. Furthermore, studies are lacking that investigate the ability of potential Mars contaminating microbes, such as isolates collected during mission preparations, to utilize electron acceptors such as iron, sulfur, or perchlorate. Determining the tolerance to radiation (both ionizing and non-ionizing), desiccation, and oxidizing environments of Mars-bound microorganisms that utilize the carbon and energy resources available on Mars is of utmost importance for determining whether the forward contamination of Mars is plausible. Results from such studies could provide NASA with data on the types and traits of microorganisms being launched into space, which will ultimately be of value for developing protocols to ensure the protection of

planetary bodies from contamination with Earth microbes in future missions. Therefore, a better understanding is needed of the kinds of microorganisms that persist on spacecraft surfaces through assembly, test, and launch phases, and whether those microorganisms can survive on Mars.

It is a matter of great concern that our search for current or past life on Mars or other planets and moons may be hindered if Mars is or was inadvertently colonized by Earth microorganisms carried to the surface on spacecraft. Although bioburden reduction measures are applied to Mars-bound spacecraft at various stages of preparation, the fact remains that microbial contamination of spacecraft persists. Because of the potential for contamination, our efforts have been curtailed to search for life in pristine areas on Mars at sites that undoubtedly hold the greatest promise for containing evidence of life, like areas where methane plumes exist. Currently there is not enough experimental data accumulated about the microorganisms that survive the bioreduction treatments applied to spacecraft to determine whether they could survive on Mars or other planetary bodies and interfere with future life detection missions. One of the primary outcomes from such studies would be an appreciation of the microorganisms that survive spacecraft pre-flight preparations. Development of a genetic inventory ("passenger list") of microbes on launch(ed) spacecraft would assist space agencies to develop/validate innovative technologies and cleaning protocols to ensure planetary protection specification values are met (NASA NPR 8020.12C appendix B), and would be of value during the implementation of challenging planetary protection missions (ie. *in-situ* life detection; exploration of Mars special regions; Mars Sample Return missions; missions to Europa, Enceladus). The information gained could also be shared with international space agencies, which may accelerate or enhance our ability to search for past or present life on Mars or elsewhere in the Universe.

This review will focus on forward contamination risks and the search for life on planetary bodies. Some information is available on the survival of spore-forming microbes in a Mars-like environment, but much more information is needed regarding survival potential of different types of microorganisms that may be transported on spacecraft, including those that

were collected from spacecraft that were launched in past missions. Without a clear understanding of the potential for Earth microorganisms to colonize other solar bodies, we cannot fully understand or predict the hazards of human space exploration.

2. Planetary Protection

2.1 Overview

Planetary protection is regulated by the United Nations Outer Space Treaty which was signed by space faring nations in 1967 (UN 1967). The International Congress of Scientific Unions, which is part of the U.N., formed the Committee on Space Research (COSPAR) to guarantee that nations adhered to the Outer Space Treaty. COSPAR ensures that compliance by space faring nations is met, and the voting body sets planetary protection policies. In the U.S., the NASA Office of Planetary Protection is responsible for implementing COSPAR planetary protection policy and sets NASA adherence and procedural policies for the NASA centers to ensure that the NASA centers are complying with the set policies for each mission. NASA centers are also responsible for reporting to the Planetary Protection Officer (PPO) on mission categories, activities and compliance efforts. Ultimately, the NASA PPO is responsible for reporting to COSPAR on their activities and compliance with COSPAR policies and COSPAR reports back to the UN on all outer space activities from all space faring nation. An overview of the organization of planetary protection can be seen in figure 2.1.

The NASA Office of Planetary Protection can request special studies on the current research in the field from the Nation Research Council (NRC) of the National Academy of Sciences, which may alter or affect current planetary protection policies. The NRC will then report back to NASA's PPO with a report on the latest research and recommendations. The NASA PPO may then choose to alter NASA's PP policy based on the findings of the report, but often funding restrictions may prohibit the ability of the PPO to make the changes suggested by the PPO.

2.2 History

Early space exploration efforts brought concern by the scientific community that exploration might result in the contamination of other planetary bodies and moons. The National Academy of Sciences, in 1957, considered the possible harmful effects of contamination by space exploration and brought these concerns and their recommendations to the International Congress of Scientific Unions (ICSU) in February 1958. In October of the same year, the ICSU formed COSPAR, the Committee on Space Research. COSPAR was responsible for overseeing all aspects of space research including what has come to be known as Planetary Protection (NASA 1973). The preamble of the COSPAR Planetary Protection Policy states:

> "States Parties to the Treaty shall pursue studies of outer space, including the Moon and their celestial bodies, and conduct exploration of them so as to avoid their harmful contamination and also adverse changes in the environment of the Earth resulting from the introduction of extraterrestrial matter, and where necessary, shall adopt appropriate measures for this purpose" (UN 1967).

COSPAR is responsible for maintaining and promulgating planetary protection policies on an international level. The intent is to prevent contamination of planetary bodies, including Earth, and to ensure State's compliance with the UN Space Treaty also known as the Outer Space Treaty (COSPAR 2002). The COSPAR planetary protection panel develops, maintains and promulgates planetary protection knowledge, policy and plans through symposia, workshops, and topical meetings at COSPAR assemblies. The panel then informs the U.N. Committee on the Peaceful Uses of Outer Space (COPUOS) regarding the policy consensus in the area of planetary protection (COSPAR https://194.199.174.76/scientific-structure/ppp).

At the COSPAR assemblies, policy changes are voted on by those in attendance at the assembly. The last revision to the COSPAR Planetary Protection Policy occurred at the 34th COSPAR assembly held in Houston, Texas in October 2002, and the revised policy is still in place at the time of this writing. The policy extended its recommendations to include that

COSPAR members should provide information to COSPAR, within 6 months post launch and again in 1 year, of the procedures and computations used for planetary protection for each flight and information about the target areas which may have been subjected to contamination. This recommendation was added to already existing COSPAR policies (COSPAR https://194.199.174.76/scientific-structure/ppp). Additional COSAR Planetary Protection Policy is discussed below.

2.3 COSPAR Policy and Categories

The current COSPAR policy states:

> " The conduct of scientific investigations of possible extraterrestrial life forms, precursors, and remnants shall not be jeopardized, In addition, the Earth must be protected from the potential hazard posed by extraterrestrial matter carried by spacecraft returning from an interplanetary mission. Therefore, for certain space mission/target planet combinations, controls on contamination shall be imposed in accordance with issuances implementing this policy" (COSPAR 2002).

COSPAR then lists five categories based on the mission type and target body of the mission and these categories are based on the best scientific information available at the time. An overview of each category can be found in Table 2.1 and the full COSPAR Planetary Protection Policy is found at https://194.199.174.76/sites/default/files/pppolicy.pdf.

Category I includes missions to target bodies which are not of interest for determining the origin of life or chemical evolution. This category does not require any planetary protection efforts to be put into place. Missions that may be listed under category I include those which do not involve landing at any site, such as a flyby or orbiter, or landers with a target of an undifferentiated, metamorphosed asteroid.

Category II includes all missions where there is significant interest concerning the origin of life and chemical evolution, and there is a likely chance that if contamination were to occur it could compromise investigations in these areas. Under Category II missions, COSPAR

policy requires preparation of a planetary protection plan that discusses impact targets, impact strategies, and a Post-encounter and End-of-Mission Report which would provide information and location of the impact if it occurred. Such missions could include flybys and orbiters but also includes landers to sites such as Venus, Jupiter, Saturn, Uranus, Neptune, the Moon, comets, and carbonaceous chondrite asteroids. Additional sites include the dwarf planets Pluto and Ceres, the moons Ganymede, Callisto, Titan, Triton, and Charon, and Kuiper-belt objects.

Category III is comprised mainly of flyby and orbiter missions to a target body where there is a higher likelihood that contamination could compromise investigations into chemical evolution or the origin of life. Target sites which fall under category III missions include flybys and orbiters being sent to Mars, Europa and Enceladus. These missions require more documentation than that of category II missions in addition to inventory of bulk organics, trajectory biasing, cleanroom assembly and bioburden reduction as deemed necessary. Bulk organics includes an inventory of all organic material on a spacecraft which are present in a total mass >1 kg and includes all organics that may be released into the environment of a protected body. Trajectory biasing has been defined by COSPAR as the probability of impact being less than or equal to $1x10^{-4}$ for a time period of 50 years post launch. Additionally, COSPAR has set a guideline that the likelihood that contamination of a protected body will occur, must be less than $1x10^{-3}$ over a 50 year period once a spacecraft has arrived at its target.

Category IV missions require more detailed documentation than category III missions, plus an enumeration of bioburden, analysis to determine probability of contamination, analysis of bulk organics, and an increase in implementation procedures. These implementation procedures would include a requirement for cleanroom assembly, bioburden reduction including sterilization of direct contact hardware and a bioshield for said hardware, and trajectory biasing in addition to the requirements laid out under category II. Missions falling under category IV would include landers, probes, and possibly some orbiters with a target site of Mars, Europa, and Enceladus. Historically, the Viking Landers, Mars Science

Laboratory, and the Mars Exploration Rovers belong to this category. Category IV missions are further subdivided into 3 additional categories for Mars missions and will be discussed in more detail below.

Category V missions are comprised of all missions where there would be a return back to Earth where protection of the Earth and the Moon from contamination would be required. An example of a category V mission is the proposed Mars Sample Return Mission which involves caching samples collected from Mars for return back to Earth for examination. Some return missions would be exempt such as those deemed to contain no indigenous life. Such missions would only have to adhere to the planetary protection requirements under categories I and II and would include missions to such places as the Moon and Venus. All other missions, including those to Mars and Europa, would adhere to the strictest levels of containment including absolute prohibition of a crash impact upon return to Earth, containment throughout the whole return phase of exposed hardware that had contact with the original target body, and absolute containment of any samples were returned as would be the case in a Mars Sample Return Mission. Strict containment of samples would likely require a Biosafety Level 4 facility (BSL-4) unless the sample was sterilized prior to handling. Additionally, COSPAR states that, if during a category V mission, there is a change in the mission classification or a mission failure and safe return of the sample cannot be assured, or the sample containment has been compromised and sterilization is impossible, then the sample must be abandoned. Given these criteria, additional safeguards would have to be put into place that would allow for abandonment of a sample or the entire spacecraft prior to reentry of Earth.

Besides the categories listed above, COSPAR has additional guidelines and category specifications for a handful of target bodies including Mars, Europa, and small solar system bodies. For all category III, IV, and V missions to Mars there are specific bioburden constraints as delineated by the category of the mission. The bioburden on a spacecraft is defined as the number of aerobic microorganisms that survive heat shock at 80°C for 15 minutes (ie. spores) when grown on tryptic soy agar (TSA) at 32°C for 72 hours. Mars

orbiters in category III missions do not have to meet lifetime requirements so long as their entire surface, mated and encapsulated bioburden levels are $\leq 5 \times 10^5$ spores.

Category IV missions are further divided into in 3 subcategories, IVa, IVb and IVc. Category IVa missions include landers that are not on life detection missions. These missions must contain a surface bioburden level on average of ≤ 300 spores per m^2 and a surface bioburden level of $\leq 3 \times 10^5$ spores. Category IVb landers are life detection missions and either 1) the entire lander is restricted to ≤ 30 spores or to levels based on the nature and sensitivity of a specific life detection system, or 2) the systems involved in the acquisition, delivery and analysis of samples must be sterilized to these levels to prevent recontamination of the subsystem or material being analyzed in its place. Category IVc missions are missions to Mars that are involved in the designation of special regions regardless as to whether the mission is classified as a life detection mission. If a mission falls under this category then it must meet all of the requirements of a IVa mission along with these additional requirements: 1) the entire landed system must have a bioburden level ≤ 30 spores, or 2) the subsystems in direct contact with the special region must be sterilized to ≤ 3 spores and a method must be enacted to prevent recontamination prior to accessing the special region.

In the case of Mars, COSPAR has specifically defined a special region as a region where terrestrial organisms are likely to replicate and there is high potential for the existence of martian forms. These regions are further defined as places where there is sufficient water activity (0.5-1.0) and there has been sufficient warmth ($\leq$ -25°C) within a period of 500 years. COSPAR has also listed a series of geological features which are considered special regions, such as gullies, pasted-on terrains, the subsurface >5 m, possible geothermic sites, fresh craters with hydrothermal activity, modern outflow channels and sites of recent seismic activity. Further information regarding special regions on Mars will be discussed later in this review.

Additional category requirements for missions to Europa have been outlined by COSPAR. For category III and IV missions to Europa, bioburden reduction must occur to a level where

the probability of inadvertent contamination of an europan ocean is $< 1 \times 10^{-4}$ per mission. The probability is calculated in part on the total bioburden at launch, the survival of contaminating organisms including survival in a radiation environment, the probability of landing on Europa, mechanisms and timescales of transport to the europan subsurface, and the survival and proliferation of a microorganism before, during and after subsurface transfer. Bioburden reduction will likely have to occur on orbiters as well as landers and spacecraft assembly will require the use of cleanroom technologies, cleaning of parts prior to assembly, and bioburden monitoring of spacecraft and assembly facilities. Furthermore, survival of extremeophiles from Earth in an europan environment would need to be considered to ensure eradication of organisms most likely to survive in such a hostile environment.

2.4 NASA Planetary Protection Policy

Each space agency has their own policies although they are required to adhere to the COSPAR Planetary Protection Policy at minimum. The NASA document NPD 8020.7G states "It is NASA's policy to comply with planetary protection provisions in support of U.S. obligations under the 1967 Outer Space Treaty" (NASA 1999). The policy further states:

"The conduct of scientific investigations of possible extraterrestrial life forms, precursors, and remnants must not be jeopardized. In addition, the Earth must be protected from the potential hazard posed by extraterrestrial matter carried by spacecraft returning from another planet or other extraterrestrial sources. Therefore, for certain space-mission/target-planet combinations, controls on organic and biological contamination carried by spacecraft shall be imposed in accordance with directives implementing this policy."

The NASA policy outlines the responsibilities of the associate administrator, or designee, of the Science Mission Directorate and the Planetary Protection Officer to administer NASA'a planetary protection policy. The responsibilities of the Planetary Protection Officer include in part: 1) that all measures have been taken to assure that NASA policy, regulatory agencies, Office of the General Council and office of External Relations objectives and policies are met, and 2) conducts reviews, inspections and evaluations of plans, facilities, equipment,

personnel procedures and practices of NASA and its contractors to carry out directives. Program managers are responsible for meeting the biological and organic contamination control requirements, implementing documents as required during research development, test, preflight, and operation activities, and for allowing the Planetary Protection Officer to conduct reviews, inspections, and evaluation in accordance with the directive. The directive also states that specific constraints for spacecraft are dependent on the nature of the mission and the target body. The specific requirements are outlined in NASA document NPR 8020.12. It further states that NASA must take into account current scientific knowledge and recommendations from advisory groups including the Space Studies Board of the National Academy of Sciences (NAS). A NAS review titled "Preventing the Forward Contamination of Mars" was published in 2006 and will be discussed later in this review. Document NPR 8020.12 also defines requirements for robotic planetary flight programs for fulfilling the requirements set forth in NPD 8020.7. The document addresses controlling both forward and reverse microbial contamination. The specific planetary protection requirements for a mission are determined by the NASA Planetary Protection Officer (PPO). The project manager or principal investigator of the mission submits a request to the PPO for the mission categorization during mission design. The PPO will then confirm the appropriate planetary protection categorization and may add supplemental conditions as deemed necessary for all NASA Missions.

If the mission is a non-NASA mission in which NASA is participating, then the planetary protection compliance is the responsibility of the lead agency. However, if NASA is responsible for the launch of the mission then the lead agency must show documentation that they have complied with planetary protection requirements consistent with the US's obligations as put forth in the 1967 Outer Space Treaty. If NASA is not responsible for the launch of the spacecraft, but is providing resources such as hardware, data, or funding, then the NASA supported organization must submit a planetary protection plan to NASA's PPO.

General NASA planetary protection requirements state that the probability that a planetary body will be contaminated during biological exploration must be no more that $1x10^{-3}$ and the

period of biological exploration must extend at least 50 years after a category III or IV mission arrives at its target. Furthermore, for all launch vehicles leaving Earth's orbit the probability of impacting Mars must be less than $1x10^{-4}$ over 50 years. However, if a spacecraft is crossing Mars en route to other target bodies then the probability of impact is increased to $1x10^{-2}$ over 50 years. These probabilities set forth by NASA are on par with those outlined by COSPAR.

2.5 NASA Requirements for Mars Missions

NASA document NPR 8020.12D lists requirements specific for Mars missions. Category III and IV missions require that an assessment of the probability of impact covering the first 50 years post launch must be provided with cruise stages, flybys and orbiters. An impact with Mars must be avoided within a probability ≥ 0.99 for 20 years post launch and ≥ 0.95 for 20-50 years post launch. If the spacecraft cannot adhere to the impact probabilities required, then they are limited to having no more than 5×10^5 spore forming units (sfu) on the entire spacecraft which includes counts on all exposed, mated and encapsulated surfaces. Landers that are not classified as life detection missions must have no more than 3×10^5 sfu on the entire spacecraft and must average no more than 300 sfu per m^2 of exposed spacecraft surfaces. Landers categorized as life detection missions must meet even stricter requirements with a bioburden of no more than 30 spores on the entire spacecraft or the subsystems involved in sample handling must be sterilized to these levels. Missions to special regions have additional requirements. Specifically, missions to these regions must have a bioburden limit ≤ 30 spores on the entire spacecraft and there must be a protocol in place to prevent recontamination of the spacecraft before it enters the special region.

As stated earlier, bioburden has been defined by NASA as the "number of aerobic microorganisms that survive a heat shock of 353° Kelvin (80° C) for 15 minutes and are cultured on Trypticase Soy Agar at 305° Kelvin (32° C) for 72 hours". All organisms meeting these requirements are considered by NASA to be spores. Given NASA's definition of bioburden, it does not take into account other organisms such as non spore forming microbes, archaea or fungi. This is of concern as studies are showing that a number of

organisms associated with spacecraft are non-spore formers that have managed to survive the heat shock process.

2.6 NASA Requirements for Icy Satellite Missions

NASA has outlined additional requirements for Category II, III and IV missions to icy satellites for all flybys, orbiters and landers. NASA states in NPR 8020.12D that the probability of contamination of an ocean or other liquid water body must be less than 1×10^{-4} per mission. The calculated probability must take into consideration the bioburden at launch, cruise survival for contaminating organisms, survival of organisms to the radiation adjacent to the target body, mechanisms and timescales of transport of organisms to the subsurface and the chance of organism survival and proliferation before, during and after subsurface transfer. Many of these additional considerations may be hard to calculate without detailed studies of the organisms that are inhabiting the spacecraft prior to launch. Such studies would require the identification and of organisms and their limit of survivability before a launch could take place.

2.7 NASA Requirements for Small Solar System Bodies

Most of the missions to small solar system bodies currently falls under planetary protection categories I and II and do not require controls on forward contamination unless otherwise warranted by a change in mission type (NASA 2011). NASA document NPR 8020.12D currently requires additional considerations only if the mission is classified as a Category V with a sample return to Earth. Given the current limit of missions to small solar system bodies, it is likely that future exploration of the solar system, along with increased technologies to explore such bodies, may require changes to NASA's policies in this area in the future.

2.8 Role of the National Research Council

As previously mentioned, NASA's PPO can request a special independent study from the National Research Council (NRC) to provide the PPO with the latest research pertaining to Planetary Protection. Additionally, the NRC's report provides the PPO with

recommendations for improvement of planetary protection policy. The latest planetary protection report from the NRC was published in 2006 and addressed prevention of the forward contamination of Mars (NRC 2006). This report will be discussed in further detail throughout this review. Once the NRC has completed the study and released the report to the PPO, the PPO will meet with the planetary protection subcommittee, and vote on what recommendations from the NRC report should be adopted and incorporated into current policy via policy changes.

3. The Martian Environment

Since the research reported later in this dissertation has been performed on microbes collected from spacecraft destined exclusively for Mars, I will focus mostly on the Martian environment. Although Mars is considered to be at the outer edge of the habitable zone of our solar system, the idea that there could potentially be life on Mars, especially in the subsurface, is not unfathomable. Although it can be expected that different areas of Mars would have somewhat different environments dependent on location, overall the Martian environment is quite inhospitable to most life as we know it. Average temperatures on Mars can range from -10° C to -76° C with an average surface temperature of -65˚C although temperatures can fluctuate from as high as 25˚C to -123˚C (Schofield 1997, Horneck 2008, Crawford 2008).

Mars is considered to be quite dry, but recent information suggests otherwise. Studies of the Gale Crater by the rover Curiosity found hydration of soils to be as much as 2.25 wt%. This finding was consistent with findings by both Viking 1 and 2 and the Mars Odyssey (Meslin 2013). What is unknown is if there is an underground source of water. Geophysical and geochemical features on Mars indicate that there may have been water on the surface at some time in the past but it is unknown to what extent surface water would have existed. Features include alluvial fans in craters, dendritic valley networks, and the presence of specific minerals thought to only form in the presence of water. One hypothesis is that hydrothermal environments associated with craters from impacts and volcanism could have easily provided a source of liquid water on Mars (Westall 2013).

The Martian atmosphere is much different from that on Earth. Mars has primarily a CO_2 atmosphere (95.3%) compared to the CO_2 content in Earth's atmosphere (0.03%). Earth's atmosphere consists mainly of N_2 (78.1%) while there is only 2.7% N_2 in the Martian atmosphere. The O_2 concentration on Earth is 20.9% whereas Mars' atmosphere contains only about 0.1% O_2 (Horneck 2008). Studies by Mumma et.al (2009) showed the presence of methane in extended plumes that appeared to be released from discrete regions on Mars. One of the principal plumes contained as much as 19,000 metric tons of methane, an amount comparable to that of a massive hydrocarbon seep in Santa Barbara, California. However, analyses by the Mars rover Curiosity found no detectable atmospheric methane. Although results are contradictory, it is possible that the location of the rover was too far from the methane seeps and prevented the detection of methane in the atmosphere.

The surface of Mars is subjected to both cosmic ionizing radiation and solar UV radiation. Ionizing radiation on Mars is believed to be 100X higher than on Earth, ranging from 100-200 mSv/a compared to Earth's 1-2mSv/a (Horneck 2008). The UV-B and UV-C fluxes on Mars are nearly 5X higher than they are on Earth with fluences of 361 kJ/m^2 and 78 kJ/m^2 respectively (Cockell 2000). On Mars, the high atmospheric concentration of CO_2 neutralizes incoming UV radiation < 200nm, however wavelengths >200nm still reach the Martian surface (Horneck 2008). Of note is that some of the UV radiation may be attenuated at times by the presence of dust storms in a particular region.

Data collected during the Viking missions showed that the surface of Mars was highly oxidized compared to the Mars atmosphere (Slesak 2012). Mapping of hydrogen peroxide (H_2O_2) on Mars using infrared high-resolution imaging spectroscopy indicated H_2O_2 abundance on Mars is 15± 10 ppb although prior mapping showed concentrations as high as 40 ppb (Encrenaz 2008). The formation of peroxides could occur in the presence of hematite, trace amounts of water, and UV radiation (Horneck 2008). A more likely scenario is that radiolysis of ice or water would create a larger amount of peroxide formation. It has been reported that the surface ice of Europa contains as much as 1,300,000 ppb H_2O_2 which is

generated from radiolysis of ice (Johnson 2003). Additionally, perchlorate, a strong oxidizing agent, was found by the Phoenix Lander to be present in Martian soils in concentrations of 2.1-2.6 mM (Hecht 2009).

Martian soils contain few nutrients to support life as we know it, and the soils themselves pose a harsh environment. Martian soils were expected to be acidic, but the Phoenix Lander showed that the soils at its landing site were mildly basic with a pH of 7.7 ± 0.5 (Hecht 2009). Salt tolerance would be required for life to survive and grow on Mars due to the high salt concentrations found in Martian soils in the form of NaCl, $MgSO_4$, $CaSO_4$, $FeSO_4$, MgCl and $CaCl_2$ (Crisler 2012). The lack of water, the intense radiation and oxidative conditions make the Martian surface quite inhospitable to life.

4. Special Regions on Mars

4.1 Introduction

Mars Special Regions are regions where organisms are likely to survive. NPR 8020.12D defines these areas as regions that have a high potential for the existence of extant Martian life forms, have sufficient water activity (0.5-1.0 aw) and have sufficiently warm temperatures (-25° C lower limit) to permit replication of Earth organisms. Areas that have observed features that may be associated with the presence of water must also be classified as Special Regions. It is noted that these parameters may need to be changed as our understanding of Mars and life on Earth evolve and as our technological capabilities improve (NRC 2006, Kminek 2010).

4.2 Formulating Special Regions

The COSPAR colloquium on special regions stated that “Preventing terrestrial biological contamination from becoming established and widespread on Mars is essential to our ability to protect high-priority science goals on Mars” (Kminek 2010). The current standards are based solely on protecting science goals and not on protecting Mars in of itself. The NRC study takes a precautionary principle approach by stating that there is insufficient data to determine which regions of Mars should be considered “special” and that all of Mars should

be considered “special” until it can be proven otherwise (Kminek 2010, NRC 2006). The COSPAR disagreed and concluded that there was sufficient data to arrive at a conclusion as to which areas of Mars would be defined as “Special Regions” (Kminek 2010).

The COSPAR colloquium concluded with the enactment of the standards that are currently in NPR 8020.12D. Two main standards, water activity and temperature, are the basis for determining which regions should be taken into consideration. One area in need of additional research is that of microbial growth and reproduction at low temperatures. It was noted that most of the work in this area has been performed on laboratory isolates and more environmental data is needed to begin to define the lower temperature for life. It was concluded that investigations were needed to determine if microbial reproduction at water activities of lower than 0.6 is possible, that more studies are needed using Mars simulated environments, and that knowledge of reproduction of communities rather than isolates is essential to improve our understanding of life. COSPAR also noted that a larger phylogenetically diverse array of organisms needs to be studied and diurnal, seasonal and long-term variations in the Martian surface need to be better understood (Kminek 2010).

The NASA PPO made some initial suggestions to try to define special regions. The parameters were set as: 1) the existence of liquid water in “pure” form or in strong brines up to 5.5M $CaCl_2$; 2) regions of current or active volcanism or enhanced heat flow which is yet unknown; 3) permafrost through 100% water ice, including segregated ground ice, ice-rich frozen ground, polar caps and subsurface ice; 4) subpermafrost groundwater; and 5) any gully system that may be indicative of recent water activity within the last <50,000 years. The Special Regions Science Analysis Group (SRSAG) determined that regions should be defined as non-special if the temperature remains below -20° C or the water activity remains below 0.5 for a period of 100 years after spacecraft arrival (Rummel 2009). Ultimately, the SRSAG developed a map of regions that are considered “significant” and of interest for determining special region boundaries (Figure 2.2). Of note, is that the current definition of special regions mostly takes into account the known and sets the water activity and temperature parameters slightly below what is currently known.

Cockell and Horneck (2006) argue that protections for planetary bodies should extend beyond preserving just the scientific value of a region and should take into account ethical reasons for preservation. They suggest the creation of "Planetary Parks" similar to designated Wilderness Areas on Earth. They state that 29 of the 30 arguments used to protect land on Earth can be applied to protecting planetary bodies. These arguments include land as a source of new species, ecosystems that provide vital functions, land provides biological transformations necessary for survival, disease sequestration and that organisms are a part of a global biological system and should be preserved. An additional 22 arguments do not depend upon life but are equally important for conservation. One major point they make is that land has its own value and its value as an object can exist independently of the human value placed on it. They also argue that even if a land is not used for ourselves it should be reserved for future generations. Cockell and Horneck conclude that application of an environmental ethic to other planetary bodies allows for preservation through a single system, and incorporates both "utilitarian and intrinsic value arguments". They also suggest that formulation of a planetary parks system could encourage commercial exploration outside non-park areas (Cockell and Horneck 2006).

4.3 Planetary Protection Requirements and Considerations

Any missions to these special regions must have a bioburden limit ≤ 30 spores on the entire spacecraft (NASA NPR 8020.12D). To date, the closest space faring agencies have come to sterilizing a spacecraft was the use of dry heat with the Viking 1 and 2 landers. Sterilization of Viking landers occurred by heating the spacecraft at 112°C until 110°C was reached at the coldest point (such as in deep crevices) which was obtained in approximately 23-30 hours (DeVincenzi 1998). It was estimated that dry heat sterilization would reduce the number of spores by an order of 4 magnitudes thus leaving a total bioburden on each spacecraft well below the ≤ 30 spores threshold. Bacterial spore numbers on the Viking 1 and 2 landers prior to dry heat sterilization was determined to be $1.6 \times 10^2/m^2$ and $9.7 \times 10^1/m^2$ respectively (Puleo 1977). For Curiosity, the total bioburden (sfu) on the spacecraft was determined to be 2.78×10^5 prior to launch (private communication). If dry heat sterilization was used on the

MSL one would expect the total bioburden level post sterilization to be approximately 28 spore forming units, just below the level required to enter special regions.

There are certain considerations that must be taken into account when depending on heat sterilization of a spacecraft to reduce bioburden to an acceptable level for special regions. In the case of the Viking Landers, each lander was encapsulated in a bioshield before heating, thus the total bioburden remaining on the spacecraft was based on prior experiments to estimate that a 4 magnitude reduction would be obtained using this method (DeVincenzi 1996). The bioburden was not actually verified post heat sterilization. Second, it has been estimated that 1:1000 spores at any given time are able to withstand extreme environments and bioburden reduction methods. Given this, it may be possible that the spores remaining on the spacecraft prior to heat sterilization are the "super-spores" and may not be as labile to the heat treatment as standard spores (Horneck 2007). Third, one must also consider that these bioburden levels are only preserved up until the time of launch thus recontamination of the spacecraft would have to be avoided to keep the bioburden at these reduced levels. Last, the bioburden counts on spacecraft only take into consideration organisms that form spores and can grow on TSA at 30° C within 72 hours. There is no experimental data to indicate the effectiveness of the heat sterilization processes on non-spore forming organisms, archaea, fungi or other organisms that were not detected by showing growth on TSA at 30°C. Thus it is quite likely that the bioburden on the entire spacecraft, even after heat sterilization, may be much higher than anticipated.

5. The Relationship between Planetary Protection, Life on Earth, and the Potential for Life on Mars

5.1 Introduction

So far this review article has mostly focused on planetary protection and the environment on Mars, but ultimately neither would be of concern without the existence of microorganisms. The entire planetary protection requirements, in regard to forward contamination, are focused purely on decreasing the number of spore forming units on spacecraft. This focus is based on the notion that spore forming bacteria are the hardiest of microorganisms and are most likely

to survive a trip to Mars and potentially flourish in the harsh Martian environment. The fact that spores are dormant and can stay that way for an extended period of time makes them of utmost concern. For example, a 1995 report described the revival and identification of bacterial spores retrieved from the abdominal contents of extinct bees preserved in 25-40 million year old amber (Cano 1995). However, as I will discuss below, many non-spore forming organisms have been identified as surviving in extreme environments.

Special regions on Mars have been defined as regions where there is sufficient water activity (0.5-1.0 aw) and sufficiently warm temperatures (-25° C lower limit). As we will see, this is a very narrow definition for determining the absolute requirements of life. There have been assumptions made that the harsh UV and ionizing environment on the surface of Mars would likely kill any organisms remaining on the spacecraft as it enters Mars' thin atmosphere. Furthermore, the focus of current planetary protection requirements excludes consideration of other microorganisms that may be on spacecraft such as fungi, archaea, or viruses. As our knowledge expands about microorganisms and the extremes that they can withstand, it is quite likely that the planetary protection requirements will need to be modified to prevent forward contamination of Mars. It is estimated that we have only identified and studied 1% of all microorganisms that are in existence, which leaves a huge gap in our knowledge of the limits of life and our own microbial communities.

5.2 Life in Extreme Environments

Despite our limited knowledge of microbes on Earth, everywhere we have looked for microbes they have been found. It appears that life inhabits all places on Earth including some of the most extreme environments imaginable. Microorganisms have been discovered surviving and reproducing in hot springs, at terrestrial depths exceeding 2 km, in the most arid of deserts, and in hydrothermal vents on the ocean floor. Microbial life has been found in extremely cold places such as in Antarctica and Greenland, and microorganisms have been described as reproducing and thriving at temperatures as low as -15°C. Many microorganisms can grow in salt at concentrations exceeding 20% NaCl, or 2M $MgSO_4$, and others thrive in either very acidic or very alkaline environments. Microbes can conserve

energy by respiring some of the most extreme compounds, such as U, Mn, Se, As, S and Cl based molecules. Life at either high or low pressures has been described, as well as organisms that are highly resistant to radiation and oxidative conditions. Most of the organisms surviving in these types of environments have a symbiotic relationship with other organisms in the same community. For example, methane oxidizing archaea (MOA) are known to live in symbiosis with sulfate reducing bacteria (SRB) in deep hydrothermal vents on the ocean floor. MOAs break down methane to CO_2 and H_2, and the H_2 is then utilized by the SRBs to reduce SO_4^{-2} to HS^- (Valentine 2000). These types of relationships between organisms are far from uncommon.

Earth microorganisms have developed physiological and biochemical mechanisms to be able to survive in a variety of extreme niches. As previously stated, it would not be unreasonable to expect niches on Mars, although considered extreme, to support microbial life of some sort as well. In the remainder of this review I will discuss what is known about how microorganism survive some of these extreme environmental conditions and how this information is relative to the search for life on Mars and the potential risk to planetary protections. Although this portion of the review will focus on bacteria, it should always be kept in mind that many of the topics discussed apply to archaea and fungi as well.

5.3 Survival to Heat

The ability of bacteria to survive heat exposure is of upmost importance for 2 reasons: 1) Dry heat is the standard used by NASA and was used on the Viking missions to sterilize spacecraft for life detection missions, and 2) Organisms must survive during entry into the Martian atmosphere if they have a chance to thrive on Mars. In regard to the latter, heat shields are placed on spacecraft to protect the spacecraft from the heat exposure upon entry into the Martian atmosphere. The heat shield on the MSL was designed to withstand up to 2760° C to protect the spacecraft and instruments on board (http://www.nasa.gov/centers/ames/ research/msl_ heatshield.html). Even with the heat shield it could be expected that the spacecraft may heat up to a more tolerable temperature

that would not necessarily be detrimental to microbes that may be on the surface of the spacecraft.

The biggest concern in regard to microbial survival of heat is the ability of microbes to withstand heat sterilization. As previously discussed, any missions to special regions or to detect life must have a bioburden limit ≤ 30 spores on the entire spacecraft, and dry heat sterilization of the spacecraft is the standard for meeting these bioburden limits (NASA NPR 8020.12D). It is estimated that the total bioburden level on the MSL post sterilization would be approximately 28 spore forming units if sterilization had occurred, which is just below the level required to enter special regions or perform life detection missions. It has been estimated that 1 in 1000 spores can withstand extreme environments and bioburden reduction methods thus any spores remaining on the spacecraft prior to heat sterilization may be the "super-spores" and may not be as labile to the heat treatment as standard spores (private communication).

Based on the dry heat sterilization method used for the Viking Landers, microbes would need to survive dry heat exposure >112°C for 30 hours. Research by Lovely and Kashefi describe an Fe(III) reducing microorganism that is able to grow at temperatures ranging from 85-121°C which is well above the temperature used for the Viking landers (Kashefi 2003). Though the organism is an archaean, it emphasizes that the sole focus on spore forming units in regard to planetary protection is misguided. Additionally, other studies have identified microorganisms within marine thermophilic and acidophilic communities which can also survive temperatures up to 120° C (Pikuta 2007). Studies by Kempf *(*2008) looked at the lethality rate constants and D-values (time required to kill 90% of the population at a given temperature) of *Bacillus atrophaeus* spores exposed to dry heat. The results showed that the lethality rate using dry heat was faster than that of ambient room humidity (36-66% Rh) at temperatures of 115 and 125°C. However, at higher temperatures (150-170°C) the ambient humidity constant rate was actually faster than that of dry heat (Kempf 2008). Thus it would not be unreasonable to expect the existence of some microorganisms on spacecraft after dry heat sterilization. Since the bioburden levels on spacecraft are focused purely on culturable

spore forming units, it is yet unknown if there is potential for other microorganisms to remain on the spacecraft post dry heat sterilization. and posing a risk to planetary protection.

Research has shown that the killing of spores via dry heat occurs in part through DNA damage of the spore. The dry heat results in DNA damage through direct damage to the molecule as well as mutations occurring in the DNA. It has also been noted that spores from thermophilic spore formers are no more resistant than spores originating from mesophilic organisms. DNA protection appears to be dependent on the presence of α/β small acid soluble proteins (SASP) which appear to play a direct role in dry heat resistance. Spores lacking these small acid soluble proteins are more susceptible to dry heat sterilization and show an increase in DNA damage (Nicholson 2000). It has been suggested that the SASPs act by protecting against depurination of the DNA (Setlow 1995). Given this data, it might be more beneficial to determine survival to dry heat depending on the SASP concentration of a given population rather than studies focusing purely on a small, controlled population.

5.4 Survival at Low Temperatures

Average temperatures on Mars can range from -10° C to -76° C with an average surface temperature of -65˚C although temperatures can fluctuate from as high as 25˚C to as low as -123˚C (Schofield 1997, Horneck 2008, Crawford 2008). For an organism to be able to thrive on Mars it would need to be able to grow and reproduce in these frigid temperatures. An exception would be a subsurface environment that was geothermally heated though no such areas have been discovered on Mars. Regardless, an organism being transferred from Earth to A number of psychrophilic (cold-loving) organisms have been isolated from many regions of the Arctic and Antarctic where there are polar ice sheets, glaciers and permafrost. Additionally, microorganisms are known to inhabit the ocean floor where temperatures are ≤ 4° C (Pikuta 2007). These organisms are comprised of representatives from the Eukarya (algae, fungi and yeast), Bacteria, and Archaea. Morozova *et al.* (2007) identified several methanogenic archaea that were able to survive not only low temperatures ranging from -75° to 20° C, but could also simultaneously survive low humidity and a 95.3% CO_2 atmosphere. The methanogens that survived best under these conditions were isolated from permafrost.

Six isolates from permafrost and 9 known species of *Carnobacterium* were found to grow not only at 23° C but also at 0°C and under low pressure and a CO_2 enriched anoxic atmosphere (Nicholson 2013)A strain of *Serratia liquefaciens*, a common mesophilic organism often found as a contaminant in bathtubs, was shown to be capable of growth at 0°C as well as at low pressure and CO_2 enriched anoxic atmospheres (Schuerger 2013). Mykytczuk et al. (2013) identified a *Planococcus* isolate that grows and divided at -15° C and is still metabolically active at -25°C.

Despite these organisms being interesting in themselves, what is even more interesting is the ability of these organisms to make both physiological and biochemical modifications to survive in such environments. *Psychrobacter arcticus* 273-4, a bacterium capable of growing at temperatures as low as -10° C, was found to down regulate genes related to energy metabolism and carbon incorporation, and up regulate genes required for maintenance of membranes, cell walls, and nucleic acid motion. Furthermore, this organism turns on the expression of a cold shock DEAD-box RNA helicase A, a protein that may be key for maintaining life in cold temperatures (Kuhn 2012). *Planococcus halocryophilus* Or1 grew at subzero temperatures by forming encrustations around the cell and increasing the ratio of saturated to branched fatty acids in the cytoplasmic membrane (Mykytczuk 2013). This is unique because often growth at lower temperatures results in a higher content of unsaturated, polyunsaturated, and methyl-branched fatty acids to increase membrane fluidity at these temperatures. In many organisms, enzymes involved in transcription, translation, protein folding and stabilization of DNA and RNA show activity at very low temperatures and are adapted to life in cold environments. Antifreeze-like proteins have been seen in Antarctic lake proteins and trelahose and exopolysaccharides might also provide cryoprotection for psychrophiles (D'Amico 2006). Although scientists are far from having a full understanding at life in cold-temperatures, studies like the ones above provide insights as to how these organisms adapt to their extreme environment. Additionally, the microbes are models to further our understanding of how organisms may survive on Mars, and can be useful as we continue the search for life on cold planets and moons. Though it is unlikely that true psychrophilic organisms would be present on a Mars-bound spacecraft since true

psychrophiles would die at room temperature where the spacecraft is assembled, the studies above demonstrate that it could be possible to have psychrotolerant organisms survive the temperatures of the cleanroom yet still survive the frigid climate in space and on Mars.

5.5 Tolerance to High Salt

Due to the high salt concentrations found in Martian soils in the form of NaCl, $MgSO_4$, $CaSO_4$, $FeSO_4$, MgCl and $CaCl_2$, salt tolerance would be required for life to survive and grow on Mars (Crisler 2012). Salts can be chaotropic as they influence water activity, affect cell turgor, and are major stressors of cellular systems (Hallsworth 2007). It is estimated that $1/4^{th}$ of the Earth's land is covered by salt and salt water makes up the majority of Earth's water. On Mars, it is estimated that sulfurous salts are more common than chlorinated salts by a ratio of 3:1. On Earth the most common type of salt is NaCl but many brines also contain $MgCl_2$, $MgSO_4$ and other salts (Crisler 2012; Hallsworth 2007). Studying hypersaline environments from Earth increases our understanding of how organisms can adapt to these extreme environments.

Since *Bacillus* sp. are known to reside on Mars-bound spacecraft, they are of special interest in regard to growth under high salt conditions. Previous studies in our laboratory have shown that many different species of *Bacillus*, including *pumilus, licheniformis, horti, mannailyticus* and *cellulosilyticus,* as well as species belonging to other genera including *Paenibacillus, Amphibacillus* and *Alkalibacterium,* could grow under salt concentrations as high as 10% NaCl. Several of these organisms also showed growth in media containing 20% NaCl. These isolates were collected from the Alvord Basin in Oregon where the soils are known to have elevated salt concentrations (Smith 2009). The ability of *Bacillus* sp. to grow under these conditions is not uncommon and many organisms which have been identified as non-spore formers can also grow in high NaCl concentrations.

A diversity of prokaryotes was discovered residing in deep hypersaline anoxic basins in the Mediterranean Sea, basins that are nearly saturated with $MgCl_2$ (5M). In addition to growing in extremely high concentrations of $MgCl_2$, the microorganisms were involved in sulfate

reduction and methanogenesis, and contributed to the cycling of carbon (Van der Wielen 2005). Furthermore, overall microbial community was unique because the bacteria and archaea identified were not related to organisms normally found in seawater, and the archaea branched deeply within the Euryarchaeota indicating they comprised a new order.

It is estimated that the majority of salt on Mars would likely be $MgSO_4$, with lower concentrations of NaCl and $CaCl_2$. Studies by Crisler *et al.* (2012) focused on the growth of microorganisms under high $MgSO_4$ concentrations using microorganisms collected from the Great Salt Plains in Oklahoma. Though the microbes were not identified, it was found that 35% of the organisms from the bacterial collection could grow in medium containing 2M $MgSO_4$ and at least 80% could grow in the presence of 10% $MgSO_4$ (Crisler 2012). , Studies using the MSL isolates testing growth under high $MgSO_4$ concentrations showed that a large percentage of the organisms from the MSL were able to grow in media containing 1M or 2M $MgSO_4$ (Smith, unpublished).

Although scientists are still learning more about how life survives in these extreme, high salt environments, we do know that the cell must make have special psyiological and biochemical properties to survive such environments. The primary factors for surviving these conditions are the amount of energy generated during dissimilatory metabolism and the mode of osmotic adaption utilized (Oren 2011). A review of studies from 1999 concluded that aerobic respiration, denitrification, and both oxygenic and anoxygenic photosynthesis can occur under the highest salt concentrations but autotrophic oxidation of ammonia and nitrate, some forms of methanogenesis and sulfate reduction were never found at salt concentrations > 100-200 gl^{-1} (Oren 2011). Processes identified as occurring, albeit poorly, at salt concentrations >200 gl^{-1} included fermentation, aerobic autotrophic oxidation of sulfur compounds, sulfate reduction by incomplete oxidizers and some other forms of methanogenesis.

Oren hypothesized based on his findings that life at high salt concentrations is energetically expensive, and the upper salt concentration limit at which dissimilatory processes occur is

determined partly by bioenergetics constraints. Given this, the main factors that determine whether a certain type of organism can make a living at high salt concentrations arethe amount of energy gained during its dissimilatory metabolism and the mode of osmotic adaptation used. Based on his review of halophiles, Oren stated that the energy cost costs associated with salt exclusion and pumping ions out was unfavorable and that the "salt-in" strategy was energetically favored. Given this the following types of metabolism most likely to occur under high salt concentrations are: i.) Those that use light as the energy source, ii.) Aerobic respiration, denitrification, and other highly exergonic dissimilatroy processes coupled with large production of ATP, and iii.) Types of metabolism performed by organisms that use the "salt-in" strategy even when the amount of ATP obtained in their dissimilatory processes is low (Oren 2011).Oren hypothesizes that the salt-in option would be energetically favorable to organisms, and it is clear that organisms have made adaptations to their molecules to thrive under high salt conditions and allow for the "salt-in" option. Studies by Tehei et al. (2002) identified a malate dehydrogenase and tRNA molecules, from the archaeon *Haloarcula marismortui*, that are protected in the presence of high salt. The salt protected the tRNA molecules from thermal degredation while the malate dehydrogenase was protected from thermal denaturation. While studying the lipid composition of *Halobacillus halophilus*, Lopalco *et al.* (2013) found that the organism increased the number of shorter chains and incorporated unsaturated chains in the lipid core structures. It was believed that these changes compensated for an increase in phospholipid packing and rigidity, and sulfoglycolipid polar heads. It is believed that these changes allowed for homeostasis of membrane fluidity and permeability under high salt stress conditions.

Although many more studies need to be conducted to have a full understanding of how organisms from Earth survive these high salt environments, these studies do show that life under these conditions is possible and even, in some cases, protective. Given this, it would not be unreasonable to think that such microorganisms would be able to thrive on Mars in the salty Martian soils. Oren includes organisms using light as the energy source, however this would be unlikely on Mars since organisms on living on this planet would also have to survive other conditions on the surface such as desiccation, and high radiation (to be

discussed later). It is more likely that organisms on Mars would utilize exergonic dissimilatroy processes or utilize types of metabolism which allowed for the "salt-in" strategy (Oren 2011).

5.6 Tolerance to pH Extremes

The ability of organisms to withstand alkaline pH is a factor to consider when discussing both life on Mars and planetary protection efforts. Initially, it was thought that the Martian soil was likely to be acidic but results by the Phoenix Lander showed that the soils at that site were mildly basic with a pH of 7.7 ± 0.5 (Hecht 2009). Although the pH at the Phoenix Lander study site was only slightly basic, it is possible that other soils on Mars are more basic. Regarding planetary protection, it has been hypothesized that organisms on Mars bound spacecraft may be resistant to the alkaline cleaning agents often used in the cleanroom environment and on spacecraft hardware. The hypothesis stems from the fact that the majority of the isolates collected from the MSL grow in alkaline media.

Alkaliphiles are organisms which grow above neutral pH whereas extreme alkaliphiles generally grow in the pH range of 10.0-14.0. Studies on alkaliphilic organisms have mostly focused on *Bacillus* sp. with the most extensive studies having been performed on *B. halodurans* and *B. pseudofirmus* (Krulwich 2011). The biggest hurdle facing alkaliphilic organisms is the ability to maintain homeostasis and maintain chemiosmosis. Alkaliphiles use transporters to help catalyze proton transport and these transporters include proton-pumping respiration chains, proton-coupled ATPases, and secondary active transporters. Often the uptake of protons is unequal where $2H^+$ are exchanged for one Na^+ ion. Studies have shown that even in extreme alkiliphiles, the pH remains relatively neutral to slightly alkaline in the cytoplasm even though the surrounding medium might be extremely alkaline. There is still much to be learned but it is clear that organisms have easily adapted to alkaline environments thus it would not be difficult for Earth organisms to grow in Martian soils or potentially survive exposure to the cleaning agents used in the spacecraft assembly facility.

5.7 Surviving Desiccation

Surviving desiccation is absolutely necessary if a microorganism can be considered a potential contaminant on Mars. First, the organisms residing on the spacecraft must be able to endure months to years in a cleanroom environment and be able to survive the 9 month trip to Mars. Second, the organisms must be able to survive the desiccating environment on Mars until they can come into contact with a water source suitable for growth. Only after transfer of the organisms to the Martian surface and finding suitable water activity, such as a polar ice cap or potential subsurface water sources, could the organisms then potentially become active. These steps must occur before an organism could be considered a real threat to causing forward contamination of Mars.

As previously discussed, Mars is considered to be quite dry, and soils contain only 2.25 wt% water (Meslin 2013). However, this analysis was performed on soils on the Mars surface so we do not know what the soil water content is at deeper depths. It is not known if there is a source of subsurface water, but geographical features of Mars indicate that there may have been water on the surface at some time in the past. It is not unreasonable to think that the water would have seeped into the subsurface and may still be present to some degree. Additionally, hydrothermal environments on Mars associated with craters from impacts and volcanism could have easily provided a source of liquid water, and crater impacts generating water are a potential concern today (Westall 2013). It may be possible for an organism from Earth to be transferred to the desiccating surface of Mars, remain dormant for an extended period of time, then flourish after a wind storm has transferred the organism to a water source or water flows from a crater impact .

Several studies have shown that desiccation resistance in microorganisms is far from rare, and not only includes spore-forming microorganisms such as *Bacillus*, but non-spore-forming organisms such as *Moraxella* and *Staphylococcus* as well (Kubota 2012; Chaibenjawong 2011). Overall, dehydration of cells leads to severe cell damage by causing structural changes to lipid membranes and proteins, cross linking and polymerization of DNA molecules, inhibiting or altering enzyme activity, changing membrane permeability,

and altering or mutating genetic information. DNA in the cell is at most risk to the desiccating environment since loss of water can lead to partial DNA denaturation (Horneck 2010). Spore-forming organisms such as species belonging to the genera *Bacillus* and *Clostridium* are more likely to resist desiccation as the spore coat provides protection against a desiccating environment. The water content of spores is reduced to 25-45% of the cell's wet weight causing proteins to become immobile and ceasing enzymatic activity altogether (Horneck 2010). However, the overall resistance of the spore to the desiccating environment is mostly due to protection of the dehydrated core by the cortex and spore coat layers while the DNA is protected by small proteins which protect the DNA from chemical and enzymatic reactivity (Horneck 2010).

Many non-spore-forming organisms have been shown to be resistant to desiccation. Studies by LaDuc *et al.* (2007) identified several isolates of *Pseudoaltermonas*, *Psychrobacte*r, and *Acinetobacter* that survived a 7 day incubation at an Rh of 18 ± 3%. Several *Moraxella* sp. have been shown to survive a 30° C incubation for 35 days under dry conditions (Kubota 2012). *Staphylococcus aureus* can survive on dry plastic surfaces for more than 1097 days, more than enough time to survive time spent in a spacecraft assembly facility and a 9 month trip to Mars (Chaibenjawong 2011). The methanogens, *Methanobacterium wolfeii*, *Methanosarcina barkeri*, and *Methanobacterium formicicum* survived desiccation for 90-120 day incubation periods (Kral 2013). Studies on Amazonian Oxbow Lake sediments showed that desiccation for 1 year at 4° C not only increased the overall abundance of *Methanocellales* and *Methanosarcinaceae*, but also increased the rates of CH_4 production after rewetting (Conrad 2013).

Alhough it is clear that the spore coat protects spore-forming organisms from a desiccating environment, it is relatively unclear how non-spore-formers survive similar environments. Studies by Goffau *et al.* (2011) have shown that cells can maintain intracellular water activity above that in their environment as long as the microbes can generate more water metabolically than is lost to the environment. However, this would require that the organisms were metabolically active which would be questionable under most desiccating environments

such as the case of *Staphylococcus aureus* residing on a dry plastic surface where there would be little to no nutrients (Chaibenjawong 2011). Studies by Chaibenjawong and Foster (2011) showed that the mutants *clpX*, *sigB*, and *yjbH* were required for desiccation resistance in *Staphylococcus auerus*. *ClpX* and *yjbH* are both important for protein turnover while *sigB* plays a role in overall stress resistance (Chaibenjawong 2011). It is likely that there are several factors involved in the desiccation resistance of non-spore- forming organisms but more studies on these unique organisms will need to be performed before we have a comprehensive understanding of these systems.

5.8 Exposure to an Oxidative Environment

Data from the Viking missions showed that the surface of Mars was highly oxidized compared to its atmosphere (Slesak 2012). Additional studies of Mars have shown that H_2O_2 abundance can range from 15± 10 ppb to 40 ppb (Encrenaz 2008). The formation of peroxides can occur in the presence of hematite, trace amounts of water, and UV radiation, and radiolysis of ice or water can create even larger amounts of peroxide formation approaching 1300 ppm as seen on Europa (Johnson 2003, Horneck 2008). For an organism to survive on Mars it would need to have mechanisms to protective itself from this oxidizing environment. However, the ability of organisms to survive H_2O_2 exposure on Mars pales in comparison what an organism would be potentially exposed to in a spacecraft assembly facility. H_2O_2 is currently being considered as a possible sterilant for spacecraft hardware since it can be used on spacecraft materials, components and electronics without causing the potential damage that is generated by heat sterilization used during the Viking missions era (Kempf 2005).

A number of microbes collected directly from spacecraft assembly facilities or the spacecraft itself are highly resistant to 5% H_2O_2 (1.5M H_2O_2). An isolate of *Acinetobacter radioresistens*, collected from the Mars Odyssey spacecraft, showed only a 2 log reduction after exposure to 100mM H_2O_2. Even after exposure to 320mM H_2O_2 there was still incomplete killing of all of the microbes (McCoy 2012). Studies by Kempf *et al*. (2005) have shown recurrent isolation of H_2O_2 resistant *Bacillus pumilus* from the JPL spacecraft

assembly facility. Both vegetative cells and spores of these isolates survived exposure to 5% H_2O_2. Spores were less susceptible to killing showing only a 1-5 log reduction compared to vegetative cells which experienced a 5-8 log reduction. The examples just mentioned are far from a comprehensive list of organisms that have resistance to H_2O_2, but they demonstrate that organisms are able to withstand these types of exposures.

There have been numerous attempts to try to understand how microorganisms protect themselves from H_2O_2 exposure. Most of these studies have been performed in *Bacillus* species although there is some knowledge overall about how bacteria cope with this stress. Three well studied mechanisms are the peroxide responsive regulators OxyR, PerR and OhrR that also act as transcription regulators. OxyR and PerR are mainly involved in the detection of H_2O_2 whereas OhrR is involved in the sensing of organic peroxides and sodium hypochlorite. When exposed to peroxides, specific cysteine residues on OxyR and OhrR and histidine residues on PerR are oxidized by an Fe-catalyzed reaction. These transcriptional regulators are not only involved in H_2O_2 sensing, but also serve in the formation of biofilms, host immune response evasion and antibiotic resistance (Dubbs 2012).

Beyond general sensing of H_2O_2, genes involved in protein protection, such as groES, dnaK and clp tend to be upregulated thus also serving to protect the cell (Mols 2011). These proteins may be important for stabilizing the enzymes involved in the actual conversion of H_2O_2 to water and O_2, including catalases, peroxiredoxins, and peroxidases. (Gioia 2007). Studies in *Bacillus subtilus* have identified σ^B-dependent stress genes that are also involved in resistance to oxidative stress. Ultimately, the work performed by Reder *et al.* (2012) identified 47 general stress response genes that were required for survival to superoxide, 6 genes required for protection from H_2O_2 stress and 9 genes that were required to protect against both.

Studies of the highly resistant strain, *Bacillus pumilus* SAFR-032, collected from JPL's spacecraft assembly facility, have identified many genes involved in H_2O_2 resistance overall (Gioia 2007). Checinska *et al.* (2013) looked further into the role of two manganese catalase

proteins in the SAFR-032 spore coat, YjqC and BPUM_1305, which had been previously identified by others. It was concluded that the synergistic activity of YjqC and BPUM_1305, along with other coat oxidoreductases, contributes to the increased resistance of SAFR-032 to H_2O_2 over other *Bacillus pumilus* strains. Although this work has improved our knowledge on the resistance of SAFR-032 to H_2O_2 it is most likely that there are many other factors involved in the resistance of this strain beyond the maganese catalases.

Our understanding of how organisms survive H_2O_2 is expanding but there is still much that needs to be learned before H_2O_2 can be effectively used as a spacecraft sterilant. Currently, the use of 5% H_2O_2 would likely work well to reduce the overall bioburden on spacecraft components to meet general bioburden requirements for non-life detection missions. However, it is very unlikely that using H_2O_2 as a sterilant to meet the requirements of <30 spores on the entire spacecraft for life detection missions or exploration of special regions would be more rigorous than the heat sterilization methods used currently without increasing the concentration of H_2O_2 used or somehow eliminating microbial resistance to H_2O_2.

5.9 Exposure to Radiation

The ability of an organism to survive radiation is paramount if the organism is to survive near the surface of Mars and pose a planetary protection threat. The radiation exposure on Mars is much more intense than it is on Earth because Mars lacks a magnetic field to deflect incoming charged particles and the atmosphere is <1% that of Earth (Hassler 2013). There are 2 major types of radiation to be concerned with both on route to Mars and on Mars. The first type of radiation, Galactic Cosmic Rays (GCR), originates outside of our solar system and is formed from events such as supernovas. The second type of radiation, Solar Cosmic Radiation (SCR), originates from the sun and consists of both a constant flow of radiation as well as brief bursts (Horneck 2010, Hassler 2013). In the past, the overall radiation level on Mars has been based solely on calculations and modeling. New studies using data collected from the MSL found that the radiation in flight to Mars is approximately two times higher than the radiation on the surface of Mars (0.21 mGy/day vs. 0.48 mGy/day). The lower radiation level on Mars surface is due in part to some atmospheric shielding by the Martian

atmosphere, which is not provided to the spacecraft en route, and because radiation from GCR is modulated by SCR(Hassler 2013).,

SCR can consist of both ionizing (e.g. gamma radiation) and non-ionizing radiation (eg. UV radiation). This section of the review will focus mostly on UV radiation since that has been the focus of the majority of previous studies. It is of note that ionizing radiation can be of more concern since it can penetrate through the Martian soils thus potentially making the first meter of soil inhabitable (Hassler 2013). Solar UV radiation is divided into 3 spectral ranges; UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (200-280 nm). UV-B and UV-C radiation are of the most concern since DNA has high absorption at those wavelengths and can be mutated leading to cellular inactivation (Horneck 2010). Radiation of biological cells can cause breaks in molecular bonds including single and double strand breaks in DNA and photolysis of amino acids (Dartnell 2010). Calculations have suggested that DNA weighted irradiance on the Martian surface would be 3 orders of magnitude greater than that on Earth meaning that microbes would need to be resistant to much higher levels of UV radiation to sustain life on the surface of Mars (Rontó 2003).

Most of the research on radiation resistance and/or survival of microorganisms have been performed on spore-forming organisms since they are of the most interest to planetary protection and tend to be hardy due to their protective spore coat. Studies by Wassman *et al.* (2012) exposed *Bacillus subtilis* spores to low Earth orbit and simulated Martian conditions for 559 days aboard the ESA's EXPOSE-E facility. Although results showed that there was 100% survival of *Bacillus subtilis* MW01 spores to simulated Martian conditions (UV $\lambda \geq$ 200 nm), only a $\leq$ 8% of spores survived low Earth orbit conditions (UV $\lambda \geq$ 110nm). Studies on *Bacillus pumilus* spores showed 10-40% viability on the EXPOSE facility versus a survival rate of 85-100% under dark simulated Martian atmospheric conditions. However, when the same studies were performed on the super tolerant *Bacillus pumilus* SAFR-032 strain, a 7 log reduction in viability was observed (Vaishampayan 2012). Overall SAFR-032 spores showing UVC resistance remain viable even after exposures up to 2000 J/m^2 (Link 2004). Comparative proteomic studies showed that superoxide dismutase was present in

higher concentrations in the space exposed isolates and exhibited higher UV-C resistance than the ground control counterparts (Vaishampayan 2012). Tauscher *et al* (2006) studied the effects of *Bacillus subtilis* spores exposed to simulated Mars solar radiation for an equivalent of 42 minutes of Mars solar radiation. Radiation exposure reduced spore viability by 3 logs but measure of germination metabolism was only reduced by < 1 log. They concluded that the spores can retain the ability to initiate germination associated metabolic processes and produce viable signature molecules despite being rendered nonviable.

It has been estimated that spores are 10-50 times more resistant than growing cells to UV radiation at 254 nm. This is due to a difference in the UV photochemistry of the DNA as well as error-free repair of any photoproducts formed by the UV light. Instead of forming thymine dimers as a photoproduct, spores tend to form thymine adducts instead; furthermore, small acid soluable proteins (SASPs) appear to suppress cyclobutane pyrimidine dimers (Nicholson 2000). Relative to gamma radiation, spores are significantly more resistant due to the decreased levels of water in the spore coat compared to vegetative cells which may reduce the amount of hydroxyl radicals formed overall (Moeller 2014). SASPs do not appear to play a role in γ-radiation resistance (Nicholson 2000).

Many non-spore forming organisms have also been identified as being UV resistant. Studies by Montero-Calasanz *et. al.* (2013) identified an isolate of *Geodermatophilus tzadiensis* that showed resistance to UV light at 254 nm. A highly radiation resistant isolate from the *Moraxella-Acinetobacter* group showed increased survival after a repeated exposure to UV light. Ultimately, this isolate was able to withstand a UV dose of 5940 J/m^2 with a 48% survival rate (Keller 1982). Antarctic Dry Valley bacteria closely related to *Brevundimonas, Rhodococcus,* and *Pseudomonas,* all showed resistance to γ-radiation. Surprisingly, these organisms, along with *Deinococcus radiodurans*, all showed increased resistance to γ-radiation when irradiated at -79° C (Dartnell 2010).

Although the ability of non-spore forming organisms to survive radiation appears to be poorly understood, there are some studies which have given clues to how these organisms

survive. Keller *et. al*. showed that the UV light resistance mechanism for survival was not associated with increased mutagenesis when the *Moraxella-Acinetobacter* isolate was repeatedly exposed to UV (Keller 1982). Studies on several strains of *Staphylococcus aureus* showed that UV-C resistance increased as the organisms entered into stationary growth phase, a characteristic that was attributed in part to the expression of σB during this phase (Gayán 2013). Exposure of the lipids and proteins of *Acinetobacter* sp. PT511.2G and *Pseudomonas* sp. NT511.2B to ultraviolet radiation caused an increase of methyl groups that were associated with lipids, causing lipid oxidation, and alterations in lipid composition in addition to changes in propionylation, glycosylation, and/or phosphorylation of cell proteins (Santos 2013). The authors concluded that these changes may account for differences in UV sensitivity.

Ultimately, there are many microorganisms, both spore forming and non-spore-forming, that are able to survive exposure to radiation and could potentially survive on Mars and the trip to Mars. For example, *Deinococcus radiodurans* would only be eradicated from the top several meters of Martian soil after a period of a few million years based on the radiation that currently reaches Mars. However, if the organism were to start growing again, then the clock would start over, and organisms could continue to stay dormant and survive up through today. This has implications not only for the potential for life to exist on Mars, but the impact of potential life that has been brought to Mars already on past missions.

5.10 Conservation of Energy

Unlike Earth, the Martian environment provides very little nutrients to sustain life. Any microbes that either survived transit to Mars, or were already on Mars, would have to make a living using the limited nutrients that are available. As previously discussed, Mars has a mostly CO_2 atmosphere (95.3%) with low amounts of N_2 (2.7%) and O_2 (0.1%) (Horneck 2008). However, studies by Mumma et.al (2009) have shown the presence of methane in extended plumes that appeared to be released from discrete regions containing as much as 19,000 metric tons of methane. Additionally, previous studies have shown high amounts of salts including $MgSO_4$, and $FeSO_4$ (Crisler 2012). Two of the most abundant compounds on

Mars are Fe and S and there is evidence that there are large concentrations of sulfur in the Martian regolith (Schulze-Makuch 2008). Perchlorate, a strong oxidizing agent, was shown by the Phoenix Lander to be present in Martian soils in concentrations of 2.1-2.6 mM (Hecht 2009). All of these compounds are potential chemical energy sources that can be used by microorganisms to survive.

The large methane plumes on Mars are of unknown origin. These plumes seasonally fluctuate but the amount of methane produced is on par with methane plumes on Earth that are known to be of biotic origin. Although the Mars rover Curiosity has found no detectable atmospheric methane, it is possible that the location of the rover prevented the detection of methane in the atmosphere since these methane plumes have been seen at polar regions rather than mid-latitude regions. Methanogenesis has become a well know method for microorganisms to conserve energy. Many archaea, such as *Methanosarcina*, can use various carbon compounds to produce methane (Smith 1978). H_2 could be readily be oxidized with the large amounts of CO_2 in the atmosphere to generate energy via methane production (Kral 2004). Once this methane is available, it could be oxidized by methanotrophic archaea in the presence of sulfate reducing bacteria to complete a methane cycle which would support at least 3 types of organisms (Caldwell 2008). An overview of the reaction might look something like this:

$2H_2 + CO_2 \rightarrow CH_4 + O_2$ (Methanogenic Archaea)

$2CH_4 + 2H_2O \rightarrow CH_3COOH + 4H_2$ (Methane Oxidizing Archaea)

$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$ (Sulfate Reducing Bacteria)

The electron donor H_2, could easily be generated by photochemical dissociation of water (Krasnopolsky 2001) and it has already been determined that there are large amounts of sulfate, especially in the form of $MgSO_4$, and $FeSO_4$, in the Martian soils (Schulze-Makuch 2008, Crisler 2012).

More likely energy sources fairly abundant in near surface soils on Mars are inorganics

such as iron or sulfur (Crawford 2008). An electron donor such as H_2 could be used to reduce Fe(III) or sulfate during respiration, with utilization of CO or CO_2 as a source of carbon. Sulfate and iron reduction by organisms on Earth have been very well studied. These organisms play very important roles in the biogeochemical cycling of carbon, nitrogen, sulfur and other metals (Zhou 2011). Studies by Karr et al. (2005) identified a group of sulfate reducing bacteria residing in the permanently frozen freshwater lake, Lake Fryxell, in Antarctica. These organisms are able to utilize the reduction of sulfate to conserve energy under very cold conditions (4° C). There have also been studies showing that Fe respiration under alkaline conditions is possible. Studies by Williamson et al. (2013) identified organisms that could easily reduce Fe(III) at pH 10. These studies show that it is possible for these reactions to occur under cold or alkaline conditions. Once Fe or S has been reduced it is available for oxidation by other organisms.

Perchlorate, detected in soils by the Phoenix Mars Lander, is one of the more interesting potential electron acceptors recently discovered on Mars (Hand 2008; Hecht 2009; Smith 2009). More than 50 microorganisms on Earth are known to respire perchlorate coupled to the oxidation of H_2 or small organic acids, a metabolism that has been intensely studied over the past decade (Coates 1999; Coates 2004). This group of organisms is quite diverse and many have been found in environments that might seem, on the surface, to be inhospitable such as paper mill waste. Studies by Ju *et al.* (2008) bacteria in sludge that were capable of oxidizing both Fe° and S° while reducing perchlorate. The enrichment culture was also able to oxidize S^{2-} and $S_2O_3^{2-}$ to support the reduction of perchlorate, and they also confirmed the disproportionation of S° to S^{2-} and SO_4^{2-}. Thus perchlorate reduction would tie in neatly to both the Fe and S cycles.

Although Mars seems inhospitable and lacks an abundant supply of nutrients, there are plenty of nutrients available to support anaerobic life on the red planet. The studies discussed above show that the organisms could work together to supply nutrients for one another within a complex ecosystem. Additionally, many of the organisms discussed above can survive in

extreme environments on Earth while still making a living as evidenced by many of these processes still taking place at low temperatures or in alkaline environments.

6. Conclusions

This review has shown the complexities of not only planetary protection but the difficulty in determining the potential for life on Mars. There is still much that needs to be learned before any definitive conclusions can be made, but until a definitive conclusion can be reached, space faring nations must proceed with caution if they wish to preserve, at minimum, their ability to conduct accurate science on the red planet. One thing that was not discussed extensively in this review is that many different types of microorganisms have been isolated from both the spacecraft assembly cleanrooms and the spacecraft themselves. JPL currently holds approximately 2500 organisms, collected from spacecraft dating from the Viking mission to the current MSL Curiosity mission, in an archive. We have just begun to examine these organisms in depth and the results of this examination will be discussed in the next 2 chapters. Many of these organisms have been found to not only survive the bioburden reduction methods utilized by NASA, but also show growth under extreme environmental conditions. Many of these conditions are analogous to those found on Mars.

With regard to NASA's planetary protection policies, the number of organisms that are currently considered as a threat is minimal, and does not encompass the entire potential threat. The NASA standard assay quantifies the number of spore forming organisms on a spacecraft that are able to grow on TSB at 30° C within 72 hours. They do not take into account any organisms which may not be able to grow on TSB, prefer higher or lower temperatures, or take longer than 72 hours to grow. Additionally, these numbers of microorganisms do not take into account the high number of non-culturable organisms that are likely to be residing on the spacecraft, nor do they take into account other microorganisms such as archaea and fungi. Archaea are known to survive in some of the most extreme environments on Earth and it is reasonable to think that they might actually prove to be a bigger threat to planetary protection because of their hardiness.

There is still much that needs to be learned about microorganisms and the potential for life on Mars before we can fully understand the implications of the special regions criteria that have been set. The SRSAG developed a map of areas which show boundaries they consider significant on Mars (Figure 2.2) based on the criteria they set for determining special regions. Ultimately, given our limited knowledge of Mars, it is possible that every lander or rover could potentially enter a special region unknowingly. For example, there was controversy over the current Curiosity mission, when the engineers removed a sterile drill bit from its box prior to launch and inserted it into the drill thus exposing it to the cleanroom environment. The planetary protection regulations for that mission required sterilization of any part of Curiosity that would touch the surface of the planet, including the drill bits (Sahagun 2012). Although water or ice was not likely present at the Gale Crater, it is still relatively unknown if the drill bit would come into contact with subsurface water. If it did come into contact with water, then it could potentially create an environment which could cause release of microorganisms from the drill bit into subsurface water thus nullifying planetary protection efforts.

Although the planetary protection requirement for special regions is ≤ 30 spores on the entire spacecraft, the spores remaining after numerous bioburden reduction and sterilization efforts would be the hardiest of spores. The efforts to remove the spores would leave behind the ones that would be most likely to survive an environment such as that found on Mars. Additionally, since the lack of bioburden cannot be confirmed post sterilization, it brings into question how much bioburden is actually remaining on the spacecraft after heat sterilization. To date, heat sterilization is the only approved method applied to spacecraft despite efforts to develop better sterilization techniques. A commonly used analogy in the planetary protection community is the arrival of Europeans and the introduction of smallpox to the indigenous people. If there is life on Mars, it is not unreasonable to think that the hardiest of organisms could easily alter an indigenous population.

Ultimately, we are currently exploring the areas which are least likely to contain life, since a mission to a protected region has yet to occur. Ideally, we would be able to sterilize our

spacecraft with absolute confidence so that we could enter a protected region, where life is most likely to exist, and perform life detection analysis. Arguments within the scientific community waver back and forth from "We have already contaminated Mars" to "There is no way organisms on spacecraft could survive the trip or the environment on Mars". These arguments alone reflect our lack of understanding of organisms from Earth and the absolute limits for life. In order to answer these questions we must have a better understanding of life on Earth.

The idea of protecting Mars just because it is intrinsically worth protecting is an idea which should be further explored as well. Maybe a consensus could be formed, in which certain special regions were open for exploration, and others would remain untouched. This would allow for life detection missions where life is most likely to exist while preserving the remainder of the special regions. Regardless, we must still answer the questions of "What is on our spacecraft?" and "Is there any chance that these organisms can contaminate Mars?". We must also look at organisms beyond just the spore forming units that are currently counted in NASA's standard assay.

The NRC published recommendations to NASA's PPO in 2006 after a request was placed by the PPO to conduct a study on planetary protection of Mars. These recommendations were constructed after an extensive study of literature on microorganisms and the environment on Mars. The NRC's final report included a list of 17 recommendations based on the outcome of their extensive studies. The condensed recommendations listed by the NRC are as follows (NRC 2006):

1. NASA should work with COSPAR and other appropriate agencies to consider whether planetary protection policies for Mars should be extended beyond protecting the science to include protecting the planet.
2. NASA should establish and budget adequately for a coordinated research initative and infrastructure to research, develop and implement improved planetary protection procedures.

3. Future missions to Mars should plan for the effective implementation of planetary protection requirements at the earliest stages of the mission and instrument design and engineers should be provided with a selection of effective, certified tools for bioburden reduction.
4. NASA should establish an independent review panel to consider the latest scientific information about Mars and Earth organisms and recommend to NASA appropriate modifications to the planetary protection implementation requirements. This panel should also prioritize measurements needed on Mars to inform future assessments and modifications to planetary protection requirements.
5. NASA should require the routine collection of phylogenetic data to ensure that the diversity of microbes in and on all NASA spacecraft sent to Mars is reliably assessed. NASA should also require systematic archiving of environmental samples from the assembly, test and launch operations (ATLO) and spacecraft.
6. NASA should sponsor research on those classes of microorganisms most likely to grow in potential Martian environments.
7. NASA should ensure that research is conducted and appropriate models developed to determine the embedded bioburden in spacecraft materials.
8. NASA should sponsor research of bioburden reduction technologies that can be used as alternatives to dry heat sterilization.
9. NASA should sponsor research on non-living contaminants of spacecraft.
10. NASA should assign high priority to defining special and non-special regions.
11. NASA should transition toward a new approach to assessing bioburden on spacecraft including transitioning from the use of spore counts to the use of molecular assay methods
12. Replace categories IVa through IVc to IVn and IVs (Table 2.2).
13. NASA should treat all direct contact missions as Category IV missions until special regions can be defined.
14. NASA should ensure that all Category IV missions meet at least level 2 bioburden reduction requirements (Viking level pre and post- sterilization levels, Table 2.2).

15. NASA should sponsor research on how to implement level 3, 4 and 5, bioburden reduction requirements in practical ways (Table 2.2).
16. Any missions to Mars that will access regions where it is suspected to have long-lived liquid water should satisfy level 4 bioburden reduction requirements (Table 2.2).
17. NASA should take new approaches to prevent contamination of Mars Category III missions.

As previously mentioned, NASA's PPO can choose whether to implement the recommendations of the NRC or disregard them in part or all together. Given the current funding state of NASA, it is very unlikely that these recommendations will be put into place. The costs of the recommended studies, let alone the implementation of additional planetary protection measures, would increase the cost of missions significantly. Such cost incurrences in these financial times would likely result in a.) A significant reduction in the number of planned missions; b.) Major delays in upcoming missions until the new criteria could be researched and implemented; and c.) Loss of the United States leadership in space exploration. Although these recommendations should be implemented, Congress would need to allocate a large influx of funding to NASA in the area of planetary protection before any of these recommendations could be put into place. What has happened instead is that funding allocations to planetary protection have been cut. The microbial archive that has been in place since the Viking missions will no longer be funded after the end of FY 2014 (private communication).

Ultimately, what is concluded is that there is a serious and real potential for the contamination of Mars by Earth organisms traveling on spacecraft. Review of the literature by the NRC confirms that this is a real possibility and one that must be considered until the scientific community has deemed otherwise. This contamination could potentially annihilate an indigenous population of microorganisms on Mars, or at the very least, alter our ability to conduct adequate and accurate research on Mars without potentially altering the scientific data that is collected. The studies show that organisms from Earth have the ability to potentially survive and thrive on Mars. Until more questions are answered it will be difficult

to truly determine the impact of any of our space exploration on potentially indigenous populations on other planets and moons thus a precautionary principle approach should be taken.

In regard to the potential for indigenous populations on other planets and moons, research has shown repeatedly that life can exist in the harshest of environments. Although this was not covered in depth in this review, life has been found in some of the most dry or frigid environments on Earth such as the Atacama Desert or Antarctica. It is not unreasonable to believe that microorganisms, similar to those found on Earth, could be thriving on locations such as Mars or Europa, especially in the subsurface where radiation would be lower and there would be a better chance for the existence of liquid water. While searching for life on other planets and moons, we look for the signs of life that are already known such as the presence of carbon and water. It may be possible that if we find life in these distant places that we may discover new limits to life in extremis.

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Table 2.1. Planetary Protection Requirements by Mission Category. Adapted from COSPAR Planetary Protection Policy (2002).

	Category I	**Category II**	**Category III**	**Category IV**	**Category V**
Mission Type	• Flyby • Orbiter • Lander- ie. asteroid	• Flyby • Orbiters • Landers at specific sites	• Flyby • Orbiters	• Landers • Probes • Some orbiters	• Earth return missions

Target Body	• Bodies where no protection is warranted such as an undifferentiated, metamorphosed asteroid	• Venus, Jupiter, Saturn, Uranus, Neptune
		• The Moon
		• Comets
		• Carbonaceous chondrite asteroids
		• Pluto, Ceres
		• Ganymede, Callisto, Titan, Triton, Charon
		• Kuiper-belt objects
		• TBD
			• Mars
			• Europa
			• Enceladus
			• TBD
				• Mars
				• Europa
				• Enceladus
				• TBD
					• Restricted Earth return from:
					  ○ Mars
					  ○ Europa
					  ○ TBD

Concerns	None	• Calculation of impact probability • Contamination control	• Limiting impact probability • Passive bioburden control	• Limiting impact probability • Active bioburden control	If restricted: • Absolutely no impact on Earth or Moon • Sterilization of returned hardware • Sample containment

Requirements	None	• Planetary Protection Plan • Pre-launch report • Post-launch report • Post-encounter report • End-of-Mission report	Category II documentation plus: • Contamination control • Inventory of bulk organics • Trajectory biasing • Cleanroom usage and assembly • Bioburden reduction	Category II documentation plus: • Probability of contamination analysis plan (P_c calculation) • Microbial reduction and assay plan • Bioburden reduction • Partial sterilization of contacting hardware • Bioshield • Bioburden monitoring • Cleanroom assembly • Organics inventory • Trajectory biasing	*Outbound:* • Same category as target body *Inbound Restricted:* Category II documentation plus: • P_c analysis plan • Microbial reduction and assay plan • Trajectory biasing • Sterile and/or contained hardware • Continuous monitoring • Advanced studies and research *Inbound Unrestricted:* • None

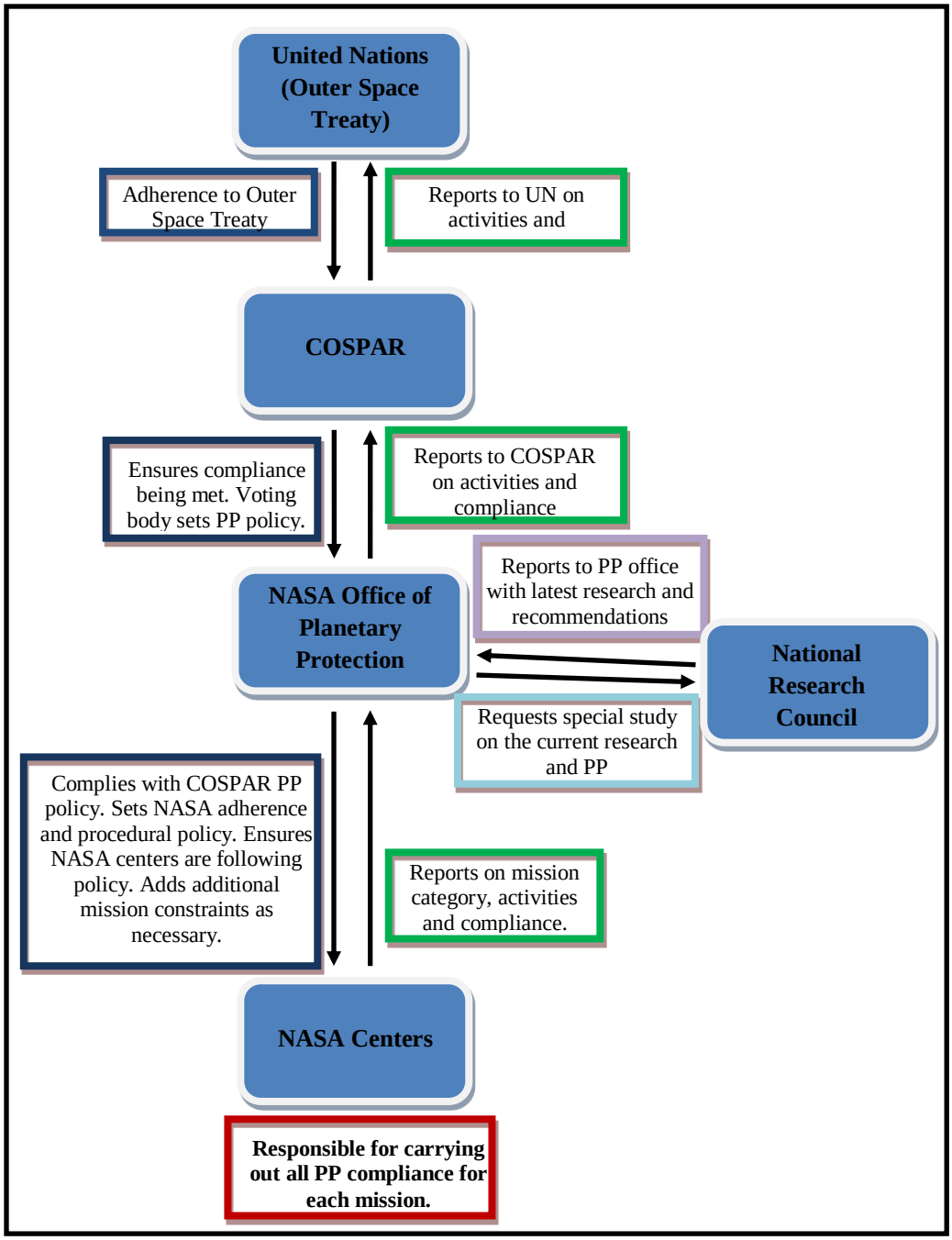

Figure 2.1. Organization of Planetary Protection. The flow chart shows the organization of policy making in planetary protection and an overview of compliance and reporting structure.

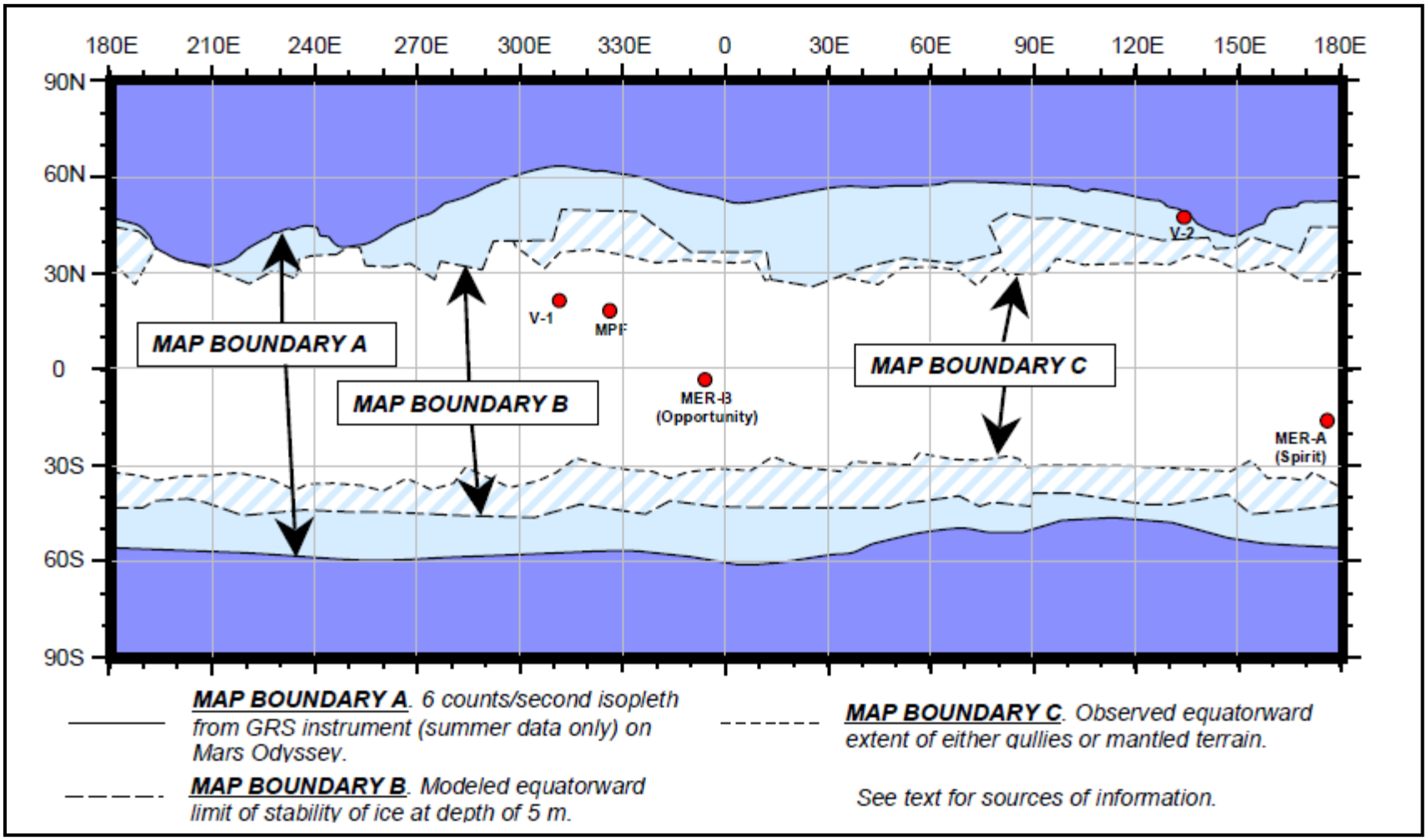

Figure 2.2. Potential Special Regions on Mars. This map shows boundaries of significance for determining possible special regions on Mars (Beaty 2006)

Table 2.2. NRC's Recommended Levels of Bioburden Reduction for the Interim Period. This table was produced by the NRC (2006) as a recommendation for new bioburden levels.

Level	Requirement	Representative Scenario
1	Viking lander pre-sterilization total bioburden (fewer than 3×10^5 total surface spores) and 300 spores per square meter.	Category IVn
2	Viking pre-sterilization levels required for the bulk spacecraft plus Viking post-sterilization on all exposed surfaces. The latter is to be understood as an areal (surface density) measurement. Explicitly, Viking post-sterilization levels correspond to a reduction of 1×10^{-4} times the Viking pre-sterilization upper limit of 300 spores per square meter.	Category IVs
3	Viking pre-sterilization levels required for the bulk spacecraft plus Viking post-sterilization on all surfaces, including those not exposed under nominal (e.g., no-crash) conditions. Explicitly, Viking post-sterilization levels correspond to a reduction of 1×10^{-4} times the Viking pre-sterilization upper limit of 300 spores per square meter.	Category III missions that do not meet existing requirements for probable orbital lifetime
4	Viking post-sterilization bioburden reduction for the whole spacecraft. Currently, this would likely mean baking the spacecraft in a manner similar to that employed in the Viking mission, although the committee encourages NASA to investigate other technologies to this same end.	Category IVs missions accessing locations determined to have long-lived liquid water
5	The committee cannot currently specify the technology that could become available to attain zero microorganisms on Mars-bound spacecraft. Bioburden reduction techniques more effective than those applied today may be or may soon be available for use on spacecraft. A level 5 bioburden reduction level would represent the implementation of these techniques, to achieve bioburden reduction significantly more rigorous than that obtained for the Viking landers.	Category IVs missions accessing locations determined to have long-lived liquid water

Chapter 3: Identification, Characterization and Survival of Isolates Collected from a Mars-bound Spacecraft

Abstract

Mars is considered a likely place to look for extraterrestrial life, given its proximity to Earth, the presence of carbon and other essential elements, and the presence of water in some form. Of current debate is whether microorganisms can inhabit Mars by surviving the intense radiation, high oxidation potential and extreme desiccation present on the Mars surface. Knowing if microorganisms survive in conditions simulating the Mars surface is extremely important because it addresses the issue of whether microorganisms from Earth, traveling on spacecraft, pose a risk to future life detection missions. To ensure international planetary protection requirements are met regarding the microbial bioburden transported into space, Mars bound rovers such as the Mars Science Laboratory (MSL) endure strict implementation sampling campaigns to assess bioburden. The objectives of the study are to 1) Identify cultivable microbes collected from the surfaces of the MSL during the assembly, launch, and test operations; 2) Distinguish those microorganisms that can utilize electron acceptors known to be available on Mars, and 3) Determine microbial survival after exposure to Mars-like surface conditions.

Organisms were collected during the sampling phase of MSL's planetary protection implementation campaign and further isolated and identified using culturing and molecular techniques. Preliminary results show that a significant portion of the 400 organisms studied are related to members of the *Bacillus* genus. Surprisingly, many of the organisms belong to non-spore-forming genera. Identification of five of the isolates indicates that they may be novel organisms based on low sequence similarity to known organisms. Data suggests that 21 of these organisms are able to reduce potential growth substrates, such as perchlorate and sulfate, found on Mars and other planets and moons. Many isolates have shown resistance to desiccation, and UV-C radiation. Moreover, 20% of the isolates can grow in the presence of elevated salt conditions (20% NaCl) and 35% grow at low temperatures (4°C). Results from this study are yielding details about the microbes that inhabit the surfaces of spacecraft after sterilization. On a broader level, this study will help gauge whether microorganisms from Earth have the potential to survive and grow on Mars should they reach the surface, a finding

that could negatively impact future life detection or sample return missions. The overall outcome of this study will benefit the development of cleaning and sterilization technologies designed to prevent forward contamination.

Introduction

The search for extraterrestrial life is bolstered by our long-standing quest to determine if we are alone in the Universe. Mars and Europa are two likely candidates to target in the search for extraterrestrial life, since both have carbon, potential energy sources, and water in some form (Malin 2000; Malin 2003; McKay 2004). The focus to search for life on Mars is supported by the fact that although Mars is quite cold and dry, current conditions are thought to be analogous to conditions on early Earth when single-celled life was gaining a foothold (Rasmussen 2009). Furthermore, because there is a diversity of microorganisms known to thrive in the most inhospitable habitats on Earth, it is not unreasonable to think that microorganisms could live on Mars. Thus there exists a real possibility that microorganisms from Earth transported to Mars on spacecraft may be able to live in the harsh Mars environment. Therefore, a better understanding is needed of the kinds of microorganisms that persist on spacecraft surfaces through assembly, test, and launch phases, and whether those microorganisms can survive on Mars.

While continuing Mars explorations confirm that all of the basic necessities for microbial life are present, it remains unclear whether microorganisms that are metabolically capable of living on Mars can actually survive in the Mars environment. The Mars surface presents a very inhospitable habitat for life because of the intense radiation, highly oxidizing conditions, concentrated evaporative salts, and extremely low water activity. Determining if microbes can survive those surface conditions is vital to discussions regarding the potential contamination of Mars by microorganisms transported on spacecraft launched from Earth. Microorganisms have been isolated from various clean room facilities used during mission preparations, despite the precautions that were taken to reduce the microbial load (LaDuc 2004; Kempf 2005; LaDuc 2007b).

One of the primary issues hindering assessment of the likelihood of the forward contamination of Mars is that there is not enough experimental information accumulated

about the survival of Earth microorganisms in the Mars environment. Previous studies testing survival of Earth microorganisms in simulated Martian conditions have focused on spore-forming microorganisms, particularly bacteria of the *Bacillus* genus, since spores are the hardiest forms of terrestrial life that we know of that can survive Mars-like conditions (Horneck, 1993; Newcombe 2005; Nicholson 2000; Riesenman 2000; Schuerger 2003; Link 2004; Tauscher 2006; Zenoff 2006; Fajardo-Cavazos 2010). Several studies have reported on the survival of spore-forming *Bacillus* species isolated from clean room facilities after exposure of microbes to specific Mars-like environmental conditions (Setlow 2001; Nicholson 2002; Setlow 2006), but recent culture independent analyses show that spore formers are not the only types of microorganisms present in assembly and clean room facilities (LaDuc 2007; Moissl 2008). Other studies have focused on testing survival of extremophilic microbes that have the metabolic capabilities to inhabit Mars (La Duc 2007; Morozova 2007). Few studies have been done using non-extremophilic, non-spore forming microbes to address survival of cells exposed to Mars-like environmental conditions (Berry 2010; La Duc 2007b; Osman 2008), and only a few studies have used isolates collected directly from Mars-bound spacecraft hardware. Furthermore, studies are lacking that investigate the ability of potential Mars contaminating microbes, such as isolates collected during mission preparations, to metabolize electron acceptors such as iron, sulfur, or perchlorate, substrates present on the Mars surface. Determining the tolerance of Mars-bound microorganisms that utilize the carbon and energy resources available on Mars to radiation (both ionizing and non-ionizing), desiccation, and oxidizing environments is of utmost importance for determining whether forward contamination of Mars is a reality.

Protection of Mars is governed by international planetary protection policy, since microorganisms transported on the surface of spacecraft to Mars could hinder the search for past or present life on Mars. This policy restricts the spacecraft's exposed surface areas, mated surface areas, and total encapsulated volume to a bioburden level of less than or equal to 5×10^5 spores (NASA NPR 8020.12D). During the preparation stages of a spacecraft such as the Mars Science Laboratory (MSL), a microbial sampling campaign is undertaken to assess the microbial bioburden actively throughout the mission build-up and testing phases.

The organisms that are enumerated from these campaigns are typically isolated and preserved for future study.

The goals of this study were to 1) identify organisms isolated and preserved from the surfaces of the MSL, and 2) investigate the potential of these organisms to withstand extreme conditions and utilize energy sources potentially present on Mars. The information collected from this study should improve the knowledge base for predictive risk assessments for the survival of organisms to Mars and provide information as to whether organisms residing on the MSL are likely to survive Mars-like conditions.

Materials and Methods

Sample Collection

Samples were collected and processed as follows by JPL's MSL Planetary Protection Implementation Team (NASA 2010). Cotton swabs were used to collect samples (~25cm^2) from exterior surfaces of the MSL. Swabs were then placed into water and sonicated to liberate microorganisms. Samples were heat shocked at 80˚C for 15 minutes, plated onto Tryptic Soy Agar (TSA), and incubated for 3 days at 32˚C. Colonies were re-streaked for isolation and resulting isolates stored in 50% glycerol at -80˚C. The glycerol stocks were subsequently shipped to the University of Idaho for identification and further studies.

Identification of Isolates

A sample from the glycerol stocks was streaked onto TSA and incubated for 24-48 hours at 30˚C. Resulting colonies were re-streaked to ensure purity before proceeding with identification. Once pure, a colony was inoculated into tryptic soy broth (TSB) and grown overnight. Cells were collected by centrifugation at 4000 x g for 7 minutes and the cell pellet was suspended in 250 µl of dH_2O. Cells were lysed by undergoing 3 cycles of freezing at -80°C for 15 minutes then heating at 80° C for 15 minutes. Silica beads were added to the cells and the suspension was mixed by vortexing for 30 seconds. Cell debris was pelleted by centrifuging cells at 4000 rpm for 3 minutes. The 10 µl of the supernatant was used for subsequent PCR. Alternatively, chromosomal DNA was extracted from cells using a Wizard

SV 96 Genomic DNA Purification System (Promega, Madison, WI) or an Ultra Clean Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA).

For each isolate, a 16S rRNA gene amplification was performed using universal bacterial primers 8F and 1525R (Reysenbach *et al.*, 1994; Suzuki *et al.*, 1996). A Polymerase Chain reaction (PCR) was performed as previously described by Smith *et al.* (2009). Briefly, PCR reagents were added to each tube in the following volumes per 50 µl reaction: 25 µl of Dream Green Taq 2X Master Mix (Fermentas-Thermo Scientific, Glen Burnie, MD), 2.5 µl of 12.5 µmol primer 8F, 2.5 µl of 12.5 µmol primer 1525R. Water was added to bring the final volume up to 50 µl after the addition of either lysate or chromosomal DNA. Chromosomal DNA was added as either the lysates (10 µl) or as purified DNA using the DNA purification kits described above (1 µl). PCR conditions were as follows: an initial denaturation step at 94 °C for 5 min was followed by 32 cycles of 95 °C for 1 min, 51.4 °C for 1.5 min, and 72 °C for 1.5 min. Completion of the 32 cycles was followed by a final elongation step at 72 °C for 5 min. PCR-amplified fragments were purified using Exonuclease I (10 U) and Antarctic Phosphatase (2 U) per 5 µl of PCR product. The reaction was heated at 37˚C for 15 minutes then the enzymes heat inactivated at 80˚C for 15 minutes.

Treated 16S rRNA PCR products were sequenced using the primer 27F (Lane 1991). The 16S rRNA gene sequences were analyzed using the rRNA analysis pipeline (HiSTA; available at http://www.ibest.uidaho.edu/tools) as previously described by DeGelder et al. (2005). Briefly, the sequences that were at least 500 bp long were analyzed using BLAST (Altschul *et. al* 1997) to search for similarity among eubacterial type strains in the Ribosomal Database Project (RDP) (Cole et al. 2003). The RDP sequence of the closest relative of each input sequence was retrieved and included in all subsequent analyses. All input sequences analyzed in BLAST, sequences of their closest relatives, and a selected sequence used as an outgroup were aligned using ClustalW (Thompson et al 1994). Genetic distances were calculated by the Jukes and Cantor method (Jukes and Cantor, 1996). Isolates with a unique sequence, or a representative of isolates having the same sequence, were chosen for further sequencing to obtain a nearly full length 16S rRNA gene sequence. Additional primers used to obtain nearly full-length sequences were 518F and 1492R (Muyzer 1993, Frank 2008).

Contiguous sequences were assembled using Vector NTI Advance 11(Invitrogen, Carlsbad, CA). Sequences were re-analyzed as described above.

Anaerobic Growth Determinations

Colonies were inoculated into 200 µl TSB in 96 well plates. Plates were incubated with shaking at 30°C for 24 hrs. The plates were centrifuged at 5000 x g for 12 min and the supernatant was removed. Cell pellets were suspended in 260 µl of phosphate-buffered saline (PBS) and inoculated into modified ATCC #2106 medium in an anaerobic chamber (Table 3.1). All determinations were performed in triplicate. Growth using perchlorate or arsenate was determined by measuring turbidity at 600 nm at 0, 7, 14, 21 and 28 days. Growth of cultures on selenite and selenate was determined visually by the formation of a red precipitate (Se˚). Growth of cultures by the reduction of insoluble Fe(III) to Fe(II) was determined visually by a color change of the solid phase iron from brown to black.

Aerobic Growth Studies (Temperature , pH, and NaCl)

Cells were grown overnight and suspended in PBS as described for anaerobic determinations. To study growth of isolates in medium containing NaCl, aliquots of washed cells were inoculated into TSB containing 0.5, 5, 10 or 20% (w/v) NaCl. To determine growth of isolates at alkaline pH, cells were inoculated into buffered TSB media at pH 7-12. Buffers (100 mM) used in the pH range of 7-10 were previously described by Nielsen et al. (1995). Buffers used at pH 11 and 12 were 1M Na_2HPO_4 and 1M KCl respectively. Cells were grown at 30˚C and turbidity monitored at 600nm (OD_{600}) at 0, 1, 3, 7, and 14 days. To determine growth of isolates at low temperature washed cells were inoculated into TSB, incubated at 4˚C, and OD_{600} readings were taken at intervals 0, 1, 3, 7, 14 and 28 days.

Desiccation Studies

Selected isolates were inoculated into TSB in 96 well plates and incubated at 30˚C overnight. Cultures (30 µl) were added to 96 well flat bottom plates, covered with a gas permeable film and left in the biosafety cabinet overnight to allow evaporation of the medium to occur. The plates were placed into a desiccation chamber containing silica gel desiccant and left to dry for 14 days. At the end of 14 days plates were removed and cells were rehydrated with 200 µl

of TSB. Initial OD_{600} readings were taken immediately after rehydration and again at 24, 48, 72 and 96 hours to determine growth.

Peroxide Tolerance Experiments

Peroxide tolerance assays were performed using a modified method of that described by Riesenman and Nicholson (2000). Cells were grown overnight in TSB then transferred into PBS. Hydrogen peroxide was added to cell suspensions (final conc = 5%) and cells were incubated at room temperature with gentle mixing for 1 hr. Following peroxide exposure, a 100 µl aliquot of the sample was removed and 900 µl of bovine catalase (100 µg/ml) was added to the sample. After incubation for 1 hour, treated cells (10 µl) were inoculated into 240 µl of TSB and OD_{600} readings were taken at 0, 24 and 48 hours. Catalase activity was confirmed a second time by placing cells on microscope slides, adding 3% and 5 % H_2O_2 and observing for the formation of O_2 bubbles (Lim et al 2004).

Results

Identification

Approximately 1000 isolates were collected from the MSL surfaces and preserved as frozen glycerol stocks during the assembly and testing phases. The isolates used in this study only consisted of the 377 isolates collected prior to May 2010 from the JPL spacecraft assembly facility. These isolates were revived from the glycerol stocks for identification and further study. Of the 377 isolates revived, 341 isolates were identified by comparative sequence analysis of the 16S rRNA gene. The majority of the isolates are spore-formers (69%) and most (90%) belong to the *Bacillus* genus. The most commonly identified *Bacillus* species were *pumilus* (16%), *amyloliquefaciens* (11%) and *megaterium* (7%). Overall, there were 27 different species represented within the *Bacillus* genus with an additional 88 *Bacillus* isolates that have not been identified to the species level. Remaining spore-forming isolates belonged to the genera *Paenibacillus* (5%), *Brevibacillus* (1%), *Sporosarcina* (2%), *Oceanobacillus* (0.3%) and *Gracibacillus* (0.3%).

The remaining isolates (31%) are most similar to non-spore forming microorganisms. The most common non-spore forming isolates belonged to the *Staphylococcus* genus (11%). Of

those identified to the species level, the majority of *Staphylococcus* isolates belonged to the species *epidermidis* (41%) and *warneri* (16%). There were 7 different species represented by the genus *Staphylococcus*. The non-spore-forming group were diverse with over 19 genera represented despite their relative low abundance overall. Some of the genera represented include *Acinetobacter* (11%), *Streptococcus* (11%), *Moraxella* (5%), *Leclercia* (5%), *Pseudomonas* (4%), *Enhydrobacter* (2%), *Leuconostoc* (1%) and *Stenotrophomonas* (1%).

Five isolates had low sequence identity to known organisms (<97%). Two of these isolates were most closely related to the *Bacillus* genus (85 and 89%). Two isolates were most closely related to the *Brevibacillus* genus (86 and 93%) while the remaining isolate was most closely related to *Enhydrobacter* (95%). Near full length 16S rRNA gene sequences were obtained for 4 of the 5 isolates with the exception being one of the isolates related to *Bacillus*.

Anaerobic Growth Studies

The 377 isolates were tested for their ability to utilize ClO_4^-, SO_4^{-2}, As^{+5}, Se^{+4}, Se^{+6} and Fe^{+3} as terminal electron acceptors. Table 3.1 shows the various combinations of electron donors and acceptors that were tested. Only 19 isolates (5%) were able to utilize any of the substrates as shown in Table 3.2. Seven isolates grew in the presence of ClO_4^- as a terminal electron acceptor coupled with either acetate or lactate as the electron donor. Only 2 isolates showed growth in the presence of acetate and ClO_4^-, whereas 5 different isolates showed growth in the presence of lactate and ClO_4^-. None of the isolates showed growth on ClO_4^- with both acetate and lactate. Isolates able to utilize perchorate as a terminal electron acceptor belonged to either the *Bacillus* (6) or *Gracibaillus* (1) genera.

Only 2 isolates were able to grow utilizing SO_4^{-2} as a terminal electron acceptor and both isolates were identified as belonging to the *Bacillus* genus. Both isolates grew in the presence of lactate and SO_4^{-2} and neither could grow in medium containing acetate. Ten isolates (3%) grew in the presence of lactate and As^{+5} but none of these isolates were able to grow in arsenic media containing acetate in place of lactate. No growth of the isolates was detectable

in media containing Se^{+4}, Se^{+6} or Fe^{+3} as the terminal electron acceptor. No isolates grew using more than one of the terminal electron acceptors tested.

Aerobic Growth Studies (Temperature, pH, and NaCl)

All isolates were tested for their ability to grow at 4°C, a pH range of 7-12, and in media with elevated salt . Growth at 4˚C was demonstrated by 131 (35%) of the isolates. All isolates identified as belonging to the *Acinetobacter, Stenotrophomonas, Leclercia, Oceanobacillus, Williamsia, Arthobacter, Gracibacillus* or *Streptomyces* genera were able to grow at 4°C. Only 35% of all *Bacillus* species and 14% of *Staphylococcus* species grew at this low temperature. Growth differences between spore-formers and non-spore-formers was minimal with 36.5% of spore formers and 32.5% of the non-spore-formers showing growth at 4° C.

The majority of isolates (97%) could grow in medium containing 5% NaCl but this percentage decreased as the salt concentration increased. Growth in 10% NaCl was accomplished by 354 (94%) of the isolates, while only 80 isolates (21%) could grow in medium containing 20% NaCl. The number of non-spore formers that could grow in 20% NaCl far outweighed the number of spore-formers by 45% to 17% respectively. Comparison of *Staphylococcus* isolates with *Bacillus* isolates showed that while 78% of isolates identified as *Staphylococcus* could grow in elevated salt conditions, only 17% of isolates belonging to the *Bacillus* genus could do the same.

All isolates grew at pH 7 since this is the pH of the medium used in the NASA Standard Assay procedure. Of the 377 isolates, 304 (81%) grew at pH 8, and 215 (57%) grew at pH 9. The number of isolates growing in media with a pH higher than 9 decreased significantly. Results showed that only 12 isolates (3%) could grow at pH 10 and only 1 isolate grew in media at pH 11. No isolates showed growth in media at pH 12. All of the organisms that grew at pH $\geq$10 were spore formers with one isolate identified as belonging to the *Paenibacillus* genus and the remainder belonging to the *Bacillus* genus.

Desiccation Studies

A total of 185 isolates were tested for their ability to withstand a 2 week desiccation period. Isolates from 14 different genera were chosen at random for testing with 144 isolates from spore-forming genera and 37 isolates from non-spore-forming generaDesiccation results by genera can be seen in Table 3.4. Of the isolates tested, 144 (78%) were resistant to dessication during the 2 week exposure period while 14 isolates (8%) showed no growth after 2 weeks of desiccation. Desiccation experiments were performed using representative isolates from 3 spore-forming genera, *Bacillus* (115), *Paenibacillus* (7), and *Gracibacillus* (1). Results showed that 94% of the *Bacillus* isolates and 86% of the *Paenibacillus* survived. The one *Gracibacillus* isolate also survived. Of the non-spore-forming isolates, 83% (25) survived while 17% showed no growth after 2 weeks.

Hydrogen Peroxide Tolerance Studies

Tolerance to hydrogen peroxide was performed on all isolates. Only 19% of isolates (72) were able to grow in TSB after exposure to 5% H_2O_2 for 1 hour. Some of these isolates can be seen in Table 3.4. The catalase assay was used to confirm that the isolates testing positive were able to tolerate H_2O_2 at both 3% and 5% and to check that O_2 bubbles were generated after addition of the H_2O_2 to the cells. Results showed that both spore-forming and non-spore forming isolates were tolerant to 5% H_2O_2. However, of the 70 isolates that showed growth after exposure to hydrogen peroxide, 83% of the isolates were spore-formers with 71% representing the *Bacillus* genus. Hydrogen peroxide tolerance was seen in isolates from 8 different genera, *Bacillus, Staphylococcus, Acinetobacter, Brevibacillus, Streptococcus, Paenibacillus, Moraxella*, and *Sporosarcina*.

Isolates Showing Survival to Multiple Conditions

Table 3.3 shows that a small number of the isolates (11%) could grow in more than one extreme condition such as high salt, high pH, growth at 4°C, and survival of H_2O_2 exposure. Of the organisms identified growing under multiple extreme conditions, 24% were non-spore formers, and 76% belong to spore forming genera. Of the 41 isolates tested, 14 could survive at least 2 of the extreme conditions and 20 isolates could survive 3 of the 4 conditions listed above. Six isolates were identified that can survive all 4 extreme environmental growth

conditions. One of these organisms belongs to the *Staphylococcus* genus, 1 belongs to the *Paenibacillus* genus, and the remaining 4 have been identified as *Bacillus* species.

Conclusions

The purpose of these studies was to identify isolates collected from the MSL prior to launch, and to determine their ability to withstand extreme environmental conditions and utilize substrates for growth that are found on Mars and other planets and moons. These experiments provided a baseline to determine the hardiness of these isolates and to assess their potential to pose a risk to planetary protection and life detection missions. This study is one of the only comprehensive examinations of isolates collected from pre-launch spacecraft with the exception of the Viking studies performed in the 1970's (Puleo 1977).

Until now, it was largely unknown what organisms inhabited the surface of the MSL. Since the NASA Standard Assay requires isolates be heat shocked at 80° C for 15 minutes before plating and enumeration, it was believed that the isolates were all likely spore formers. This study has shown that 31% of all of the isolates are non-spore-formers. Additional studies will need to confirm that these isolates survived NASA standard assays and are truly heat resistant, or if they are the result of downstream sample handling.

Identification of organisms in samples collected off of the MSL has resulted in the identification of isolates representing as many as 25 genera and 65 different species. Five of the isolates are potentially novel based on the low 16S rRNA gene sequence identity (< 97%) to known organisms. Since these isolates were collected post NASA standard assay, they represent only a portion of the total community of organisms on the spacecraft suggesting there is an even greater diversity of organisms when taking into account the total bacterial, archaeal, and fungal community present. At this time, international policy is mostly concerned with the number of spore forming isolates on board the spacecraft, since these isolates are most likely to be able to survive the trip to Mars.

Of the isolates tested to date, the anaerobic growth studies have shown that 21 isolates can grow using the electron acceptors employed. Perchlorate was shown to be present on Mars

by the Phoenix Lander in concentrations of 2.1-2.6 mM (Hecht 2009) and 7 isolates showed growth in medium where perchlorate was the sole terminal electron acceptor. Sulfate has been shown to be a major constituent of Martian soils and sulfate systems exhibit higher water activity thus being favorable to life (Crisler 2012). Two of the isolates in this study showed growth using sulfate as a terminal electron acceptor. Although selenium and arsenic compounds have not been reported to be on Mars, these compounds are somewhat common on Earth and are used by Earth microorganisms to conserve energy. Given this, it is not unreasonable to expect that they may be found on Mars or other planetary bodies. This study identified 10 organisms which grew in media where arsenic was the only terminal electron acceptor available. To further elucidate the isolates potential survivability on Mars, follow-up studies will be conducted with these isolates to verify that reduction of various compounds is directly linked to microbial growth.

It is expected that microbes growing in Mars soil would need to tolerate specific physical and chemical pressures. Salt tolerance would be required if microorganisms were to survive and grow on Mars due to the high salt concentrations found in Martian soils (Crisler 2012). The majority of the isolates collected from the MSL were able to grow in elevated salt. (94% of the isolates; ≥10% NaCl). Some Martian soils are mildly basic with a pH of 7.7 ± 0.5 as determined by the Phoenix Lander (Hecht 2009). Most of the isolates from the MSL (81%) were able to grow with an elevated pH of 8 and over half of the isolates could grow in media with a pH ≥ 9, thus showing that the majority of these isolates could survive and thrive in the alkaline Martian soils. Although the average temperature of Mars can range from -10° C to -76° C, based on Mars Pathfinder data, we chose to test all organisms at a baseline of 4° C (Schofield 1997). About a third of the isolates could grow at 4°C, and these isolates will used to test for growth at even lower temperatures. Notably, only 11% of the isolates were able to thrive more than one single environmental condition. Although the growth on Mars-simulated conditions (e.g. anaerobic substrates, salt, temperature and pH) provide a means to determine the potential ability to proliferate on Mars, the tolerance to harsh environmental stresses is equally important for the organism's survival, both in transit to Mars and on Mars.

The Mars surface is arid and highly oxidizing. Over 78% of the isolates could survive desiccation for a period of 2 weeks and further studies to determine the maximum length of time each of these organisms can survive desiccation are needed since an organism traveling on spacecraft to Mars would have to endure a 9 month trip without water. Up to 19% of the MSL isolates survived exposure to 5% hydrogen peroxide indicating that many of these isolates would survive bioburden reduction methods that utilize hydrogen peroxide. Additionally, it has been reported that the surface ice of Europa contains as much as 0.13% hydrogen peroxide which is generated from radiolysis of ice (Johnson 2003). Thus organisms traveling to and surviving on distant planetary bodies would need the means to protect themselves from strong oxidants such as hydrogen peroxide. The ability of organisms to survive highly oxidizing conditions and UVC radiation make them more likely to survive on a spacecraft destined for Mars or other planetary bodies.

The results of this study enhance our current understanding of the microorganisms present and associated with spacecraft surfaces. This study also provides further detailed information regarding the physiological traits of those microorganisms and their ability to survive extreme environmental conditions analogous to those on Mars. We expect that continued studies will further identify organisms that exhibit unusually high resistance to stresses specific to the Mars environment (e.g., UV radiation). Currently, it is not known how the microbes adjust to the bioburden reduction technologies used by NASA and other space agencies, and how the human-controlled environment may influence the overall evolution of the microbial population within this environment. The information collected from these studies will allow us to assess the current cleaning procedures of spacecraft components and will provide information on the ability of these isolates to withstand extreme conditions similar to those experienced during space travel and on Mars.

Acknowledgements
This work was funded by the Idaho Space Grant Consortium through the NASA ISGC Research Fellowship Program and the ISGC Research Initiation Grant Program. Additional funding is provided by NASA EPSCoR award # NNX11AQ30A. Computational resources were supported by grants from the National Center for Research Resources (5P20RR016448-

10) and the National Institute of General Medical Sciences (8 P20 GM103397-10) from the National Institutes of Health. We wish to thank the non-authored MSL implementation team members, F. Morales, B,. Koukol. , M. La Duc and G. Kazarians, for the collection of these isolates, and J. Spry for his for his encouragement and management support for the work conducted at JPL. We extend our gratitude towards P. Grunther, and K. Buxbaum for their mentorship, programmatic oversight, and monetary assistance.

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Table 3.1. Anaerobic Media. Combinations of electron donors and electron acceptors used to test growth of the MSL isolates under anaerobic conditions.

Media	Electron acceptor	Electron donor
1	Perchlorate (10 mM)	Acetate (20 mM)
2	Perchlorate (10 mM)	Lactate (20 mM)
3	Arsenate (10 mM)	Acetate (10mM)
4	Arsenate (10 mM)	Lactate (20 mM)
5	Selenite (5 mM)	Acetate (20 mM)
6	Selenite (5 mM)	Lactate (20 mM)
7	Selenate (10 mM)	Acetate (10mM)
8	Selenate (10 mM)	Lactate (20 mM)
9	Sulfate (50 mM)	Acetate (20 mM)
10	Sulfate (50 mM)	Formate (20 mM)
11	Fe (III) (80 mM)	Acetate (20 mM)
12	Fe (III) (80 mM)	Lactate (20 mM)

Table 3.2: Isolates showing anaerobic growth. Various substrates and growth results under anaerobic conditions. Also shown are results from H_2O_2, desiccation and UV-C experiments. Ac=Acetate, Lac=lactate, For=formate, NT=not tested.

Isolate	Genus	Growth	pH	NaCl	4° C	5% H_2O_2	Desiccation	UV-C
2.1	*Bacillus*	Ac + ClO_4^-	8	10	-	+	NT	NT
8.1	*Bacillus*	Ac + ClO_4^-	8	10	-	-	NT	NT
276.1.2	*Bacillus*	Lac + ClO_4^-	7	10	-	-	NT	NT
279.1.1	*Bacillus*	Lac + ClO_4^-	7	10	-	-	NT	NT
279.1.2B	*Gracibacillus*	Lac + ClO_4^-	9	10	+	-	+	NT
285.1.2	*Bacillus*	Lac + ClO_4^-	7	5	-	-	NT	NT
286.1	*Bacillus*	Lac + ClO_4^-	9	5	-	-	NT	NT
222.1.1	*Bacillus*	For + SO_4^{2-}	9	10	+	-	+	NT
236.1.1	*Bacillus*	For + SO_4^{2-}	10	10	-	+	+	500
243.1	*Bacillus*	Lac + As^{+5}	7	10	-	-	+	NT
249.1	*Bacillus*	Lac + As^{+5}	7	10	-	-	-	NT
272.1.1	*Sporosarcina*	Lac + As^{+5}	9	10	+	-	NT	NT
272.1.2	*Sporosarcina*	Lac + As^{+5}	9	10	+	-	NT	NT
273.1.1	*Bacillus*	Lac + As^{+5}	9	10	+	-	NT	NT
273.1.2	*Bacillus*	Lac + As^{+5}	7	10	-	-	NT	NT
273.1.3	*Bacillus*	Lac + As^{+5}	9	10	-	+	NT	2000
277.1.1	*Bacillus*	Lac + As^{+5}	9	10	-	-	NT	NT
277.1.2.1	*Bacillus*	Lac + As^{+5}	9	10	-	-	NT	NT
284.1.2	*Bacillus*	Lac + As^{+5}	9	10	-	-	NT	NT

Table 3.3: Isolates showing multiple extreme growth characteristics under aerobic conditions.

Isolate	Genus	20% NaCl	4° C	5% H_2O_2	Upper pH	Isolate	Genus	20% NaCl	4° C	5% H_2O_2	Upper pH
4.2	*Neisseria*	-	+	-	8	137.1	*Bacillus*	+	+	+	9
46.1	*Sphingopyxis*	+	+	-	8	149.2	*Bacillus*	+	+	-	9
47.1	*Sphingopyxis*	+	+	-	9	152.1	*Oceanobacillus*	+	+	-	9
66.1.1	*Bacillus*	+	+	-	7	160.1	*Bacillus*	+	+	-	8
66.1.2	*Bacillus*	+	+	-	9	160.2E	*Bacillus*	+	+	-	8
66.1.3	*Staphylococcus*	+	+	-	9	164.1.2B	*Bacillus*	+	+	+	9
68.1	*Bacillus*	+	+	+	9	166.1	*Bacillus*	+	+	+	9
72.1.1	*Staphylococcus*	+	+	-	7	170.2	*Bacillus*	+	+	-	7
73.1	*Stenotrophomonas*	+	+	-	9	173.1	*Bacillus*	+	+	-	7
100	*Pseudomonas*	+	+	-	7	195.1A	*Paenibacillus*	+	+	+	10
104.1.2	*Staphylococcus*	+	+	+	8	207.1	*Bacillus*	+	+	-	9
105.1	*Bacillus*	+	+	-	7	215.1	*Bacillus*	+	+	-	9
105.2	*Bacillus*	+	+	-	9	218.1	*Bacillus*	+	+	-	7
107	*Staphylococcus*	+	+	-	7	225.2.1	*Bacillus*	+	+	-	8
112.1	*Bacillus*	-	+	-	10	236.1.1	*Bacillus*	+	-	+	10
116.1	*Sporosarcina*	+	+	-	9	250.1	*Bacillus*	+	+	-	7
116.2	*Bacillus*	+	+	-	9	251.1	*Bacillus*	+	-	-	10
117.1	*Paenibacillus*	+	+	-	9	265.1	*Bacillus*	-	-	+	10
127.1.1B	*Bacillus*	+	+	-	9	104594	*Streptomyces*	+	+	-	9
130.1	*Bacillus*	-	+	-	10	104598	*Bacillus*	+	+	-	9
133.1	*Bacillus*	+	+	-	7						

Table 3.4: Results of isolates from the different genera subjected to desiccation. Desiccation was performed for a 2 week period and percentage of isolates that grew after desiccation studies were completed are shown below.

Genus	Number of Isolates with Positive Growth	Number of Isolates with No Growth	% of Isolates Showing Growth
Bacillus	108	7	94%
Pseudomonas	1	1	100%
Staphylococcus	9	2	82%
Streptomyces	2	0	100%
Acinetobacter	3	0	100%
Leclercia	4	0	100%
Paenibacillus	6	1	86%
Streptococcus	1	0	100%
Arthrobacter	1	1	50%
Enhydrobacter	2	0	100%
Moraxella	2	0	100%
Gracibacillus	1	0	100%
Sphingopyris	0	2	0%
Unknown	4	1	80%

Chapter 4: Conclusions to the MSL Studies in Relation to Life on Mars and Planetary Protection

In 1957 The National Academy of Sciences became concerned about the possible harmful effects of contamination by space exploration and in 1967, 10 years later, the United Nations Outer Space Treaty was finally signed and COSPAR was formed (UN 1967, NASA 1973). Yet, after 40 years of spaceflight to Mars, there is still very little information about the organisms residing on the spacecraft and whether they actually pose a risk to planetary protection. The studies on microorganisms collected from the MSL prior to launch provided a baseline of knowledge about the organisms residing on the MSL and their ability to withstand extreme environmental conditions such as those found on Mars. Coming into these studies there was an almost complete lack of knowledge about these organisms and others collected from previous missions that were stored in the archive at JPL. The only information that was available was concerning microbes that had been collected during the Viking missions and a few other organisms isolated from other spacecraft (Puleo 1977, LaDuc 2003). Most of the previous in depth studies were performed on organisms isolated from the cleanrooms where the spacecraft were assembled (LaDuc 2007, Link 2004, Moissl 2008).

The NASA Standard Assay is used to determine the bioburden on spacecraft prior to launch. As previously mentioned, NASA must only consider the number of spore forming units in their bioburden counts thus the NASA Standard Assay is used (NASA 2011). During this assay, organisms are heat shocked at 80° C for 15 minutes with the intention of killing any vegetative cells thus hypothetically leaving behind only the spore formers. The cells are then plated on TSA at 32° C for 72 hours and the number of spore forming units is counted. Using this method there are many things which are not taken into consideration or accounted for including 1) only organisms that can grow on TSA at 32° C within 72 hours are counted; 2) there is likely to be microrganisms on the spacecraft that are not being counted using the standard assay such as archaea and fungi or those organisms that cannot grow under the conditions listed; 3) only a sample area of the spacecraft is swabbed and the total bioburden is mathematically deduced; and 4) bioburden embedded in the spacecraft components is not

considered. This gives us a relatively small picture of what is on the spacecraft yet it is all we have to work with today.

One might expect that the isolates on the spacecraft would be the same isolates that are found in the spacecraft assembly facility but this is not necessarily the case. Different bioburden reduction (cleaning) agents are used on different spacecraft components depending on the type and materials of the component. Cleaning agents used in the spacecraft assembly facility may not match those actually used on the spacecraft. Additionally, these components are often built in other cleanroom or assembly facilities thus the bioburden may be different depending on the facility from which the component originated. Thus the only way to determine what types of microorganisms are on the spacecraft is to look at what is coming off of the spacecraft itself. The JPL microbial archive has provided an opportunity to take an in depth look at the organisms directly inhabiting the spacecraft. Although it is a very narrow view, since we are only looking at organisms that survived the NASA Standard Assay, it is the only view we have available to us at the moment.

Given that all of the isolates in the archive came about as a result of the NASA Standard Assay, it was believed by JPL that the organisms in the archive were all spore-forming isolates. However, it was unknown how many of these organisms were not spore-formers after all and how many of them might actually show resistance and/or survivability to extreme growth conditions. The results showed that out of the 341 isolates identified, 31% were classified as belonging to non-spore forming genera. Additionally, the non-spore-forming isolates showed the most diversity with over 19 genera represented despite their overall low abundance. In comparison, the spore-forming isolates only had representatives from 6 genera. What we currently do not know is if the non-spore-formers are the result of downstream sample handling or if they really survived the heat shock as outlined in the standard assay. These experiments will need to be performed if we are to have extremely high confidence that they originated from the spacecraft.

The temperatures on Mars can range from -10 to -76°C with an average surface temperature of -65° C. However, temperatures on Mars can fluctuate from as high as 25°C to as low as -

123°C (Schofield 1997, Horneck, 2008, Crawford 2008). In order to study the ability of the isolates to grow at low temperatures, we chose to conduct our experiments at 4° C. This temperature would provide us with a baseline for growth with the expectation that not all of the isolates would show growth at this temperature. This would us to narrow down the number of isolates for future studies at even lower temperatures. Many of the isolates collected from the MSL (35%) showed the ability to grow at 4° C. Although 4° C would be at the upper end of temperatures seen on Mars, it may be possible that these organisms, if they reached the surface of the red planet, could survive the on the cold surface temperatures if they were introduced during one of the heat fluctuations that are known to occur. We are in the process of seeking funding to further explore the growth of these organisms at colder temperatures down to -15° C. These studies would give us a better idea which organisms are likely to thrive at these frigid temperatures.

Studies on the ability of the organisms to grow in the presence of high salt were conducted on all of the organisms. As mentioned in Chapter 2, salt tolerance would be required for organisms to grow on Mars due to the high salt content in the Martian soils in the form of NaCl, $MgSO_4$, $CaSO_4$, $FeSO_4$, MgCl and $CaCl_2$. On Mars it is expected that there would be more sulfurous salts over chlorinated salts by a ratio of 3:1 (Crisler 2012). We chose to conduct our studies using NaCl in part due to the ability of high concentrations of NaCl to be easily dissolved in media. Additionally, we were contracted by JPL to conduct studies on the same isolates using up to 2M $MgSO_4$ thus we excluded $MgSO_4$ as part of our own studies. As discussed in Chapter 3, results showed that the majority of isolates could grow in medium containing high NaCl concentrations. Many also showed growth in $MgSO_4$ at both 1M and 2M concentrations. A direct comparison of organisms that can grow in the presence of NaCl and $MgSO_4$ has not yet been conducted but we suspect that many of these salt tolerant organisms will be able to grow in the presence of either of these salts.

Oren (2011) concluded that aerobic respiration, denitrification, and both oxygenic and anoxygenic photosynthesis could occur under the highest salt concentrations but autotrophic oxidation of ammonia and nitrate, some forms of methanogenesis and sulfate reduction were never found at salt concentrations > 100-200 gl^{-1}. Processes identified as occurring, albeit

poorly, at salt concentrations >200 gl^{-1} included fermentation, aerobic autotrophic oxidation of sulfur compounds, sulfate reduction by incomplete oxidizers and some other forms of methanogenesis. He argues that it imposed too high of an energetic cost to the microorganism to be able to live under high salt concentrations and grow under certain anaerobic conditions. Interestingly, although 21% of the MSL isolates could grow in the presence of 20% NaCl aerobically, surprisingly none of the organisms that had previously tested positive for anaerobic growth, could grow beyond the 10% NaCl concentration tested under aerobic conditions. It would be interesting to see if any of these organisms can grow anaerobically in the presence of NaCl exceeding 10% but <20%.

Studies conducted by the Phoenix Lander while on Mars showed that the soils were mildly basic with a pH of 7.7±0.5 (Hecht 2009). However, one must consider that pH might vary from site to site and it may be possible for some places on Mars to be more alkaline or acidic. In these studies we only looked at the ability of microorganisms to grow under alkaline conditions. The majority of the isolates (81%) showed the ability to grow at ≥pH 8. and 57% could grow in media at pH 9. These results show that these isolates could at least grow in the alkaline soils at a pH equivalent to what was determined by Phoenix. Additionally, since many of the archived isolates grew in alkaline media (pH ≥9), it has been hypothesized that these organisms may be resistant to the alkaline cleaning reagents used to reduce the bioburden on spacecraft components or in the spacecraft assembly facility. Although these studies have not been completed, we are proposing to study this in the next funding period, because such studies may give us information as to why the organisms are surviving the bioburden reduction processes in the first place. We expect to find that many of these organisms are indeed resistant to the cleaning agents that are used at JPL both in the cleanroom itself as well as the spacecraft.

For organisms to even have a chance to pose a planetary protection threat, they must be able to survive in the spacecraft assembly facility for the duration of the building of the spacecraft, survive the 9 month trip to the red planet, then be able to survive on Mars until they come in contact with an environment that is hospitable for growth. For an organism to meet all of these challenges it must be able to survive for long periods without water. For

spore-forming organisms surviving desiccation is not considered surprising since the spore coat gives the organism protection while in a dormant stage. However, for non-spore-formers, desiccation provides a great challenge. It was expected that the 2 week desiccation period that was chosen for baseline studies would be long enough to reduce down the number of isolates to a more manageable number for later studies. It was unexpected that 78% of the isolates tested would be resistant to desiccation. It was assumed that most spore-formers would survive, since the spore coat would trap water in the cell, and homeostasis inside the cell would be maintained via the protective spore coat. Some loss would be expected since organisms may be poor at sporulation and potentially wouldn't sporulate in time to survive the initial desiccation. However, 17% of these organisms belonged to the *Staphylococcus, Pseudomonas, Acinetobacter, Leclercia, Enhydrobacter, Streptomyces* and *Moraxella* (non-spore-forming) genera. Previous studies by LaDuc *et al.* (2007) identified several isolates of *Acinetobacter* that were able to survive desiccation and desiccation resistant isolates of *Staphylococcus* and *Moraxella* have also been described (Kubota 2012, Chaibenjawong 2011) so these results are not inconsistent with previous findings. Since very little is known about the ability of non-spore-formers to resist desiccation, such a study would likely yield very interesting details.

Mars is thought to have a highly oxidative environment compared to its atmosphere but the abundance of H_2O_2 in the Martian environment pales in comparison to the amount of H_2O_2 that organisms would have to survive during bioburden reduction of the spacecraft (Slesak 2012, Encrenaz 2008, Kempf 2005). Since vapor hydrogen peroxide is being proposed to replace heat sterilization for future missions, it is important to be able to determine the efficacy of this method. Our studies showed that 19% of the MSL isolates were able to survive a 1 hour exposure to 5% H_2O_2. Other studies, such as those conducted by Kempf (2005) and McCoy (2012), have also shown that many organisms can survive H_2O_2 exposure up to 5% concentrations. The results obtained during the MSL study indicate that 5% H_2O_2 alone would not be as effective as heat treatment for sterilization of the spacecraft. Currently, JPL is considering the use of a vapor hydrogen peroxide (VHP) system to use for terminal sterilization (Rohatgi 2001). This process would use a solution of 59% H_2O_2 in vapor form to effectively kill spores. This process is still under development and has yet to be approved for

use. However, current studies being conducted in collaboration with JPL have shown that many of the organisms isolates from Mars bound spacecraft are resistant to VHP as well (unpublished). These studies indicate that it is very unlikely that VHP can be used for terminal sterilization to meet the thresholds required for entry into special regions on Mars.

For organisms to be able to survive on Mars, they would need to be able to utilize the limited resources for growth and to conserve energy. We chose to test several substrates that we knew would be available on Mars including perchlorate, sulfate, and iron (Schulze-Makuch 2008, Hetch 2009, Crisler 2012). We also tested the ability of organisms to utilize arsenic or selenium to conserve energy. Although arsenic and selenium species have not been identified as part of the minerology on Mars, it is possible that these compounds may potentially be available similar to what is found on Earth, especially since they are analogs for phosphorous and sulfur respectively. The results from the MSL study showed that 21 isolates were able to grow using the limited carbon sources and electron pairs that were employed. Seven isolates showed growth where perchlorate was provided as the sole electron acceptor. Two isolates were able to utilize sulfate and ten organisms were able to grow in the presence of arsenic. We did not identify any organisms able to utilize selenite, selenite or Fe(III) to conserve energy. Studies showing reduction of the various electron acceptors in conjunction with increased microbial growth were not performed and should be included in any further studies to verify that reduction is actually occurring and linked to growth of the organism. Also, it is possible that we may have identified more organisms able to grow under anaerobic conditions if more electron donor and carbon source pairs were utilized. Regardless, this study did show that some of the organisms isolated from the MSL are able to grow anaerobically using substrates available on Mars.

Ultimately, we discovered that 11% of the isolates were able to thrive under multiple environmental conditions including elevated pH, elevated salt concentrations, peroxide resistance, desiccation resistance, growth at low temperatures, and/or growth under anaerobic conditions. For any of these organisms to be able to survive the journey to Mars and then thrive on Mars, they would at minimum need to be able to thrive under these extreme conditions. Although we tested each condition individually, many of these organisms were

able to thrive under the majority of the conditions it was subjected to. For example, isolate 236.1.1, identified as a *Bacillus* sp., was able to grow in medium containing 10% NaCl, at pH 10, and survived exposure to 5% H_2O_2 , and desiccation. In addition, this organism also showed growth utilizing SO_4^{2-} as a terminal electron acceptor. Even more remarkable, isolate 222.1.1 was able to grow in medium containing 10% NaCl, at pH 10, showed growth at 4˚C and survived exposure to 5% H_2O_2 , and desiccation and also showed growth utilizing SO_4^{2-} as a terminal electron acceptor. It is these types of organisms which are most likely to not only survive the bioburden reduction processes, but are also most likely to pose a threat to planetary protection.

An absolute conclusion cannot be made from these studies. We still do not know for sure if any of these organisms could truly survive the bioburden reduction methods *and* the trip to Mars. What we can conclude is that the organisms on the MSL are quite hardy and many can survive multiple extreme environmental conditions singularly. We still need to learn if these organisms can survive multiple extreme environmental conditions simultaneously and we have proposed to proceed with these studies during the next grant funding period. These studies have also indicated that many of the organisms inhabiting the spacecraft are resistant to bioburden reduction methods. Further studies on these organisms may help us learn the best methods for reducing their numbers on the spacecraft. Ultimately, it would be advantageous if we could identify the resistance mechanisms and find a way to circumvent their resistance and reduce the overall bioburden on the spacecraft.

The organisms which are currently under study do not reflect the total bioburden on the spacecraft. NASA's Standard Assay is extremely biased and does not take into account organisms that may be on the spacecraft but are not detected using the standard methods. Studies have been undertaken to determine the absolute bioburden on the spacecraft using molecular techniques, but these studies only tell us who are the organisms and not what they can do. At this time, we do not know how to cultivate every single microorganism that is identified using molecular methods. This puts us at a disadvantage for determining the true threat of whether these microbes can contaminate MarsThe more we learn about the types of

microorganisms residing on spacecraft, the more we realize that our knowledge is extremely limited.

What we can deduce from these studies is that there is a very real threat to planetary protection until proven otherwise. Extensive literature reviews by the NRC (2006) show that the threat persists to contaminate Mars. Everywhere we look for life on Earth, it has been found. It is not unreasonable to expect that microbial life has adapted to the extreme environment on Mars. The same holds true for the microbes inhabiting spacecraft. The results obtained in my studies of the organisms from the MSL indicate that some of these organisms can survive exposure to extreme conditions, and by default, they have most likely survived the bioburden reduction processes employed by NASA.

It is imperative that we obtain a broader understanding of the microbes inhabiting spacecraft before launch. We need to go beyond just looking at the identity of the microbes using molecular techniques. We need to cultivate organisms, and not just those that have survived the NASA standard assay, and look at the ability of these organisms to survive harsh environmental condition analogous to those found on Mars. A good start would be to employ different types of media and different growth conditions to allow for a greater diversity of microbes to grow. Although we would be unable to capture and study all of the microorganisms inhabiting the spacecraft, it would at least provide a less biased view than what we have based on the methods used currently.

Until we can get a better understanding of what is on our spacecraft then we should be taking a more precautionary principle approach. Currently, bioburden reduction methods seem to be selecting for the hardiest of organisms, which theoretically are the organisms most likely to survive the trip to Mars. Until we can ensure the cleanliness of our spacecraft we should not be entering special regions where life is most likely to exist. However, this greatly limits our exploration of Mars since current missions target areas where life is least likely to exist in the present or in the past. New bioburden reduction methods will be essential to get spacecraft to the level of cleanliness required to explore these special regions. It may be that we may need

to take a compromised approach in which some regions are deemed off-limits while opening up very limited region for further exploration.

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Chapter 5: Selenium: Environmental and Toxicological Hazards, Regulation and Remediation

Abstract

Selenium is a metalloid that is naturally occurring in the environment. Although it is a trace element required for cellular function, it is toxic to animals in high concentrations. Anthropogenic activities, such as use of seleniferous fertilizer for agricultural crops, agricultural runoff, and mining operations, has resulted in the release of selenium into the environment leading to selenium bioaccumulation and toxicity in macrofauna. The purpose of this review is to discuss the impact of selenium release into the environment and associated toxicological hazards, describe the efforts to regulate selenium release into the environment and discuss potential methods to attenuate and remediate selenium in impacted areas.

1. Introduction

Selenium (Se) is a metalloid that is closely related to sulfur on the periodic table of the elements (Barceloux 1999). Selenium, while an essential trace element required for cellular function in humans and other animals, can be toxic at higher doses, (Barceloux 1999, Knotek-Smith 2006) and has been referred to as the "essential toxin" (Lenz 2009). Chronic selenium toxicity results in selenosis, also referred to as 'alkali' disease in animals (Barceloux 1999). Symptoms of selenosis in humans include hair loss, fingernail brittleness, skin rash, unsteady gait, paralysis, and garlic breath. Oral exposure to toxic concentrations of selenium can lead to nausea, vomiting, and diarrhea as well as cardiovascular symptoms including chemical pneumonia and cardiomyopathy. A recent study has suggested a correlation between exposure to excess selenium and the motor neuron disease amyotrophic lateral sclerosis (Vinceti 2010). Severe cases of selenium poisoning in humans can result in death, although only a handful of fatal cases have been reported (Spiller 2007, Navarro-Alarcon 2008, Lenz 2009). In other animals, such as horses and cattle, selenosis is characterized by anorexia, weight loss, blindness, respiratory distress, muscular dystrophy (white muscle disease), and lesions on the hoofs (Koller 1986, Stowe 1992, Navarro-Alarcon 2008, Lenz 2009).

Selenium is naturally occurring in the environment. Since the chemical properties of selenium are quite similar to those of sulfur, selenium is often found associated with, but not limited to, sulfides like pyrite (FeS_2), chalcopyrite ($CuFeS_2$), and high sulfur coals, but can also be found in some varieties of slate (Lenz 2009, Barceloux 1999). Selenium is present in many forms including selenate (Se^{+4}), selenite (Se^{+6}), elemental selenium (Se(0)), hydrogen selenide (H_2Se), and selenic acid (H_2SeO_4) to name a few (Barceloux 1999, Lenz 2009). The most soluble and mobile forms of selenium are Se(VI) and Se(IV) (Rosen 2008). In water, selenium is present as a result of wet and dry deposition from the atmosphere, and by surface and subsurface drainage, especially from agricultural drainage water, and oil refining wastewater. Atmospheric selenium comes from the combustion of fossil fuels, incineration of various wastes, discharge from coal fired steam plants, and volcanic gases. Selenium found in soils is usually the result of weathering and leaching of bedrock. This metalloid is not distributed evenly through the earth's crust resulting in areas of both high and low selenium concentrations in soils (EPA 2008a). Ultimately, as much as 40% of all selenium is introduced into the environment by anthropogenic means, mostly from the use of fertilizers or from mining activities (Barceloux 1999, Lenz 2009).

There are many commercial uses for selenium including manufacture of ceramics, glass, pigments, semiconductors, photographic cells, and production of alloys with copper and steel. Additionally, selenium is found in fertilizers, dietary supplements, gun bluing solutions, fungicides, and shampoos. It is also produced as a byproduct of copper electrolytic refining as well as other refining processes (Barceloux 1999, Lenz 2009).

Agriculture practices and mining activities have resulted in excess levels of Se in many watersheds and soils. One of the most infamous incidents of selenium contamination from anthropogenic sources was at the Kesterson Reservoir in California. In the 1980's, the reservoir was a storage facility fed by an 85 mile long subsurface drain which transported agricultural runoff elevated in selenium. Eventually, much of the water entering the reservoir evaporated resulting in a selenium-rich reservoir. The elevated levels of selenium were toxic to the fish and waterfowl living in the nearby Kesterson Wildlife Refuge, and many fish and birds died or suffered deformities such as abnormal beaks, ectrodactyly (missing digits), and

anophthalmia (absence of 1 or more eyes) (Barceloux 1999). Similar incidences occurred in nearby Tulare Basin. Other examples of the negative impacts of excess selenium were reported for Belews Lake, North Carolina, and in Martin Reservoir, Texas, two basins that were used to provide cooling water for coal-fired power plants. In both instances, exchange of the cooling water with basin water resulted in large concentrations of selenium being released into the water. In Belews Lake, enough selenium was released into the water to eliminate most of the fish from the reservoir (Finley 1985, Barceloux 1999).

In southeastern Idaho excess selenium in the environment is primarily occurring as a result of phosphate mining activities. The Phosphoria Formation has been mined for over a century and stretches along portions of Idaho, Utah, Wyoming and Montana. Currently, this area is mined by several companies including Agrium, J.R. Simplot Company, and Monsanto but has also been mined by FMC Corporation, Astaris, Nu-West, Rhodia Inc., and P4 Production LLC (USHHS 2006, Hamilton 2004). The phosphorous mining process requires the removal of large amounts of selenium-rich waste material from the phosphate-rich ore (Hamilton 2004;USHHS 2006). The waste material has been used for reclamation of closed mining sites, which included planting of vegetation. Livestock that grazed on the vegetation in the reclaimed areas exhibited selenosis and some animals had to be euthanized. Currently, leaching of selenium from these soils is occurring as a result of precipitation runoff thus causing selenium to enter surrounding streams, rivers and aquatic plants and animals (Hamilton 2004).

The purpose of this review is to discuss the selenium contamination resulting from anthropogenic activities, the potential health threat to humans and wildlife, the difficulties in regulating selenium, and the utilization of research and subsequent technologies to reduce selenium concentrations in soils and watersheds and to prevent further habitat contamination.

2. Background on the Role of Selenium in Health and Toxicology

2.1 Bioavailability

Studies on the bioavailability of selenium are essential since there is a narrow range between selenium concentrations that result in selenium deficiency and selenosis. The bioavailability

of selenium is dependent on the selenium species and the organism that is taking up selenium. Generally, the bioavailability of selenium in plants is quite high while it is low in animals, and even lower in water. The seleno compounds most often found in plants are selenate, selenite, and many forms of seleno amino acids such as Se-methionine and Se-cysteine. In animals, the most common selenium forms are Se-cysteine and selenotrisulfides of cysteine, selenate and selenite, while the most common form in drinking water is selenate. Organic compounds are usually the most bioavailable compounds (Barceloux 1999, Navarro-Alarcon 2008).

Bioavailability is also dependent on many other factors such as total proteins and fats in the species absorbing the selenium, and interactions with other trace elements and metals, which can be synergistic, antagonistic, or additive. Selenium is known to decrease toxicity of some metal(loid)s such as mercury, arsenic, and lead, while tellurium and zinc interfere with the absorption or action of selenium (Koller 1986). Most selenium species obtained through dietary sources are absorbed (approximately 80%). Wheat products and meat are the major Se dietary sources to humans although bioavailability in fish is lower than that of other meats. Se-methionine is a major selenium source for animals and is readily bioavailable but in fish the selenium bioavailability is low primarily due to high concentrations of other metals such as mercury (Navarro-Alarcon 2008). Additionally, bioavailability of selenium and the species of selenium present in water are dependent on the physical properties of the water body, such as the pH and E_h and the metal species present in the water, as many metal species might interfere with Se availability (Barceloux 1999).

2.2 Physiology and Biochemistry of Selenium Utilization

Selenium is an essential component of many proteins which have various roles in animals. One of the most well known and important selenoproteins is glutathione peroxidase (GSH-Px), an enzyme involved in the protection of cell membranes and lipid containing organelles from oxidative stress. Specifically, GSH-Px acts with vitamin E to catalyze the breakdown of hydrogen peroxides by recycling glutathione which produces the peroxides. One effect caused by selenium deficiency is the two-fold increase of the activity and toxicity of glutathione-S-transferase activity and increased glutathione synthesis in the liver. Another

important selenoprotein is Selenoprotein P which is a plasma protein that functions as an extracellular antioxidant. Other selenoproteins are found in muscle, iodothyronine deidodinases which synthesize and regulate some thyroid hormones, and thioredoxin reductases which are involved in gene expression. Additionally, selenium can increase cytochrome P450 dependent drug metabolism (Koller 1986, Navarro-Alarcon 2008).

2.3 Toxicokinetics

Selenium metabolism is still not a very well understood process, though more information is becoming available through research studies. Metabolism generally involves three processes: 1) reduction of various Se species to selenide (HSe^-); 2) incorporation of selenide into various selenium containing proteins; and 3) methylation of selenides to metabolites for elimination, although demethylation to convert selenides back into inorganic selenium is known to occur as well. Selanate is more readily absorbed by the gastrointestinal tract than selenite. Both selenate and selenite can be incorporated directly into the synthesis of selenium containing proteins even though bioavailability from organocompounds is usually highest (Barceloux 1999, Navarro-Alarcon 2008). Selenomethionine is known to be absorbed through an active transport mechanism used for the absorption of methionine, and a similar mechanism may be used for the absorption of Se-cysteine. Absorption of selenite occurs by passive diffusion while that of selenate is performed by a sodium mediated carrier that is also used by sulfate. Se-cysteine is the most common form of selenium present in animal tissues, with the total amount of selenium present in humans in the range of 10-20 mg (Navarro-Alarcon 2008).

Selenium binds primarily to plasma proteins thus serum levels have been used to determine selenium concentrations in animals. Selenium is then distributed to the liver and kidneys. Barceloux (1999) reports that selenium concentrations are found to be highest in the liver and lower in skeletal muscle while Navarro-Alarcon (2008) states that half of all selenium present in the body is found in muscle tissue. Most selenium is eliminated from the body in urine, although selenium can also be eliminated in feces as a secondary path of elimination, and through the skin and respiration. However, elimination through respiration usually occurs when large amounts of selenium have to be excreted (Barceloux 1999, Navarro-

Alarcon 2008). The half-life for Se elimination in humans is approximately 12-17.5 hours depending on the amount and species of Se ingested (Barceloux 1999).

Se can be toxic to animals when consumed and absorbed in large amounts. In laboratory studies the minimal lethal dose was shown to be 1.5-3.0 mg/kg body weight (bw). Additionally, it was found that diets containing ≥ 5mg/kg of selenium resulted in chronic toxicity (Koller 1986). Rats given 4 ppm of Se in drinking water exhibited discoloration of the liver, hyperplasia, and hepatic cirrhosis. In livestock, a single dose of 2 mg Se/kg bw resulted in lassitude, dyspnea, and death within 12 hours. However, results of studies on Se toxicity in cattle have been contradictory as another report stated that 9.9-11 mg Se/kg bw was the minimal lethal dose. Ultimately, the minimal acute lethal dose in livestock has been determined to be 1-5mg/kg bw. The FDA has approved supplementation of animal feed at 0.1 ppm and the maximum dietary tolerable levels at 2 ppm (Koller 1986).

In humans, selenium intake of 3200-6700 µg/day can result in disruption of the endocrine system, diminished T_3 cell levels, impairment of natural killer cells, and hepatoxicity, while intake of 1260 µg Se/day can result in milder selenosis symptoms such as fingernail changes. Some studies have shown that individuals with Se intake of up to 853 µg Se/day did not exhibit any symptoms of selenosis while other studies have shown that dietary intake of 300 µg Se/day could have toxic effects on synthesis and function of hormones. There is currently no evidence that Se is carcinogenic, and studies actually show an inverse relationship between Se intake and cancer cell mortality (Navarro-Alarcon 2008). Given the results of the toxicological studies in humans, the US Environmental Protection Agency (EPA), under the authority of the Safe Drinking Water Act (42 USC §300g-1(b)) set the MCL in drinking water to 0.05 mg/L, (40 CFR 141.62) and The U.S. Department of Health and Human Services (HHS), Agency for Toxic Substances and Disease Registry has defined the minimal risk level in humans as 0.005 mg/kg/day (USHHS ATSDR 2003).

Uptake of selenium by fish and other aquatic inhabitants such as birds is usually through food sources rather than water as the bioavailability of selenium is much greater in food. Furthermore, bioaccumulation of Se in plants and aquatic invertebrates increases the

concentration of Se intake throughout aquatic animal food chains. The health effects of excess selenium in fish have been well studied, partly due to the fish kills in Kesterson Reservoir, Belews Lake and Martin Lake. Extensive analysis of metals, metalloids, and pesticides showed that only selenium was present at elevated levels. Selenium exposure at concentrations as low as 2.4 µg/g in diets or µg/L in water were found to be fatal. Studies have shown that selenium can reduce mercury accumulation in fish, however selenium would bioaccumulate in fish if waterborne selenium concentrations were greater than 3-5 µg/L (Hamilton 2004). In Kesterson Reservoir, excess selenium resulted in deformed embryos of water birds such as ducks. Selenium exposure concentrations of 6 µg/g in the diet or 6 µg/L in water can cause deformities in chicks while concentrations as low as 10 µg/g in food or 10 µg/L in water can cause mortality in birds. The concentrations of selenium resulting in morbidity and mortality are dependent on the form of selenium absorbed and the species of animal affected, as many closely related animals show different responses to the same concentrations and forms of selenium (Hamilton 2004).

3. Environmental Fate of Selenium

The environmental fate of selenium is dependent on many factors such as biological activity, redox conditions (E_h), pH, and the presence of other metals that can interact with selenium. Selenium enters the atmosphere when methylated selenides are volatilized by algae, microorganisms and plants. Additionally, the combustion of fossil fuels results in the formation of selenium dioxide which is then reduced to elemental selenium by interactions with sulfur dioxide (Barceloux 1999). Wet and dry deposition returns selenium species back to the soils and water. In soils elemental selenium is not soluble and therefore it is immobile, unlike selenate and selenite, both of which are highly soluble and mobile. Other forms of selenium such as selenium sulfides, and metal selenides are the predominate forms of selenium in acidic soils with high organic matter and are immobile, while selenic acids are usually found in the water present in soils and are well mobilized. Selenate salts are predominant in aerobic alkaline soils and are highly mobile due to their high solubility and low absorption, while selenite salts are less soluble due to their ability to bind to iron and aluminum oxides. In water, selenate and selenite are the predominate species under aerobic and alkaline conditions. Under acidic conditions, selenite is easily reduced to elemental Se

by mild reducing agents (Barceloux 1999). It is common to find selenium concentrations of up to 0.01 ppm (mg/L) in drinking water, which is well below the EPA established MCL of 0.05 mg/L. Under alkaline and oxidizing conditions, the bioavailability of selenium increases, as does the uptake of selenium species by plants and algae (EPA 2008a).

3.1 Environmental Selenium in Southeastern Idaho

Selenium contamination resulting from phosphate mining in southeastern Idaho has become a concern to the health and safety of wildlife and humans. Phosphate mining has been occurring in this region since 1919 and there are many active and abandoned mines in this area. The deposition of waste rock as a consequence of harvesting phosphate ore deposits has resulted in the transport of selenium from the mined areas into the surrounding watershed, including the Blackfoot, Salt and Bear tributaries. Selenium is absorbed by aquatic plants and algae, which serve as food for aquatic animals thereby leading to Se bioaccumulation in the food chain. Additionally, bioavailable selenium is present in the soils and is absorbed by a number of terrestrial plant species. Many of these plant species are food for livestock as well as wildlife such as deer and elk. Twenty-four sites, most of these in southeastern Idaho, are on the National Priorities List, and selenium is ranked 147 on the CERCLA Priority List of Hazardous Substances (USHHS 2007, Hamilton 2004).

Presser et al. (2004) conducted studies on selenium loading in the Blackfoot River watershed which receives drainage from eleven phosphate mines, three of which are currently active. Their studies were conducted in 2001 and 2002 which happened to be drought years. Their observations as well as extrapolations from historical hydrographs revealed that there was a 3.6 to 7.4 fold increase in Se loading in the watershed during the high flow season occurring April-June. Additionally, their data showed that dissolved Se species were a 50:50 mixture of selenate and organic selenide which is highly bioavailable. Ultimately, the study showed that drainage from phosphate mining caused the drinking water standards for Se (50 µg/L) and the criterion for hazardous Se waste (1000 µg/L) to be exceeded in this watershed. Studies by Lamothe and Herring (2004) showed that small but measurable elevated amounts of selenium were present in the near-ground level atmosphere in wetland areas at the base of waste rock piles. These levels were above those detected at a nearby background sampling

location. Selenium was the only trace element that was elevated, indicating that the wetland sites at the base of waste piles are a source of volatilization and release of selenium into the atmosphere.

3.2 Toxic Effects of Selenium in Animals in Southeastern Idaho

Uptake of selenium by plants is a major concern in the areas surrounding active or abandoned phosphate mines, reclaimed mine sites, and tributaries. Many of the plants located in these areas are food sources for wildlife such as deer and elk, as well as livestock. Over the years, some of the livestock that grazed on the plants in these areas exhibited selenium toxicity and had to be euthanized while others were found dead due to selenium poisoning. Fish living in the nearby streams and rivers have been shown to have elevated levels of selenium, leading to a number of studies that have been conducted on the toxic effects of selenium in fish (Hamilton 2004). Generally, the toxicity threshold has been determined to be somewhere between 4-12 µg/g. Additionally, the toxic threshold for most waterfowl eggs appears to be between 6-16 µg/g with 16 µg/g being the EC10 value (Hamilton 2004).

Mackowiak et al. (2004) embarked on a study to determine the uptake of selenium into plants. They collected plant samples at waste rock dumps as well as undisturbed sites and compared the trace element concentrations. They also collected vegetation samples from the wetlands associated with these waste rock dumps. They found that the selenium concentration was higher in plants obtained from the waste rock dumps and the associated wetlands. Legumes had the highest concentration of selenium (80 mg/kg), followed by trees (52 mg/kg), grasses (18 mg/kg) shrubs (6 mg/kg) and forbs (3mg/kg). Several plant species contained Se concentrations above the acute and chronic toxicity thresholds for livestock and wildlife.

In both 1996 and 1997 two separate groups of horses pastured on separate parcels of land, one downstream of a closed mine site and the other on a piece of mine property, were diagnosed with chronic selenosis. There have been reports of sheep grazing on phosphate mine associated lands that have also exhibited selenosis. Given these cases, along with the elevated selenium concentrations found in water, sediment, vegetation, fish and bird eggs,

there is great concern about the potential health effects on fish and other wildlife as well as the human population in the surrounding areas (Hamilton 2004).

4. Regulation of Selenium

Currently, selenium is only strictly regulated through the Public Health Service Act (PHSA) or more commonly referred to as the Safe Drinking Water Act (SDWA) (42 USC §§ 300f – 300j-26). It is under the SDWA that actual standards are set for selenium. Selenium discharge is implicated under the National Environmental Policy Act (NEPA) (42 USC §§4321-4347, the Clean Water Act (CWA) (33 USC §§1251-1387), the Endangered Species Act (ESA) (16 USC §§1531-1544) and the Migratory Bird Treaty Act (MBTA) (16 USC §§703-712) to the extent it impacts the environmental values addressed by these Acts. Selenium is generally regulated under the CWA nonpoint source provisions that call for voluntary mitigation measures, and through the mandatory NPDES permit process applicable to point source. NEPA is concerned with environmental impacts on lands that will significantly affect the quality of the human environment, the CWA regulates release from point sources, through the National Pollutant Discharge Elimination System, and ESA and MBTA directly involves protection of specific wildlife. Under the NEPA, the CWA and the CERCLA, the SDWA provides guidance in determining impact, water quality standards, or cleanup standards, respectively. Cleanup standards are generally based on standards established elsewhere, thus if the cleanup involves a drinking water source, the standards under the SDWA will be used. For example, under the ESA or MBTA, selenium may be one of many threats to a particular species so the regulatory impact would come from the protection of those species from harmful levels of contamination of a particular substance. Given the evidence that selenium poses a risk to wildlife and human health, there is a drive to further regulate selenium release through additional legislative action. However, this poses additional issues as regulation and release limits are based on the best available science, and there is often controversy as to how much selenium can be released without posing a health risk. Additionally, there is disagreement over whether the regulations should be based on selenium levels in sediment, water, or animal tissue. The following addresses some of the regulations regarding selenium discharge, the efforts that have been made to best manage

selenium release, and some of the difficulties in determining the best science available for setting criteria.

4.1 The Safe Drinking Water Act

The SDWA was originally passed into law in 1974 under Title 42 §300 f-j with major amendments added in 1986 and 1996. This law gave the EPA the authority to regulate contaminants in public water systems that may effect the health of people. Under the law, the EPA is required to specify for each contaminant the maximum contaminant level (MCL) that is economically and technologically feasible, and if not economically and technologically feasible, to determine the level of a contaminant which leads to the reduction of the contaminant (42 USC §300g-1(b)). The EPA is also required to assure that a supply of drinking water dependably complies with the stated MCLs and includes quality control and testing procedures to ensure compliance (42 USC §300f). Additionally, the EPA must publish the MCLs and promulgate regulation for contaminants if the EPA determines that 1) the contaminant has an adverse effect on the health of people, 2) the contaminant is known to occur or there is substantial likelihood that the contaminant will occur in public water systems with a frequency and at levels of public health concern, and 3) regulation of the contaminant presents an opportunity for health risk reduction to people served by the public water system (42 USC §300g-1). The EPA must regulate at least 5 new contaminates every 5 years only if they are contained on the list of unregulated contaminates under 300g-1(b)(1)(B)(ii)(I). If there is nothing on the list, then there is no requirement to act (42 USC §300g-1(b)(3)(A)) Additionally, the EPA must complete reviews of the MCLs every 6 years (42 USC §300g-1(b)(9)). The caveat is that this code only covers public water systems, defined as "a system for the provision to the public of water for human consumption . . . if such system has at least fifteen service connections or regularly serves at least twenty-five individuals" and does not include regulation of private water wells or services (42 USC §300f(4)).

Selenium was one of the original contaminants regulated in 1976 with revisions being made to the MCL in 1991 (EPA 2003). The current MCL and maximum contaminant level goal (MCLG) for selenium is 0.05 mg/L. (40 CFR 141.62). The MCLG is a non-enforceable value

based on the risk of harm to health whereas the MCLs are enforceable standards and based on the MCLG. The MCLG is based on the reference dose (RfD) where the 'no-observed-adverse-effect level' (NOAEL), 'lowest-observed-adverse-effect level' (LOAEL) or the lower confidence limit on the benchmark dose (BMDL) is divided by the uncertainty factor (UF) which includes sensitive humans, animal studies, completeness of the database, and chronic data. A factor of 10 is used as the default when the LOAEL is used in place of the NOAEL or when the LOAEL and NOAEL are derived from experimental data rather than long term data. The drinking water equivalent level (DWEL) is then calculated by multiplying the RfD by the body weight (70 kg for adults and 10kg for children), and dividing that number by the amount of drinking water consumed (2L per day for adults, 1 L per day for children). The MCLG is then calculated by determining the relative source contribution (RSC), which is based on actual exposure data, and multiplying it by the DWEL (EPA 2010a).

In March 2010, the EPA released the results of their six year review of contaminants, and determined that revision of the selenium MCL was not appropriate at that time. As of this writing, no ongoing EPA health assessments are being conducted for selenium (EPA 2010b). Currently, the tolerable upper intake level for selenium in infants, children and adults is up to 60 μg/day, 90-400 μg/ day, and 400 μg/day, respectively (ODS-NIH 2009) although it has been reported that as much as 500 μg -600 μg per day may not result in any adverse health effects (Koller and Exon 1986). The recommended dietary allowance for selenium is as low as 15 μg/day for infants and small children to 70 μg/day for adults (ODS-NIH 2009). To exceed the upper intake level for selenium, an adult would have to consume as much as 8 L of water at the MCL of 50 μg/L and/or consume excess amounts of food which contain high selenium levels.

Although the MCL under the SDWA seems reasonable and safe for human health, there is still an effort to lower the MCL to 2 μg/L since there remains a threat to public health through bioaccumulation in food sources (Hamilton and Lemly 1999). The SDWA MCL for selenium is the national criterion for drinking water. Additionally, drinking water sources often include reservoirs and lakes, which are also water sources for fish and other wildlife.

Many states' research scientists and technical review committees agree that the criterion should be set at 2 µg/L or less to protect aquatic birds and mammals (Hamilton and Lemly 1999). Since selenium has a bioaccumulation factor of 1000 (EPA 2008a), human health could be placed at additional risk when the consumption of animals exposed to selenium concentrations at the current MCL is taken into consideration. An example of a public health assessment conducted in southeastern Idaho is further discussed below but the results of the study did show that there was a potential threat to children if certain foods were consumed in excess. Additionally, most livestock are fed diets which often contain additional selenium, so consumption of drinking water from public systems could result in adverse health effects to livestock. Given the data available in the scientific community as well as widespread scientific support, the EPA may need to reconsider the potential health risk to humans when determining the MCL at the next six year review.

4.2 Aquatic Life Water Criteria and the Clean Water Act

The Clean Water Act (CWA) prohibits the discharge of a pollutant from any point source into navigable water of the United States except as in compliance with 33 USC §1311 requiring establishment of effluent limitations. "Point source" has been defined as "any discernable, confined, discrete conveyance, including but not limited to any pipe, ditch, channel, conduit, well, discrete fissure, container, rolling stock, and other items from which pollutants are or may be discharged" (33 USC §1362(14)). Under the definition of point source, agricultural storm water discharges and return flows from irrigated agriculture are excluded (33 USC §1362 (14)).

Compliance with the CWA requires a permit for discharge of a pollutant from a point source (33 USC §1342). The term "pollutant" is defined as "dredged spoil, solid, waste, chemical wastes, biological material, rock, sand, cellar dirt and industrial, municipal and agricultural waste, among other items, discharged into water." (33 USC §1362(6)). The EPA has further defined pollutant in its regulations implementing the CWA to surface runoff which is collected or channeled by man, and discharges through pipes, sewers, or other conveyances which do not lead to treatment works (40 C.F.R. §122.2).

Pollutants have been divided up into 3 major categories: 1) conventional pollutants, 2) toxic pollutants, and 3) non-conventional pollutants. Conventional pollutants include 1) biochemical oxygen demand (BOD) 2) total suspended solids (nonfilterable) (TSS) 3) pH 4) fecal coliform and 5) Oil and grease as listed under 40 C.F.R. § 401.16. Additional pollutants include phosphorous, and nitrogenous compounds. Toxic pollutants include a wide range of chemicals and these pollutants are listed under 40 C.F.R. §401.15. Waters regulated by the CWA are defined as "waters of the United States" (33 USC §1362(7)), but there is some uncertainty regarding the full reach of federal regulatory authority under the Constitution (*Rapanos v. United States*, 126 S.Ct. 2208, 547 U.S. 715, 2006). EPA has defined the stretch of federal jurisdiction to include "all waters which are currently used, were used in the past, or may be susceptible to use in interstate or foreign commerce, including all waters which are subject to the ebb and flow of the tide." This includes, "all interstate waters, including interstate wetlands, . . . lakes, rivers, streams, . . . mudflats, sandflats, sloughs, wet meadows, and natural ponds the use, degradation or destruction of which would affect or could affect interstate or foreign commerce" (40 CFR §122.2). Waste treatment systems which include treatment ponds or lagoons designed to meet the requirements of the CWA are not considered waters of the United States (40 C.F.R. §122.2). This exclusion applies only to manmade bodies of water which were neither originally created in waters of the United States nor resulted from the impoundment of waters of the United States.

The discharge of any pollutant from a point source requires a permit. This includes all storm water discharges associated with land disturbances, including the clearing, grading, and excavating of lands (40 C.F.R. §122.26). The Administrator may issue a permit under the National Pollutant Discharge Elimination System (NPDES), for the discharge of any pollutant, or combination of pollutants, upon condition that discharges will meet certain requirements under the CWA. Under 33 USC §1362(14) and 40 C.F.R. §122.26 agricultural storm water runoff does not require a permit because it is exempt from the definition of point source. This also holds true for return flows from irrigated agriculture (33 USC §1342 (l)(1), 40C.F.R. §122.3). When required, a permit may be issued by the EPA or an approved State which has been delegated authority by the EPA (33 USC §1342(c)).

Although selenium has been accepted as a toxic pollutant, it is only regulated by the nonpoint source provisions of the CWA when found in agricultural storm water discharge. Yet this is one of the major contributors to selenium release and subsequent contamination. The CWA does require an NPDES permit for all point source discharges from mining operations (33 USC §1342), however non-point source contamination from mining operations is poorly regulated under the CWA. Under the CWA it is required that states identify impacted waters and pollution sources and establish a total maximum daily load (TMDL) for each pollutant at a level necessary to implement water quality standards and ensure protection of wildlife. Impoundments to contain mine tailings and treat waste from mining and mineral processing operations would be regulated under this provision and would cover discharges from these impoundments into any waters of the United States. The EPA can draft criteria to assist the states and tribes in establishing water quality criteria for non-point sources, and is ultimately responsible for approving the water quality standards, but there has been disagreement in the scientific community as to what constitutes the best way to set criteria based on the best available science.

Lemly (1998) has argued that the best way to derive water quality criteria is to take into account each specific site and alter the criteria when warranted. He proposes a two step process which involves gathering information on selenium residues and the biological effects, then examining the degree of bioaccumulation and the relationship between measured residues and threshold concentrations for reproductive effects in wildlife, including any observed effects. From this, Lemly proposes that criteria can be raised or lowered by a proportional amount related to the hazard. This method would be more encompassing than traditional methods since water flow rates, degree of water stagnation and/or mixing of water can result in varying effects on different ecosystems and wildlife. While the method would allow for all of the variables in the biological and watershed system to be evaluated when setting criteria, it might prove difficult because each site would need to be evaluated separately and would require a significant amount of resources to perform the evaluation. However, since states are required to identify impacted waters and set TMDLs to meet water quality criteria on a water body by water body basis, this site-specific process, or the seven

step procedure for setting selenium TMDLs as described by Lemly (2002) might prove beneficial to the States as they could use these guidelines to set criteria for each specific impacted water body.

In 2004, the EPA issued a "Draft Aquatic Life Water Quality Criteria for Selenium" with the purpose of providing "guidance to States and Tribes authorized to establish water quality standards under the Clean Water Act (CWA) to protect aquatic life from toxic forms of selenium" (EPA 2004). Although this guidance doesn't impose legally binding requirements on any of the aforementioned parties, it provides guidance on appropriate water quality criteria for the protection of aquatic life. The draft presented acute toxicity data of selenite on 14 invertebrates and 20 species of fish, as well as acute toxicity data of selenate on 10 species of invertebrates and 8 species of fish in North America. The report concluded that freshwater aquatic life should be protected under the following conditions. First, the concentration of selenium in whole-body fish tissue does not exceed 7.91 μg/g dw (dry weight), the chronic exposure criterion. If whole-body fish tissue concentrations exceed 5.85 μg/g dw during summer or fall, fish tissue should be monitored during the winter to determine whether the selenium concentration exceeds 7.91 μg/g dw. Second, the 24-hour average concentration of total recoverable selenium in water seldom (e.g., not more than once in three years) exceeds 258 μg/L for selenite, and likewise seldom exceeds the numerical value given by exp(0.5812[ln(sulfate)]+3.357) for selenate. These are the acute exposure criteria. At an example sulfate concentration of 100 mg/L, the 24-hour average selenate concentration should not exceed 417 μg/L (EPA 2004). The EPA also concluded that the data indicates "that saltwater aquatic life should likewise be protected from acute effects of selenium if the 24-hour average concentration of selenite seldom exceeds 127 μg/L" since selenium may be just as chronically toxic to saltwater fish. Additionally, the EPA states that "the status of the fish community should be monitored if selenium exceeds 5.85 μg/g dw in summer or fall or 7.91 μg/g dw during any season in the whole-body tissue of salt water fishes" (EPA 2004).

Since there has been some scientific pressure to move towards development of regulations based on selenium accumulation in the tissue of aquatic species, this draft criteria provides a scientific basis for determining acute toxicity in various aquatic species. However, using

selenium tissue concentration in aquatic species produces yet another conundrum, as aquatic species would have to be sacrificed to determine if water quality standards are being met. Additionally, the report states that aquatic life should be protected if the 24-hour average concentration of total recoverable selenium in water seldom exceeds 258 µg/L for selenite or the numerical value given by exp(0.5812[ln(sulfate)]+3.357) for selenate. In their example, if the sulfate concentration is 100 mg/L then the selenate concentration should not exceed 417 µg/L, which is well above the current MCL of 5 µg/L. Furthermore, it has been noted that selenium contamination pulse events, such as the release of large quantities of selenium during one event, can have adverse consequences as much as 10 years after the release of selenium has ceased (Hamilton 1999). These issues indicate that the determination of tissue concentrations in aquatic species may not be easily studied in the field, and the criterion for protecting aquatic life may not be adequate to actually protect various species.

In October 2008, The Federal Register published a "Notice of Data Availability: The Toxicity of Selenium to Aquatic Life as Related to Developing a Recommended Aquatic Life Criterion" which announced the availability of the USEPA study titled "Effect of Selenium on Juvenile Bluegill Sunfish at Reduced Temperature" (EPA 2008b). This announcement was accompanied by a public comment period that lasted approximately one month. This report attempted to address the study design issues and lack of corroboration between the 2004 Draft Update of the Aquatic Life Ambient Water Quality Criteria for Selenium and a study published by Lemly in 1993. In the Lemly study, juvenile bluegill fish were exposed to both aqueous and dietary selenium for 180 days at both 4°C and 20°C, and selenium measurements were made at four time points. The results of Lemly's study indicated that "significant mortality" occurred in juvenile bluegill fish during winter months when tissue concentrations of selenium increased to 7.91 µg/g dw and lipid levels decreased to 6 percent. The EPA study attempted to determine tissue based effect levels for selenium exposure over a simulated winter season using a range of six selenium concentrations to determine protective effect levels, and to determine the effects on fish using a more natural food source. The fish in the Exposure Systems (ES) were exposed to selenium through two different routes. The fish in ES1 and ES3 received six aqueous doses of selenium in the form of a 1:1 molar ratio of selenite and selenate. The fish also received selenium in the form of a natural

food source, the worm *L. variegates*, which had accumulated selenium by supplementation of their food source, yeast. The fish in ES2 received one dose of aqueous selenium and one dose through commercial fish food supplemented with seleno-L-methionine .

The two exposure methods used in the EPA study are likely to lead to a more accurate ecological risk assessment as the fish in natural systems are likely to absorb selenium through the digestive and respiratory systems. Furthermore, the exposure of selenium through the food source allows one to make better inferences on the effects of bioaccumulation in this food chain. However, the study also made an assumption that the bioconcentration of selenium in the food source correlated to a 1:1 transfer to the fish. Although this was confirmed in preliminary experiments it might have been more appropriate to also study the results of selenium transfer in the actual experiment, especially in the ES2 group which was fed with a commercial fish food supplemented with selenium, as it is possible that the commercial fish food leached selenium into surrounding waters before it was consumed by the fish. This possibility was not discussed in the report and it would be best if this were addressed. Additionally, the study did not indicate how the fish were fed which might have an effect on the distribution of selenium to each fish. For example, if certain fish were to consume more food than the others, this could ultimately skew the morbidity and mortality rates within each group as there is a high probability that the fish which consumed more food would be more likely to die from selenosis.

The temperatures in each ES were held at 20°C for 30 days then decreased 2°C per week until the final predetermined temperatures were reached. The final temperature reached in the ES1 and ES2 tanks was 4°C while the final tank temperature in ES3 was 9°C. Although these conditions allow for study of the effects of winter conditions, there appears to be a flaw in the experimental design. It would have been more appropriate to add additional Exposure Systems because one cannot compare the effects between each of the exposure systems. For example, ES2 was held at the same temperature (4°C) as ES1 however ES2 was fed commercial fish food supplemented with selenium on one occasion and only received one aqueous dose of selenium while the fish in ES1 were exposed to six aqueous doses and six dietary doses through the ingestion of worms. Additionally, the ES3 fish were maintained at

9°C while fed six dietary doses of worms supplemented with selenium, but the investigators did not study the effects of feeding an ES group the commercial food supplemented with selenium while being held at 9°C. It seems that it would have been appropriate to expand the study population to determine if there was a difference at each temperature between fish fed worms and fish fed with the commercial diet so that investigators could make a direct comparison of the effects of temperature and type of food between groups.

Additionally, the study did not state what form of selenium was consumed by the worms before being fed to the fish. The researchers did conclude that the fish that received seleno-L-methionine accumulated 2.5 times the selenium accumulated by the fish which received a more natural diet. However, since the form of the selenium consumed by the worms is not stated, inferences cannot be made as to whether the form of selenium fed to the fish is likely to have the same type of bioaccumulation or toxic effects, since some forms of selenium are less toxic than others. Additionally, since the fish that accumulated 2.5 times more selenium were fed a commercial product we do not know if this result has any relevance to a natural system. Based on this data, it does not seem that the results from the fish fed the commercial product containing seleno-L-methionine should be used in determining a quality criterion for protection of aquatic life as one cannot infer an acceptable risk level or determine the margin of safety with this data.

Ultimately the study did not address or provide background information on the tolerance, biosynthesis, metabolism, storage and potential teratogenesis of selenium in this species making it difficult to accurately assess the materials and methods as well as the results of the investigation. Data presented in the EPA study often conflicted with the results presented by Lemly which makes it more difficult to determine hazardous concentrations and use the data reliably for risk management and assessment. For instance, the toxicity in juvenile bluegill in the EPA study was 1.9 times less than those observed by Lemly, and the body condition factor and lipid content in the fish did not decrease as they did in the Lemly study. Furthermore, these data did not provide information as to whether there is immediate toxicity with rapid high concentrations of selenium which might occur during snowmelt when runoff is high but temperatures are still low. Although the EPA study provides additional useful

information, further research is needed to resolve the discrepancies between the two sets of data and to provide additional information to address concerns discussed in this response. Comparison of the EPA and Lemly studies further exemplifies the difficulty in setting criteria to regulate selenium in the environment since so many variables exist in biological systems.

4.3 NEPA Assessment

The National Environmental Policy Act (NEPA) §42 USC §4332(2)(C) states that an environmental impact statement (EIS) must be prepared by any agency whose "major Federal actions significantly [affect] the quality of the human environment". Once an agency begins preliminary planning for a project the agency must address whether an EIS will be considered. There are three categories which a project may fall under and the agency action will differ depending on the project category. The three categories are 1) the project is categorically excluded; 2) the project because of size and/or impact will always require an EIS; and 3) the agency is unsure whether an EIS will be required.

The projects that are categorically excluded have no significant impact on the environment either individually or cumulatively, or they have been listed as excluded projects by definition. In the second category, an EIS will always be required, and the agency should begin an EIS at the earliest possible time. The third category deals with projects the agency is unsure will require an EIS. If the project does not fall under one of the first two categories, an environmental assessment (EA) needs to be performed to make a determination if an EIS will be required. (40CFR1501.4) The EA should provide sufficient evidence and analysis as to whether an EIS should be prepared or if there is a finding of no significant impact (FONSI) 40CFR1508.9.

In order for the US Forest Service and other federal agencies to issue permits such as mining permits to companies, an EA needs to be performed. However, this has proven difficult for federal agencies assessing the risk of selenium pollution as selenium can affect large aquatic resources, has complex cycling pathways, and exhibits a wide range of toxic effects (Lemly 2007). Additionally, as selenium pollution has increased, there has been a drain on federal

agencies and their resources from responding to land management issues to addressing violations of NEPA, CWA, the Endangered Species Act (ESA) and the Migratory Bird Treaty Act (MBTA). Furthermore, many of the violations have resulted in lawsuits which further drain public agency resources by having to allocate funds towards legal proceedings rather than land management (Lemly 2007).

In 2007, Lemly published a procedure for NEPA assessment to provide the US Forest Service a method to determine the risk of selenium pollution when reviewing mine permit applications. NEPA only provides information and any subsequent regulation would still be under one of the other statutes. The procedure consists of five major components, including assessments of geology, mine operations, hydrology, biology, and hazards. For geological assessment, core samplings and analysis of ore and overburden must be conducted. If the selenium concentration is less than 1 μg/g dw then a recommendation to issue a permit may be made as long as aquatic habitats are monitored once mining begins to ensure that environmental standards are maintained. If the selenium concentrations in the core samples are greater than 1 μg/g dw then tests need to be performed by an EPA certified laboratory to determine the likelihood of selenium leaching from exposed materials and the daily selenium loading (Lemly 2007). However, trying to determine leaching from materials in a laboratory setting may pose problems as it doesn't necessarily represent leaching occuring under natural conditions.

Mine assessments need to be made to determine the amount of excavated solid material that will be exposed to weathering, how much liquid will be produced, and whether the liquids will be discharged or retained in ponds. For the solid wastes generated, average annual precipitation needs to be ascertained to perform a series of calculations to determine the release of selenium from solid material. These two data sets will allow assessors to determine the maximum total daily selenium load generated by the mine. In the case of assessing impacts on hydrology, the surface water surrounding the mine site must be studied to identify all potential receiving waters and protect the weakest link in the hydrological unit (HU). All aquatic habitats and their relationships to one another must be mapped and the sediment types must be identified to determine the selenium retention capacity (RC) of the HU. A final

RC rating is concluded and used to estimate the concentrations of selenium that could result from the mine discharge (Lemly 2007).

Biological assessment is more straightforward. A list of fish and aquatic-related wildlife present in the HU is made, fish and wildlife use of the habitat is characterized, and endangered, threatened, or management priority species are identified including selenium sensitive species. Habitats where bioaccumulation is likely to be an issue need to be identified. The sum of these data can be factored into the TMDL and issues can be addressed to protect wildlife and habitats. Finally, hazard levels need to be calculated based on projected selenium concentrations in water and tissues. If there is little to no hazard, then issuing of permits can be recommended. If the hazard is low, moderate or high then additional procedures need to be carried out to determine the TMDL of selenium that is permissible in order to meet water quality standards under the CWA. If the TMDL can be met by the mining company then it is recommended that a permit be issued. Additionally, monitoring of water, fish, and birds needs to be performed to ensure that discharge limits are met (Lemly 2007).

Lemly's procedure was validated by performing tests at two phosphate mine sites and a gold mine site, and the tests showed that the method could accurately predict selenium hazard. Although this method was developed to address potential selenium contamination hazards at mine sites, it may also be used to determine other metal and metalloid hazards at both mining and non-mining sites. This method will allow federal agencies to meet requirements under NEPA by providing a scientifically viable method for determining the environmental impacts of activities on federal lands.

4.4 Public Health Assessment

To address public health concerns, the US Department of Health and Human Services performed a public health assessment for the southeast Idaho phosphate mining resource area which encompasses parts of Caribou, Bingham, Bannock and Bear Lake Counties (USHHS 2006). In 1996 and 1997 several horses were diagnosed with chronic selenosis, and there were many reported problems with sheep. In 1997 a voluntary committee of the Idaho

Mining Association was formed to identify mitigation methods for selenium in mining waste. Ultimately, a memorandum of understanding was signed that designated the Idaho Department of Environmental Quality (IDEQ) as the lead agency for the investigation of the area. Health assessments of selenium in livestock, fish and other wildlife, as well as a public health assessment, were conducted by IDEQ and other agencies and their contractors. Environmental data was obtained from the sites and comparison values (CV) were used to determine which contaminants at a site needed further evaluation. The CVs were determined for each of the various media and reflected an estimated contaminant concentration that is not expected to cause adverse health effects under normal conditions. Often these CVs are set much lower than levels that show no health effects to include further protections for more sensitive populations such as young children or the elderly.

The report concluded that the Southeast Idaho Phosphate Mining Resource Area has no apparent public health hazard. This conclusion was based in part on the lack of statistically higher cancer incidences, and the lack of criteria met as follows: 1) the presence of a completed human exposure pathway; 2) sufficiently high contaminant levels to result in measurable health effects; 3) a sufficient number of people in the completed pathway for the health effect to be measured; and 4) a health outcome database in which disease rates for populations of concern can be identified. The report also concluded that the levels of contaminants in the soil, water and sediment were not high enough to result in any cancer or non-cancer health risks to sportsmen, residents and children. Although the agency concluded that ingestion of beef, elk and fish from these areas were unlikely to cause any health hazards, studies of selenium present in wildlife did show that there could be potential health hazards to children if they consumed very large quantities of fish and/or elk liver. Given the data, the USHHS recommended that children should not eat more than four-4 oz. portions per month of Yellowstone Cutthroat and Brook trout from East Mill Creek due to contamination. Additionally, small children weighing up to 33 pounds can only safely consume 2 oz. of elk liver per month while children weighing 100 pounds should eat no more than 6 oz. of elk liver per month.

The purpose of the USHHS report was to identify and address any public health concerns in conflict with previous research. Previous research has suggested that there is a major health concern to livestock, fish and other aquatic inhabitants. The data has shown that the removal of soils for the acquisition of phosphate ores has resulted in the mobilization of soluble selenium species. These selenium species bioaccumulate in plants and algae which are primary food sources for several species of wildlife. Deer and elk are known to forage on legumes which were shown to contain the highest concentration of selenium in the plants studied. Consumption of large quantities of selenium-rich legumes could result in selenium toxicosis in deer and elk. In addition, there have already been several reports of selenosis in horses and sheep grazing on selenium contaminated lands. Toxicosis in livestock is very costly to livestock producers as it can result in the loss of revenue due to the inability to sell the livestock to slaughter houses, expenditures resulting from the treatment and handling of livestock exhibiting selenosis, and the loss of money spent in the raising of livestock that are unable to be sold.

Previous research also found that fish in streams receiving runoff from the impacted areas contained elevated levels of selenium. Elevated selenium levels can result in fish morbidity and mortality, which can negatively impact the people and animals which feed on them by decreasing the availability of a major food source, and because of the potential for health effects from consuming excess selenium. Although the USHHS report was comprehensive and concluded that selenium posed no apparent public health hazard, the results of the previous research studies did indicate that there was potential health risk to children and to the wildlife in the surrounding area. Although the USHHS report did not result in any further regulation of selenium, the results should strongly be taken into consideration in the development of new environmental standards. Additionally, given the potential risk to children, the cost to livestock producers, the effect on wildlife, and the impact to the watersheds, the total cost to the environment and surrounding populations should be taken into consideration when developing criteria in the future. Solutions outlined in the following sections should be contemplated as ways to resolve the issues facing selenium discharge from mining activities.

5. Selenium Attenuation and Remediation

Many treatment options are being considered for the attenuation and remediation of selenium in impacted areas. However, there are often multiple differences in conditions at different sites that need consideration. The differences, such as the total selenium concentration, the species of selenium present and biological and physio-chemical differences, make it difficult to develop a one size fits all approach (Lenz 2009). Given these difficulties, many different methods are being researched and tested.

5.1 Physical and Chemical Processes

Physical and chemical processes such as nanofiltration, reverse osmosis and ion exchange, have been considered. Ion exchange resins adsorb selenium oxyanions thus removing them from wastewaters. This is an inexpensive method but it has reduced ability to adsorb selenium in the presence of sulfate and often is inefficient at selenium removal. Nanofiltration and reverse osmosis have been applied in the treatment of agricultural drainage water resulting in the removal of 90% of the selenium. However, both methods are quite expensive, result in selenium brine wastes that still need to be disposed of, and can be hampered by the precipitation of minerals such as gypsum. Chemical reduction of selenium to Se^0 by zero valent iron (ZVI) has also been investigated. Although this method has been successful in laboratory experiments, it is of limited use in the field as there is significant interference by sulfate, phosphate, and carbonate ions (Lenz 2009).

The EPA and DOE in conjunction with MSE Technology Applications, Inc. and the Mine Waste Technology Program (MWTP) published a report in 2001 summarizing the results for various technologies to treat wastes and reduce volume, mobility and toxicity (EPA/DOE 2001). In this study they examined the removal of selenium by reduction to a solid state using either an iron surface or a cementation process. It was concluded that selenate reduction proceeded rapidly on an iron substrate, and the rate of selenium reduction was doubled by using galvanic coupling of iron and copper. Although this report showed that these removal techniques were promising, another report published by the EPA in 2001 concluded that this type of technology, using iron absorption processes, requires excessive reagent usage and is cost prohibitive. Additionally, studies showed that the mean selenium

effluent concentrations were as high as 834 µg/L and 563 µg/L for the catalyzed cementation process and ferrihydrite adsorption tests respectively. These limits are well above the National Primary Drinking Water Regulation Maximum Contaminant Level of 50 µg/L (EPA 2001). Given the data, it is unlikely that this process will be able to be used on a large-scale to remediate selenium contaminated water since this process is expensive and still results in excessive selenium concentrations .

5.2 Natural Attenuation

Natural attenuation is the degradation, diffusion, dilution, sorption, volatilization, and or stabilization of a contaminant, which reduces its concentration in the environment via natural means. The process of natural attenuation was studied by Stillings and Amacher (2004) in wetlands resulting from mine drainage in southeastern Idaho has been studied. It was noted that selenium quickly decreased in surface water as it flowed from the seeps into the wetlands, dropping from as much as 520 µg/L to as little as <5 µg/L. Most of the selenium was found to be associated with iron oxides in the sediment and it was hypothesized that iron oxides were involved in selenium sequestration. Compensation ponds are another method used to contain runoff while giving time for natural attenuation to occur. Studies on the use of compensation ponds in conjunction with agricultural evaporation basins have shown that the compensation ponds are successful in attracting waterfowl away from the evaporation ponds, thus keeping waterfowl from being exposed to selenium, while the evaporation ponds are successful in decreasing total selenium concentrations in surface water (Gao 2007). Research is continuing on the utilization of these different methods, increasing the likelihood that an inexpensive method for the attenuation and remediation of selenium waste will be developed.

5.3 Phytoremediation

Biological conversions of selenium waste have been studied as potential methods for the remediation of selenium. One such option is the use of plants to remediate contaminated areas. This is a favorable remediation method since selenium enriched plants could be harvested thus preventing selenium from entering back into the soil. Harvested plants could be used as animal feed supplements such as in livestock feed, and for production of paper

goods, building materials, or other industrial purposes (Berken 2002, Parker 2003). One particular study showed that vegetation growing in soils overlying the Phosphoria Formation tended to be higher in selenium than vegetation in undisturbed limestone or chert soils. Wetland vegetation contained lower concentrations of selenium as the distance from the mine waste rock dump increased. Legumes tended to have a higher selenium concentration than other plants such as grasses and shrubs, however grasses were the highest selenium accumulators in contaminated wetlands (Mackowiak 2004). Other studies have shown high selenate concentrations in the roots of aster plants harvested from reclaimed mine soils, thus further supporting the use of phytoremediation on selenium contaminated soils (Oram 2010).

Many plants belonging to the family *Brassicaceae* appear to be likely candidates for phytoremediation of selenium. Parker *et al* (2003) described the potential of *Stanleya pinnata* to hyperaccumulate selenium. Selenate was preferentially accumulated over selenite. Additionally, this plant is well adapted to the western United States and may prove useful for the remediation of phosphorous mining sites and agricultural run-off areas in Idaho and California, respectively. Yawata *et. al.* (2010) investigated selenium metabolism in *Brassica juncea*, *Brassica rapa* var. *hakabura* and *Brassica rapa* var. *peruviridis*, and showed that all three plants accumulated selenium in the roots and leaves.

Genetic engineering of plants has also been considered for the phytoremediation of selenium. For example, hyperaccumulating plants are favored for remediation processes but their growth tends to be slow resulting in lower biomass. One way to overcome this dilemma would be to genetically engineer plants that carry both selenium hyperacccumulation and fast growth traits. Alternatively, plants could be modified so that they could grow easily in high seleniferous soils, or could over express genes needed for selenium accumulation, or have genes inserted that function in a selenium assimilation pathway (Berken 2002). However, these methods are not yet viable under current conditions as genetically modified organisms have yet to be widely accepted around the world.

Although the use of plants to remediate selenium contaminated soils and wetlands appears promising, there are some potential issues which may hinder widespread use of

phytoremediation. Although plants may be harvested and either discarded safely or utilized for feed or manufacturing purchases, the harvesting of plants would provide an additional cost although the cost would likely be less than that of electrochemical remediation processes. Additionally, plants that have accumulated selenium might pose a risk to wildlife that forage on lands within a contaminated site. As previously mentioned, this has been an issue in southeastern Idaho and has caused a risk to the health of both wildlife and livestock.

5.4 Microbial Transformation of Selenium

There are numerous heterotrophic microorganisms in soils that are able to interact with selenium species. Many reduce selenate to Se^0 and these organisms play a major role in affecting the fate of selenium species in aquatic environments (Lovely 1993). Studies comparing the efficacy of various selenium removal processes showed that biological reduction of selenium was the most favored and cost efficient method when compared to cementation and ferrihydrite adsorption. The mean selenium concentration in effluents ranged from 8.8 µg/L to below the detection limit of 2.2 µg/L which is well below the 50 µg/L MCL. Additionally, over 70% of the samples collected over a 6 month period were below detection levels. Biological selenium reduction processes were more cost efficient ($603,999) and required less construction time (22 weeks) than that of cementation processes (29.2 weeks and $1,083,285) and ferrihydrite adsorption (30 weeks and $1,026,835). Furthermore, the net present value of US dollars per 1000 gallons treated was $1.32 compared to $8.17 for cementation processes and $13.90 for ferrihydrite adsorption (EPA 2001). The organisms used in the study consisted of a proprietary biofilm of microorganisms including *Pseudomonas* spp. and was developed by Applied Biosystems in Salt Lake City, Utah (EPA 2001).

Additional studies have also supported the use of indigenous microorganisms for the reduction of selenium. Research by Knotek-Smith et al. (2006) showed that there were many indigenous microorganisms in the soils surrounding a mine site in southeastern Idaho that could reduce selenate to elemental selenium. Other studies on microbial selenium reduction describe a consortium of *Bacillus* isolates from selenium enriched natural soils (Prakash 2010), and the isolation of a selenate respiring organism, *Enterobacter hormaechei*, from a

coal mine tailings pond sediment (Siddique 2007). Our laboratory has isolated several novel selenium respiring organisms as well as several novel selenium detoxifying organisms from seleniferous shales collected from reclamation areas within mining sites in southeastern Idaho, thus the development of proprietary biofilms may not be necessary for microbial selenium reduction in the field.

The presence of nitrate can be inhibitory for effective selenium removal by microorganisms. Some researchers have suggested the use of an algal-bacterial system in which the algae would remove the nitrate through denitrification, thus allowing more successful remediation of selenium by bacteria (Lovely 1993). This suggestion of combining different organisms to accomplish the task of nitrate reduction for more efficient selenium removal has been further supported by the research performed by Zhang et al. (2007). Their studies showed that when the nitrate reducing bacterium, *Dechloromonas* sp. HZ, was added to drainage water containing the selenate reducing bacterium *Bacillus* sp. RS1, selenate removal from the water occurred much faster. However, other studies have shown that some organisms, including *Sulfurospirillum barnesii*, are able to simultaneously reduce nitrate and selenate which suggests that the selenate and nitrate reduction pathways in this organism may be constitutively expressed (Gadd 2000). Furthermore, studies conducted by Chung *et al.* (2007) suggest that simultaneous bio-reduction of selenate, nitrate and other compounds may be possible in a biofilm reactor environment although it is possible that competition among microorganisms for available electron acceptors may be necessary to see concurrent reduction of multiple compounds.

Several selenium reducing isolates have been identified and investigated. One organism, *Bacillus beveridgei*, isolated from Mono Lake in California, is able to reduce both selenate and selenite. This organism will completely reduce selenite to Se^{0} and Se^{-2} (Baesman 2009). *Bacillus selenatarsenatis* is able to reduce 1 mM of selenate to elemental selenium through a selenite intermediate and also has capabilities to reduce both arsenate and nitrate thus making this organism a likely candidate for remediation of several compounds in the field (Yamamura 2007). Another *Bacillus* organism, strain ML-SRAO, is able to reduce selenate and can oxidize arsenite in the presence of selenate. This is the first account of such a process

and how this occurs is not fully understood (Fisher 2008). Hockin and Gadd (2005) described the removal of selenate by a *Desulfomicrobium* sp. during growth on lactate and sulfate. When sulfate was limited in the culture, selenate was reduced to selenide, but under high sulfate concentrations the selenate was reduce to Se^0. Two strains of the bacterium *Anaeromyobacter dehalogenans* are able to reduce both selenate and selenite to Se^0. However, these isolates were unable to reduce selenite under high selenite concentrations and this has been attributed to the high toxicity of selenite to organisms (He and Yao 2010). Other organisms that have been described as having the ability to reduce selenite include *Geobacter sulfurreducens, Shewanella oneidensis, Veillonella atypica*, multiple *Bacillus* species, *Stenotrophomonas maltophilia*, and *Pseudomonas* sp. (Antonioli 2007, Hunter 2009, Pearce 2009, Ikram and Faisal 2010). These studies demonstrate that numerous organisms belonging to different genera can reduce selenium oxyanions to less toxic forms.

Despite the research that has been published thus far, there is still little known about the diversity of selenium reducing microorganisms in the environment and the pathways utilized in Se reduction. To date, only one selenium reducing organism, *Thauera selenatis*, has been well studied, yet there are still many questions to be answered as to what regulates Se reduction by this organism and the role of certain genes within its Se reduction operon (Schröder 1997). Putative selenate reductases have been identified in other organisms but more studies are needed to confirm the selenate reduction pathway in those isolates (Watts 2003, Bébien 2002). We have isolated several novel Se respiring organisms, and several novel organisms believed to be reducing selenium through a detoxification pathway, from seleniferous shales collected from reclamation areas within mine sites in southeastern Idaho.

Studies within our laboratory, and other laboratories, indicate that different metabolic pathways are used by Se reducing organisms that conserve energy versus those that detoxify Se. For example, in microorganisms reducing Se for detoxification purposes, we cannot detect the presence of a gene coding for a selenate reductase subunit (*serA*) using *serA* specific primers and classical polymerase chain reaction methods (PCR) (Smith unpublished). This is significant because it means that we do not understand all the Se reduction pathways that are likely to have an impact on Se reduction in the natural

environment. Thus further studies will allow us to better elucidate these two processes so that we can better develop a strategy that encompasses all metabolic processes likely to be important for Se remediation. We have determined that the detoxifying organisms are able to reduce 1mM of Se under aerobic conditions; however, none of the organisms contain *serA* homologs as indicated by the inability to amplify the *serA* gene using *serA* specific PCR primers. This suggests the presence of an unidentified Se reduction pathway that is likely involved in detoxification of Se, a conclusion also made by He and Yao (2010). In comparison, the isolates that conserve energy by reducing selenate all contain homologs to both the *serA* gene and a selenate reductase beta subunit (*serB*) gene. Of note is that all of these organisms can tolerate higher levels of Se ranging in concentrations from 2.5 mM to >20 mM.

Although microbial reduction of selenate appears to be the most cost efficient method with high efficacy, more work is required to identify additional organisms that can reduce selenium oxyanions to less toxic forms of selenium. It is highly likely that indigenous organisms residing in selenium contaminated soils and water have inherent capabilities to reduce selenium. However, the little-studied pathways used by the microorganisms known to reduce selenium, the regulatory elements involved in these pathways, and the optimal conditions for selenium reduction in natural environments by indigenous microbes, needs further study before selenium reduction strategies using microbes can be applied in the field. Additionally, processes would have to be engineered to prevent re-oxidation from occuring.

5.5 Enzymatic Selenium Reduction

Applied Biosystems has developed an optimized mixture of bacterial enzymes from microorganisms isolated from selenium contaminated mining water and soil, and these enzymes were used to investigate the potential for enzymatic selenium reduction. These enzymes reduce both selenite and selenate to elemental selenium thus making them ideal since they greatly increase the kinetics of the reaction, nutrients are not required, and the effects of toxic process solutions can be eliminated (EPA 2001). The prepared enzyme extracts were compared to live cultures over a 2 month period. Results were initially promising as the enzymatic reduction of selenate exceeded that of the bacterial cultures;

however, over time there was a loss of stability in the enzyme preparations and the technology was not recommended for pilot-scale testing (EPA 2001). Although the enzyme preparation was not feasible, further research into indigenous bacteria residing in contaminated soils and water would likely allow for future development of the use of enzymes for remediation.

6. Microbial Oxidation of Selenium

Although it is known that microorganisms play a significant role in the reduction of selenium, very little is known about the role of microorganisms in the oxidation of reduced Se leading to the release of mobile and toxic forms of selenium. Since selenium is chemically similar to sulfur, it is hypothesized that microorganisms can oxidize selenium in a manner similar to that of reduced sulfur species (Losi 1998). An initial report by Lipman and Waksman (1923) described the oxidation of selenium by a group of autotrophic microorganisms. However, a promised detailed description of the organisms and materials and methods was never published. A later report of selenium oxidation was published by Torma and Habashi in 1972. In this report the authors describe the oxidation of CuSe to Cu^{2+} and Se^0 by *Thiobacillus ferrooxidans*; however this report appears to have been at least partially retracted (Sarathchandra 1981).

More recently, several reports on microbial oxidation of selenium have been published. The oxidation of Se^0 to Se(IV) by the bacterium *Bacillus megaterium* was initially described by Sarathchandra and Watkinson (1981). Their results showed that *B. megaterium* was able to oxidize Se^0 to Se(IV) at a rate of 208 μg/L per day for the red allotropic form of selenium and 69 μg/L per day for the gray allotropic form. They hypothesized that the different oxidation rates between allotrophic forms was probably a result of the difference in surface area rather than the difference in forms. However, Zawislanski (2003) noted in his conclusions from field studies that oxidation of biogenically produced Se^0 can occur as much as an order of magnitude faster than that of Se^0 from abiotic sources, a finding consistent with those of Losi (1998). This raises the question as to whether Se^0 is oxidized by microbes more efficiently after biogenic reduction of abiotically oxidized Se species, a process that may be of relevance to the release of selenium oxyanions from mined seleniferous shales. Sarathchandra and

Watkinson (1981) showed that abiotic oxidation of Se^0 to Se(IV) by O_2 resulted in an average rate of 11 μg/L per day which is well below the rate of microbial Se^0 oxidation by *B. megaterium*. Trace amounts of selenate were detected at the end of the six month incubation period but it only represented 1 percent of the total Se(IV) that was formed, indicating that *B. megaterium* is capable of efficiently oxidizing Se^0 to Se(IV).

A study by Losi and Frankenberger (1998) aimed to decipher the contribution of abiotic and biotic Se^0 oxidation processes in soil and liquid cultures. Results of the study showed that biotic processes played a major role in the oxidation of selenium. In the experiments, Se^0 was oxidized to both Se(IV) and Se(VI) but the amount of Se(VI) produced was dependent on the location of the soil sample, indicating that different microbes may be responsible for oxidizing Se^0 to different selenium species. Even though Losi and Frankenberger showed that biotic processes were important in selenium oxidation, they did not isolate any of the organisms responsible for the selenium oxidation in soil samples. However, both heterotrophic and autotrophic selenium oxidation was observed and it was concluded that an inorganic carbon source was favored to that of glucose. They also showed that Se^0 oxidation rates increased if the soils had been previously exposed to Se^0 indicating that soils could be enriched for Se^0 oxidizers.

Dowdle and Oremland (1998) reported on the oxidation of selenium by *Thiobacillus* ASN-1, *Leptothrix* MnB1 and a soil enrichment culture. In all cases, Se(VI) was the primary end product over that of Se(IV). Their results also showed that the rate of oxidation of red selenium was approximately twice that of gray selenium. They concluded in part that the oxidation of selenite to selenate is the rate limiting step in the reaction because Se(IV) adsorbs strongly to soil particles at neutral pH making it less available to microbes. The conclusion was further supported by results obtained with the soil enrichment cultures and with the *Leptothrix* and *Thiobacillus* cultures, all of which produced Se(VI) as the primary product. All sterile cultures showed negligible selenium oxidation, further supporting the conclusion that biotic processes play a major role in Se^0 oxidation. Overall, it was concluded that microbial oxidation of selenium was a very slow process compared to that of selenate reduction. Based on their results, Dowdle and Oremland (1998) concluded that microbial

selenium oxidation is a result of a cometabolic process rather than that of an energy yielding process. However, to date only three microorganisms have been fully confirmed to have the ability to oxidize selenium. Given this, scientists are unable to determine if selenium oxidation is a cometabolic process in all organisms or just in the organisms which have been thus far reported on.

Despite the recognition that microbes can oxidize reduced selenium species, studies have not really clarified our understanding of selenium oxidation in the context of selenium biogeochemical cycling and the role of microbes in the release of oxidized selenium species into the environment. A study published by Wright (1999) showed that Se^0 can be oxidized by NO_3^- in surface and groundwater samples. In these experiments, shales were added to water containing various amounts of NO_3^- and selenium oxidation occurred in all cases where NO_3^- was added. This experiment did not include the use of autoclaved shale samples so it is unclear if the results obtained were the result of abiotic or biotic processes. Since NO_3^- is an electron acceptor, it would seem reasonable that NO_3^- could be reduced during the oxidation of Se^0 and this is further supported by theoretical calculations showing a favorable Gibbs free energy. The conclusions by Wright (1999) did note that management of fertilizer amendments could help control the oxidation and subsequent mobilization of selenium. This could be an important process in the control of selenium release in agricultural settings. Currently it is unknown if high nitrate concentrations associated with blasting during mining of ores may also contribute to the release of selenium into the environment.

To date, very few microbes have been identified which have capabilities to oxidize Se^0 to Se(IV) or Se(VI). It is also unknown what concentrations of Se^0 are toxic, if any, to Se^0 oxidizing organisms as this may affect the ability of organisms to live in environments containing high amounts of Se^0. It appears that biologically prepared Se^0 is favored by microorganisms over that of abiotically formed Se^0, which may also indicate that microorganisms may play a limited role in the initial oxidation of Se^0 but may play a substantial role in subsequent Se^0 re-oxidation after microbial selenium reduction. Initial reports by Losi and Frankenberger suggest that Se^0 containing soils may enrich for Se^0 oxidizing organisms but this has yet to be supported by data. Finally, it has been shown that

NO_3^- plays a role in the oxidation of selenium but it is unclear whether this is due to biotic or abiotic processes. It may be that other electron acceptors can also play a role in the release of oxidized forms of selenium, but that has yet to be established.

7. Conclusion

Although selenium is necessary for human and animal health, high doses of selenium can result in morbidity and mortality. Release of selenium into the environment is mainly the result of anthropogenic activities such as mining and agriculture. There has also been speculation that indigenous microbes in selenium-rich environments may contribute to the mobilization of selenium. Currently, selenium is mostly regulated through the SDWA and more loosely regulated under NEPA, CWA, ESA and MBTA, though the MCL from the SDWA is used as the national criterion for other standards. Efforts to further regulate selenium release are underway but because there are often discrepancies in data or there is a lack of relevant information, it has proven difficult to define how much selenium can be released since regulations set forth by the EPA must be made using the best available peer-reviewed science. Funding research investigating the toxicity of selenium would likely resolve some of these discrepancies and would provide further data to support additional regulation of selenium.

Although there does not seem to be an immediate public health threat to humans residing or recreating in selenium contaminated areas, there is a health threat to the fish, wildlife and livestock populating the area. A set of standards and regulations that sets maximum allowable runoff criterion to prevent environmental and wildlife harm would be of immense benefit. Until then, continuous monitoring of impacted areas should be required to prevent further contamination and harm on wildlife. Furthermore, efforts to develop better agriculture and mining methods should be a priority. Current data available on the bioaccumulation and toxicity of selenium in birds, elk, deer, and other wildlife is lacking. Funding of such research would allow for a better understanding of the potential risks involved in the consumption of these animals by humans, and the impact of selenium toxicity as a whole on wildlife. Further research on selenium toxicity in humans and other animals would allow us

to set more reasonable and scientifically supported MCLs and lead to a better understanding of selenium toxicity as a whole.

Increases in research funding should be made available to further investigate remediation and natural attenuation of impacted sites. Additional studies in these areas could potentially provide cost effective and highly efficient methods to decrease the occurrence of contamination in mined areas, could lead to development of novel methods to clean up currently contaminated sites, and could provide better information on the persistence of high concentrations of selenium in the environment. Currently, the use of biological methods to remediate contaminated sites seems to be the most effective and the most cost efficient. Further research into the selenium reduction pathways used by microorganisms is likely to advance the development and optimization of biological remediation methods, including enzyme preparations that may ultimately prove more effective.

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Chapter 6: Analysis of a Selenate Reduction Pathway in Se-respiring *Dechloromonas* species

Abstract

Selenium (Se) is a naturally occurring element that is an essential trace element but can be toxic at higher doses. In nature, Se often substitutes for sulfur and is found associated with sulfides in coals and shales. Oxidative weathering of sulfides results in the oxidation of reduced forms of Se to the more mobile Se(VI) and Se(IV) forms which are readily transported through watersheds into aquatic habitats. Previously, we isolated several novel dissimilatory Se(VI) reducers belonging to the *Dechloromonas* genus from seleniferous shales. The objective of this study is to characterize the Se reduction pathway(s) and regulatory element(s) in Se-respiring *Dechloromonas* isolates. Primers for amplification of selenate reductase genes from the isolates were designed based on the *Thauera selenatis* selenate reductase A (*serA*) and B (*serB)* gene sequences. Amplicons were cloned from each of physiologically distinct isolates, and sequencing confirmed that amplicons contained putative *serA* and *serB* genes. Sequence comparisons showed that the putative *serA* and *serB* genes of the *Dechloromonas* isolates shared high identity to the *T. selenatis serA* and *serB* genes. A better understanding of the Se reduction enzymes and their regulatory controls in microorganisms will further aid our understanding of selenium cycling in nature and could lead to the development of bioremediation strategies to be applied in selenium contaminated environments.

Introduction

Selenium (Se) is a metalloid that is closely related to sulfur (Barceloux 1999). While it is an essential trace element required for cellular function in humans and other animals, it can be toxic at higher doses (Barceloux 1999, Knotek-Smith 2006). Selenium is naturally occurring in the environment and since the chemical properties of selenium are quite similar to those of sulfur, it is often found associated with, but not limited to, sulfides like pyrite (FeS_2), chalcopyrite ($CuFeS_2$), and high sulfur coals, but can also be found in some varieties of slate (Lenz 2009, Barceloux 1999). Selenium can be found in many forms including selenate (Se (VI)), selenite (Se(IV)), elemental selenium (Se^0), hydrogen selenide (H_2Se), and selenic acid

(H_2SeO_4) to name a few (Barceloux 1999, Lenz 2009). The most soluble and mobile forms of selenium are Se(VI) and Se(IV) (Rosen 2008).

Up to 40% of all selenium is introduced into the environment by anthropogenic means. The primary sources of selenium are agricultural runoff and mining activities (Barceloux 1999, Lenz 2009). The use of fertilizers and mining activities has resulted in the contamination of many watersheds and soils with selenium, and high concentrations of selenium in water and wetlands can be toxic to fish and waterfowl. In several regions selenium contamination is primarily occurring as a result of phosphate and coal mining activities (USHHS 2006, Hamilton 2004). Waste rock from mining activities often contains high amounts of selenium over that of unmined soils (USHHS 2006). When the waste rock is exposed to the environment, Se^0 and Se^{-2} is oxidized to the more toxic forms Se(VI) and Se (IV). The waste material is often used for reclamation of mined sites, a process that usually includes planting of vegetation. Oxidized forms of Se are bioaccumulated by plants and ultimately, livestock that grazes on the vegetation in these reclaimed sites have exhibited selenosis and many animals have had to be euthanized. Leaching of selenium from reclaimed soils has also occurred during precipitation runoff leading to high selenium accumulation in streams, rivers and aquatic plants and animals (Hamilton 2004).

Biological conversions of selenium have been investigated as methods for remediation of selenium, and the results are promising. To date, only a few Se reducing organisms have been well studied (Schröder 1997, Yee 2007, Kuroda 2011). However, there are still many questions to be answered as to what regulates Se reduction, the pathways used by various microorganisms to reduce selenium, the regulatory elements involved in these pathways, and the optimal conditions for microbial Se reduction *in situ*. The objective of the research is to identify and characterize the selenate reductase operons within five novel Se respiring bacteria that were isolated from seleniferous shales.

Materials and Methods

Selenate Reducing Dechloromonas sp.

Waste rock samples were collected from an active phosphate mine in southeastern Idaho. Selenate reducing *Dechloromonas* sp. were isolated from enrichment cultures prepared using the waste samples that showed the formation of a red precipitate, elemental selenium. Five isolates were purified and identified using comparative sequence analysis of 16S rRNA genes (Childers, unpublished). The five selenate reducers, designated *Dechloromonas* sp. A34 , *Dechloromonas* sp. L33, *Dechloromonas* sp. LK1, *Dechloromonas* sp. CMS, and *Dechloromonas* sp. E5-1, were used in this study.

Isolation of DNA

Isolates were streaked for purity onto R2A agar. Isolated colonies were picked and transferred to R2A broth and incubated at 30˚C for 24-48 hours. Chromosomal DNA was prepared as previously described by Ausubel, et al. (1994). DNA was isolated from environmental soil samples using a Promega Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's instructions.

Primer Design

Gene sequences encoding for the *Thauera selenatis* selenate reductase were obtained from the NCBI website using accession number AJ007744 (Schröder 1997). Primers were designed to target the *serA* and *serB* sequences at *T. selenatis* positions 2620 (serA 2620F), 4973 (serA 4973R), and 6136 (serB 6136R). To obtain a nearly full length sequence of the *Dechloromonas ser*A and *serB* genes, additional primers were constructed once sequence information was obtained from the *Dechloromonas* sp. The additional primers were serA 2620F, serB 4973R, and serB 6136R (Table 6.1).

Amplification and Identification of serA and serB

The *Dechloromonas serAB* gene sequences were obtained by performing gene amplification using the primers in Table 6.1. PCR (50 uL) reactions contained the following: 25 µL of 2x Master Mix (Promega), 2.5 µL of 12.5 µmol primer serA 2620F, 2.5 µL of 12.5 µmol primer serA 4973R or serB 6136 R, 19.0 µL of dH_2O and 1.0 µL of chromosomal DNA (~200ng). PCR conditions were as follows: An initial denaturation step at 94° C for 2 minutes was followed by 32 cycles of 94° C for 1 minute, 55.0° C for 1 minute, and 72° C for 3 minutes.

Completion of the 35 cycles was followed by a final elongation step at 72° C for 10 minutes. PCR-amplified fragments were purified using the Ultra Clean PCR Clean Up Kit (Mo Bio Laboratories, Inc.)

Sequencing reactions were performed on the *serA* and *serB* gene fragments generated from the cultured strains. The PCR mixture (11 uL) consisted of 5 μL of BigDye Terminator Ready Reaction Mix (Applied Biosystems), 5 μL of DNA template (~20 ng), and 1 μL of 12.5 μmol primer. The sequencing conditions were as follows: 96° C for 30 seconds, 56° C for 15 seconds, and 60° C for 4 minutes for a total of 25 cycles. Sequencing was performed by the Washington State University Laboratory for Biotechnology and Bioanalysis.

The PCR amplified fragments of the *serA* PCR-amplified fragments were purified using the Ultra Clean PCR Clean Up Kit (Mo Bio Laboratories, Inc.) Restriction Fragment Length Polymorphism (RFLP) analyses employing *Hae III* was performed on the amplicons as previously described by Smith *et al* (2009) to determine sequence variation between isolates.

Phylogenetics

Contiguous sequences were formed using Vector NTI Advance 10 (Invitrogen), and the sequences were analyzed by performing BLAST searches against the GenBank database. Sequences were aligned using the Clustal X program. Neighbor joining phylogenetic analysis were conducted using PAUP (version 4.0b10). Bootstrap analysis using 1000 replicates was performed to create confidence estimates for the nodes within the phylogenetic tree.

Genome Sequencing

Chromosomal DNA isolated from *Dechloromonas* sp. A34 was sent to the National Biodefense Analysis and Countermeasures Center (NBACC) for genome sequencing and annotation. The fully annotated genome sequence was returned to our laboratory for analysis of the selenate reductase operon.

Results

Identification of the serA gene in Dechloromonas Isolates

The primers used in this study were initially designed based on the gene sequences in the *Thauera selenatis serABDC* operon. Figure 6.1 shows a list of the primers designed and their relation to the *ser* genes of *T. selenatis*. Amplicons were obtained using *serA* primers from *Dechloromonas* sp. L33, A34, CMS, and E5-1. Initial analysis of the amplicons using RFLP indicated that the *serA* gene sequences of all of the isolates differed from the *serA* gene sequence of *Thauera selenatis* (Figure 6.2). The RFLP patterns of L33 and A34, and CMS and E5-1, were identical to one another . The restriction pattern observed for LK1 was similar to those for CMS and E5-1 but an additional fragment was seen above the 201 bp fragment.

The putative *serA* sequences were used in BLAST searches against the GenBank database to determine the similarity of sequences to *serA* from *T. selenatis*. Results showed that the *serA* sequences of L33 (92%), A34 (93%), and CMS (83%) had high nucleotide sequence identity to that of *T. selenatis serA.* Neighbor joining analysis of the sequences using 1000 bootstrap replicates showed the relationships of the *Dechloromonas* sp. and *T. selenatis serA* sequences (figure 6.3A). The *Dechloromonas* A34 *serA* sequence was most closely related to that of *T. selenatis* followed by LK1, L35 and CMS, respectively. The nearly full length gene sequence of *serA* from *Dechloromonas* sp. A34 (GQ451985.1), L33 (GQ451987.1), and CMS (GQ451986.1) were deposited into GenBank. The nearly full length sequence of *serA* from A34 was translated and compared to the *serA* amino acid sequence of *T. selenatis*. Comparison of the amino acid sequences showed only an 18 amino acid difference (figure 6.4).

Identification of the serB gene in Dechloromonas Isolates

Amplicons were obtained using *serB* primers from *Dechloromonas* sp. CMS, L33 and A34. Amplicons of the *serB* gene were obtained from three of the five *Dechloromonas* isolates. Results showed that *serB* genes of A34, L35, CMS, and LK1 had 92%, 89%, 88%, and 89% homology respectively to the *serB* nucleotide sequence of *T. selenatis* based on BLAST searches against the GenBank database. Neighbor joining analysis of the sequences using 1000 bootstrap replicates showed that *serB* genes from the 3 isolates analyzed have high homology to that found in *T. selenatis* (figure 6.3B). The sequences obtained for L33 and

A34 show that they were identical to one another. The *serB* sequence from CMS was very closely related to *T.selenatis* and slightly more distantly related to L33 and A34.

Genome sequence of Dechloromonas A34

Efforts to identify *serC* and *serD* in the *Dechloromonas* sp. using primers specific to the *serC* and *serD* genes in *T. selenatis,* or degenerate primers used in combination with the *serC* and *serD* primers, were unsuccessful. DNA preparations of *Dechloromonas* sp. A34 were sent to NBACC for full genome sequencing and annotation. Analysis of the genome sequence showed that a *serABDC* operon was present in *Dechloromonas* sp. A34. A BLAST searches against the GenBank database showed that the *ser* operon in A34 partially matched the *ser* operon in *T. selenatis*. One hit showed 91% identity at the nucleotide level to *T. selenatis serA* genes, while another hit showed 75% identity at the nucleotide level to T. selenatis *serB* genes. Further analysis of the A34 *ser* operon showed a replacement of 289 bp of the *T. selenatis ser* operon with 278 bp in the *Dechloromonas* A34 *ser* operon. The substituted nucleotides spanned the 3' terminal region of *serD* and the 5' end of *serC* and did not match any other sequences in the GenBank database (Figure 6.7). It was also noted that the *serC* gene in *Dechloromonas* A34 is 54 bp shorter than that of *T. selenatis*.

Comparison of the amino acid sequences of *serD* from A34 and *T. selenatis* show relatively high sequence homology (67.4%). However, the serD gene from A34 had an additional 2 amino acids which were not found in *T. selenatis* (figure 6.5). Comparison of the amino acid sequences of *serC* from *T. selenatis* and A34 confirmed that part of the *serC* gene found in *T. selenatis* was missing in A34. Alignment of 162 of the amino acids from A34 and *T. selenatis* show that the protein sequence is 71.1% identical at amino acid positions 59-221.

The presence of the transcription regulatory protein and sensory protein, ZraR and ZraS respectively, were identified upstream of the A34 *ser* operon based on genome annotation. BLAST searches showed that these sequences were related to the histidine kinase family of sensors. Additionally, a phosphate ABC transporter was identified immediately upstream of the *ser* operon.

Conclusions

We had previously isolated and identified five selenate reducing *Dechloromonas* sp. from seleniferous shales. We were able to initially identify *serA* genes within all five of these isolates using molecular methods and primers based on the *T. selenatis serA* and *serB* genes. Additional primers were generated based on *Dechloromonas ser* sequences obtained using primers specific to *T. selenatis* genes, and putative *serA* and *serB* genes were obtained from the isolates. Four of the *serA* amplicons from the isolates showed high sequence homology to the *serA* gene found in *T. selenatis*, indicating they were likely serA homologs. Likewise, we identified *serB* genes from the *Dechloromonas* isolates that also were highly homologous to the *serB* gene of *T. selenatis*. It was determined that the *Dechloromonas* isolates likely reduce selenate using a pathway similar to that of *T. selenatis*.

Efforts to identify homologs for *serC* and *serD* in the *Dechloromonas* isolates using both PCR and Tn-5 methods were unsuccessful. Therefore full genome sequencing of *Dechloromonas* A34 was performed. The genome sequence of *Dechloromonas* sp. A34 contained a putative *serABDC* operon. Nucleotide sequence comparison of the *Dechloromonas* A34 and *T. selenatis ser* operons showed that the operons are highly conserved between the two genera with two exceptions. The region spanning the 3' terminal end of *serD* and the 5' terminal end of *serC* was the primary exception in that approximately 280 bp were different between the sequences. The other difference is that the *Dechloromonas* A34 *serC* sequence is slightly shorter. Since *Dechloromonas* A34 reduces selenate to elemental selenium, it is concluded that these nucleotide changes do not impact the function of the chaperone-like protein *serD* or the cytochrome function of *serC*. It is unknown if these differences persist in the other Dechlormonas isolates, and work to identify homologs to *serC* and *serD* in the remaining isolates is ongoing.

Regulatory elements were found upstream of the *ser* operon in *Dechloromonas* A34. Putative regulatory and sensory proteins, ZraR and ZraS (also referred to as hydH/G) respectively (Leonhartsberger 2001), were found to belong to the histidine kinase family of sensors. Previous studies have shown ZraR and ZraS are a two-component regulatory system that controls the zinc and lead response system as well as hydrogenase 3 in *E. coli*

(Leonhartsberger 2001). We hypothesize that ZraR and ZraS play a role in the regulation of the *ser* operon (Figure 6.7). Additionally, a putative phosphate ABC transporter was identified immediately upstream of the *ser* operon and it is hypothesized that transporter is responsible for the transport of selenium in and out of the periplasm.

Further analysis of the genome was done to determine if genes for nitrate and sulfate reductases were present, as these enzymes have been shown to reduce selenate in other microorganisms. Genes encoding a putative periplasmic nitrate reductase, *napA* and *napB*, are located approximately 1.4 Mb upstream from the *ser* operon, suggesting that the nitrate and selenate reductases are distinct enzymes. Additionally, a putative sulfate reductase is located approximately 1.8 Mb downstream of the *ser* operon, suggesting that the identified ser operon operates independently in the reduction of selenate and does not play a role in the reduction of either nitrate or sulfate.

We propose that *Dechloromonas* sp. A34 contains a selenate reductase operon nearly identical in sequence and gene order to that of *Thauera selenatis*. Figure 6.8 shows a conceptual model for the interaction of the Ser proteins in *T. selenatis* and in *Bacillus selenatarsenatis*, another recently studied selenate reducer (Schröder 1997, Kuroda 2011). Based on the similarity of the *Dechloromonas* A34 ser *operon* to that of *T. selenatis*, it is proposed that the Ser proteins function similarly. It is predicted that ZraR and ZraS are involved in the sensing of selenium and the regulation of Ser expression though studies are needed for confirmation of ZraR and ZraS function.

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Table 6.1. Primers used in this study. Primers for amplification of the *serA* and *serB* genes were designed based on the *serA* and *serB* sequences of *Thauera selenatis* (AJ007744), and from *Dechloromonas* sequences obtained in this study.

Primer	Sequence (5' to 3')
serA 2620F[a]	CGACGGGCTTCATCACCCACTCCAACGG
serA 3032F	GACGTCTSTCGATGACCGGCG
serA 3300F	CGGCCATCGTCAACGTGCTGA
serA 4037F	GGCTGGATGCCCAACTGGCAGTCG
serA 4263R	GAGGTCTCGCGGATGTCCAGCT
serA 4833F	GGATGATGCGCCACCAGCGCG
serA 4940F	GGCGAGTTCTTCGCCATGGCCAAG
serA 4973R[a]	CTTGGCCATGGCGAAGAACTCGCC
serB 5260R	GGCCATCGTGCAGGTGTGGCAACCG
serB 6136R[a]	ACCTGTCCTTGTTGGTGTAGCCGAT

[a], obtained using *Dechloromonas* sequences

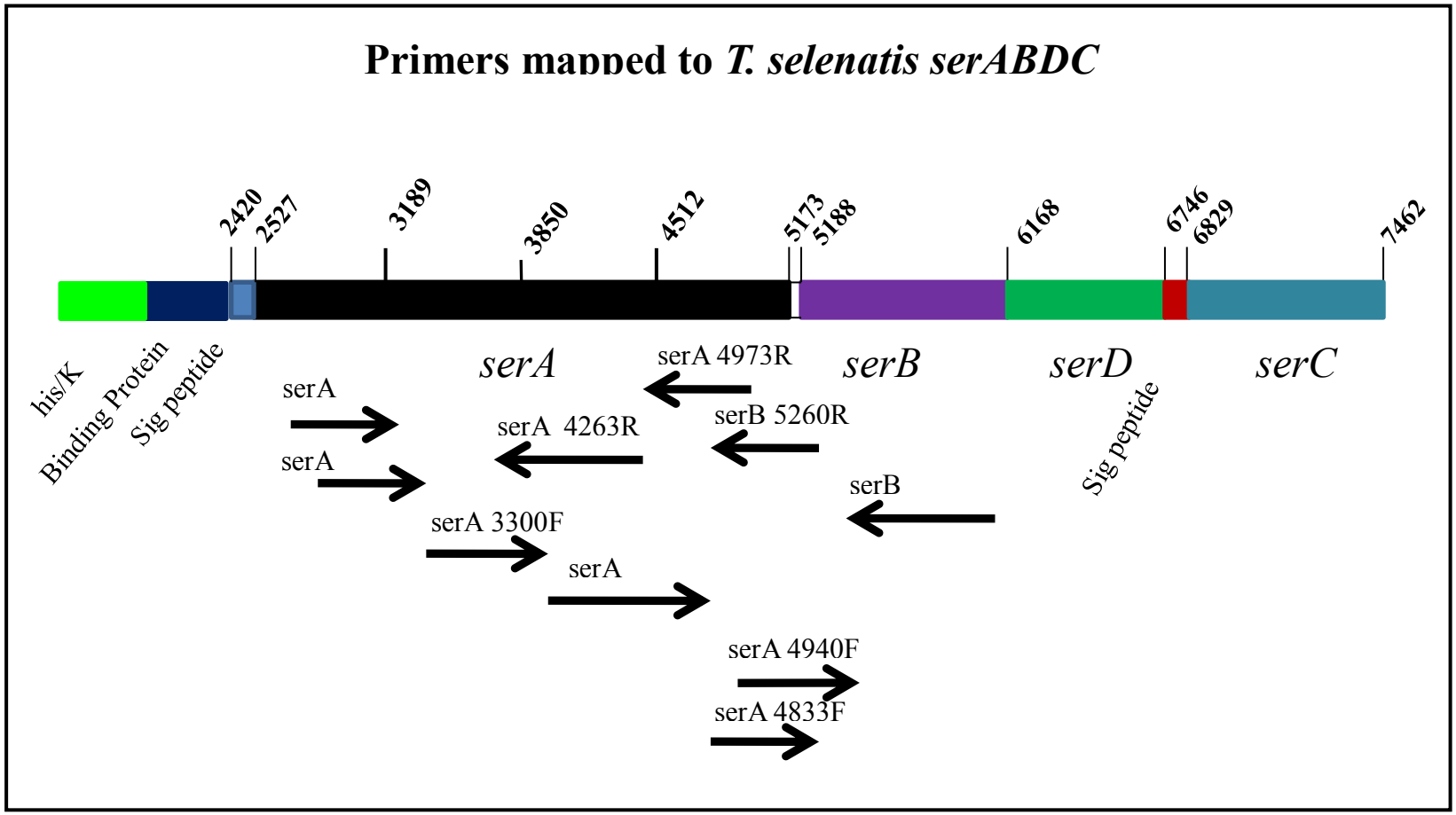

Figure 6.1. Primers targeting the *serAB* sequences of the *Thauera selenatis serABDC* operon.

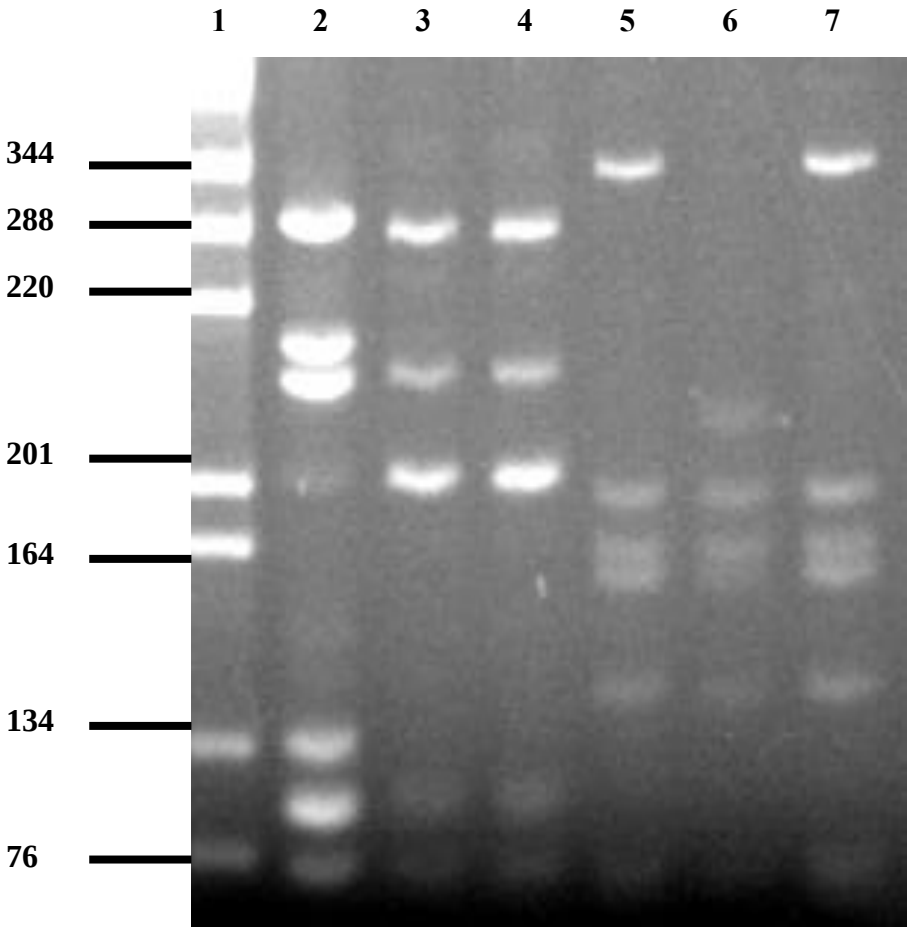

Figure 6.2. RFLP patterns of *serA* homologs. DNA was restricted with *Hae* III and digests were visualized on a 3% agarose gel stained with ethidium bromide. Numbers on the left denote sizes of fragments in the DNA ladder. Lane 1: 1 kb DNA Ladder (Invitrogen, San Diego, CA).; Lane 2: *Thauera selenatis*; Lane 3: *Dechloromonas* sp. L33; Lane 4: *Dechloromonas* sp. A34; Lane 5: *Dechloromonas* sp. CMS; Lane 6: *Dechloromonas* sp. LK1; Lane 7: *Dechloromonas* sp. E5-1

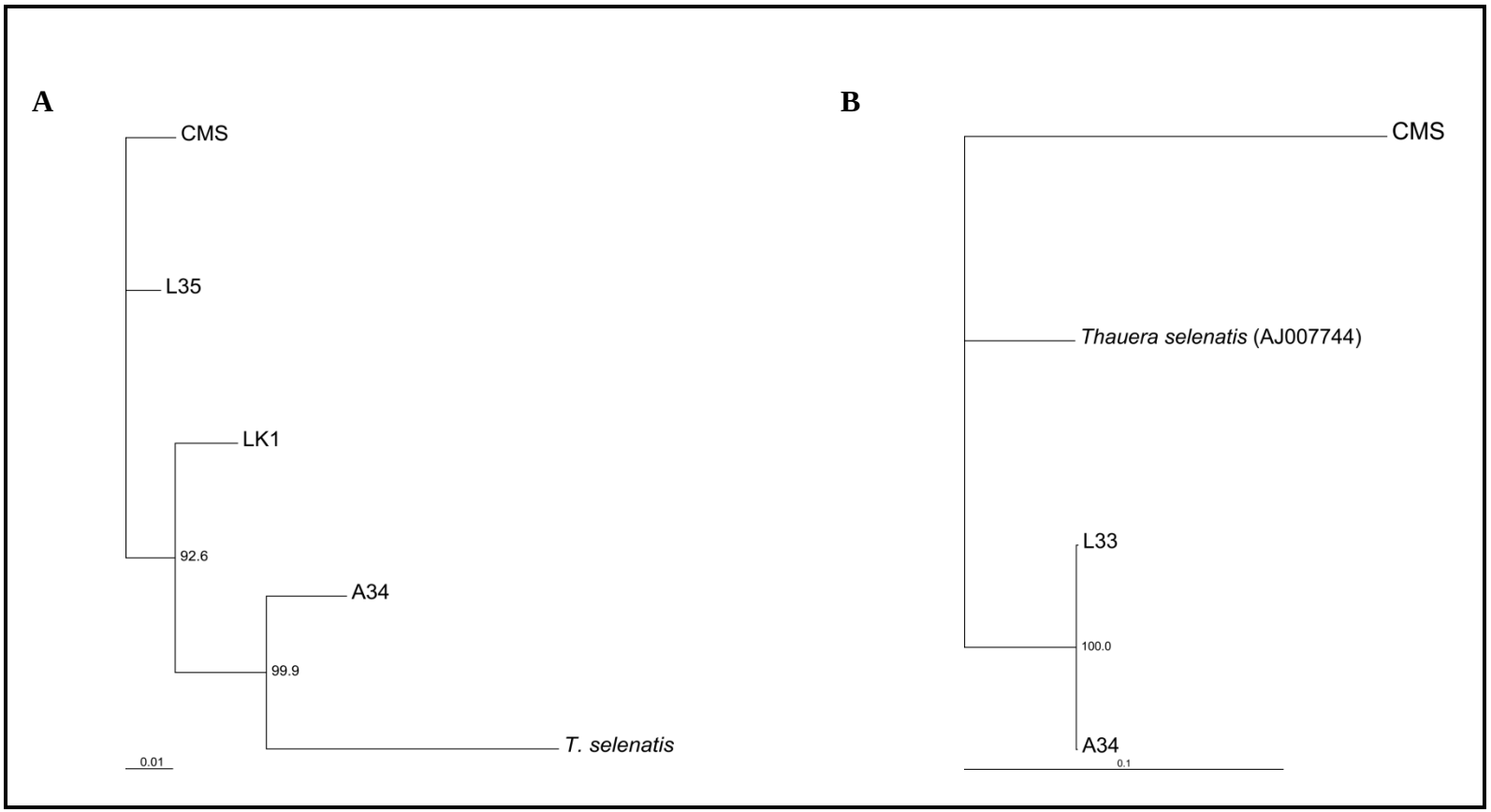

Figure 6.3. Phylogeny of *serA* and *serB* sequences. The relationship of (A) *serA* sequences of *Dechloromonas* isolates to *T. selenatis* and (B) *serB* sequences of *Dechloromonas* isolates to *T. selenatis*

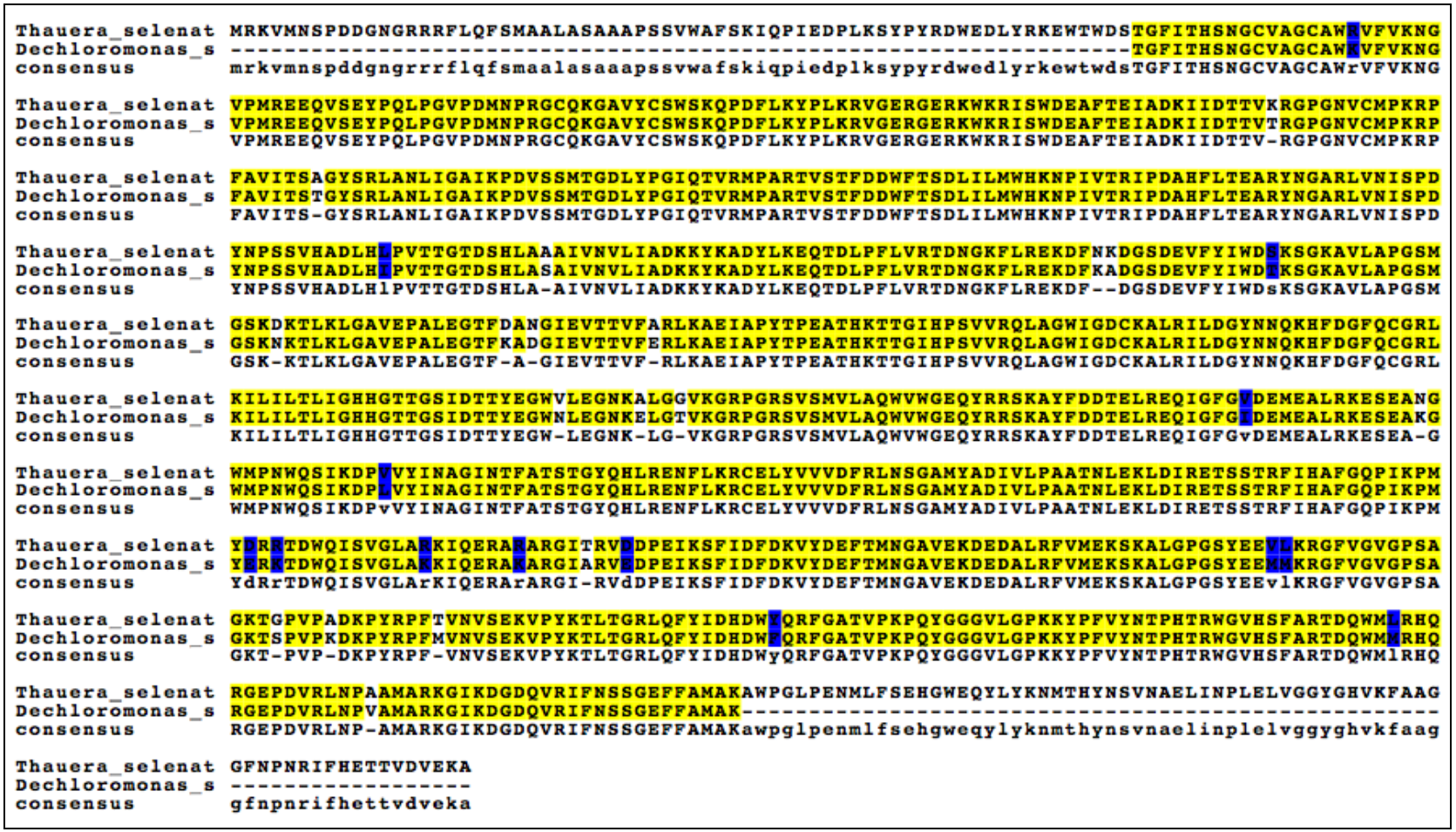

Figure 6.4. Alignment of the *serA* amino acid sequences. *T. selenatis* and *Dechloromonas sp.* A34

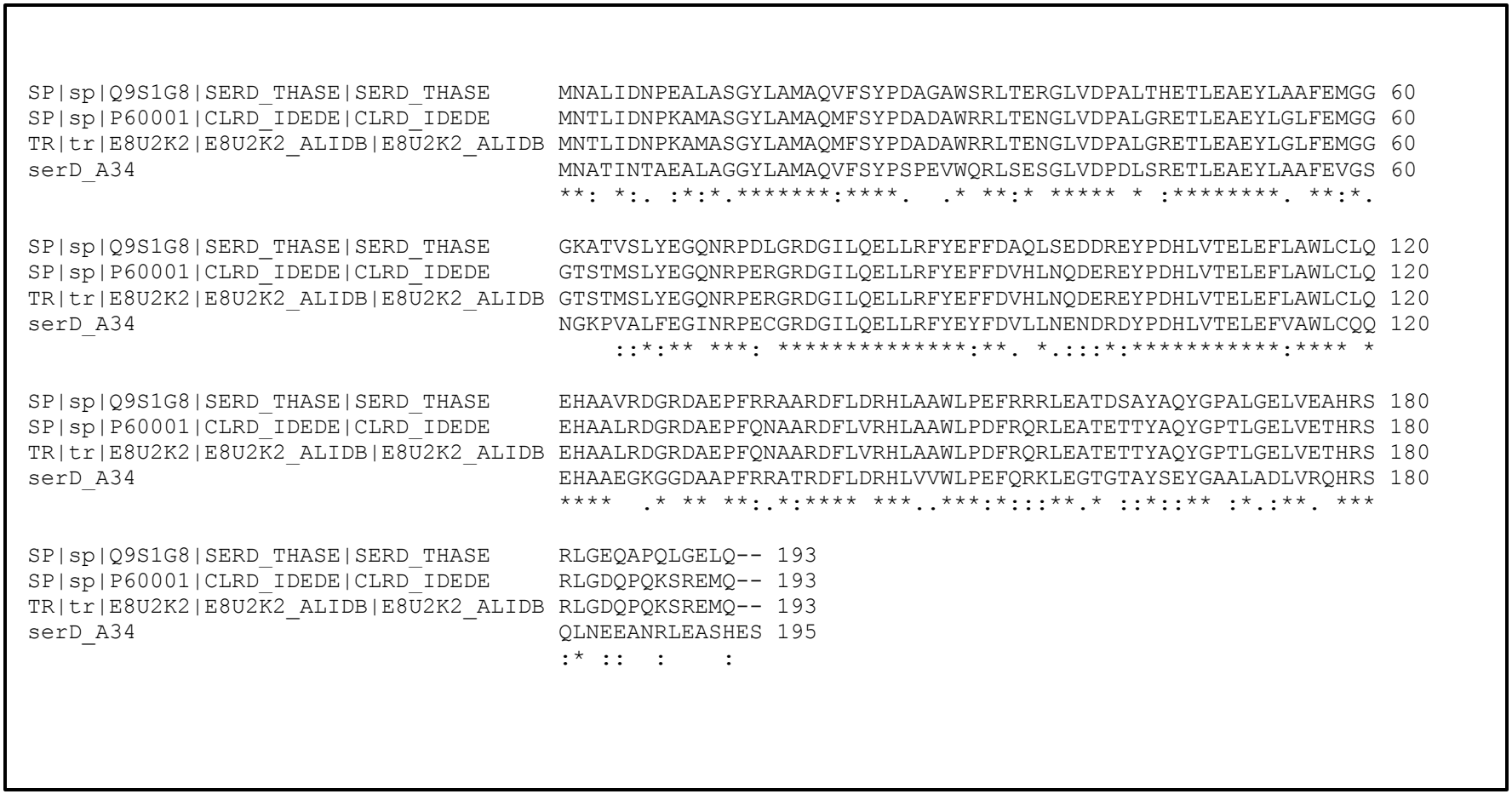

Figure 6.5. Alignment of *serD* and *clrD.* *The serD* amino acid sequences are from of *T. selenatis* and *Dechloromonas sp.* A34 and the *clrD* is rom *Ideonella*.

Figure 6.6. Alignment of *serC* and *clrC*. The *serC* amino acid sequence if rom *T. selenatis* and *Dechloromonas sp.* A34 and the *clrC* from *Ideonella.*

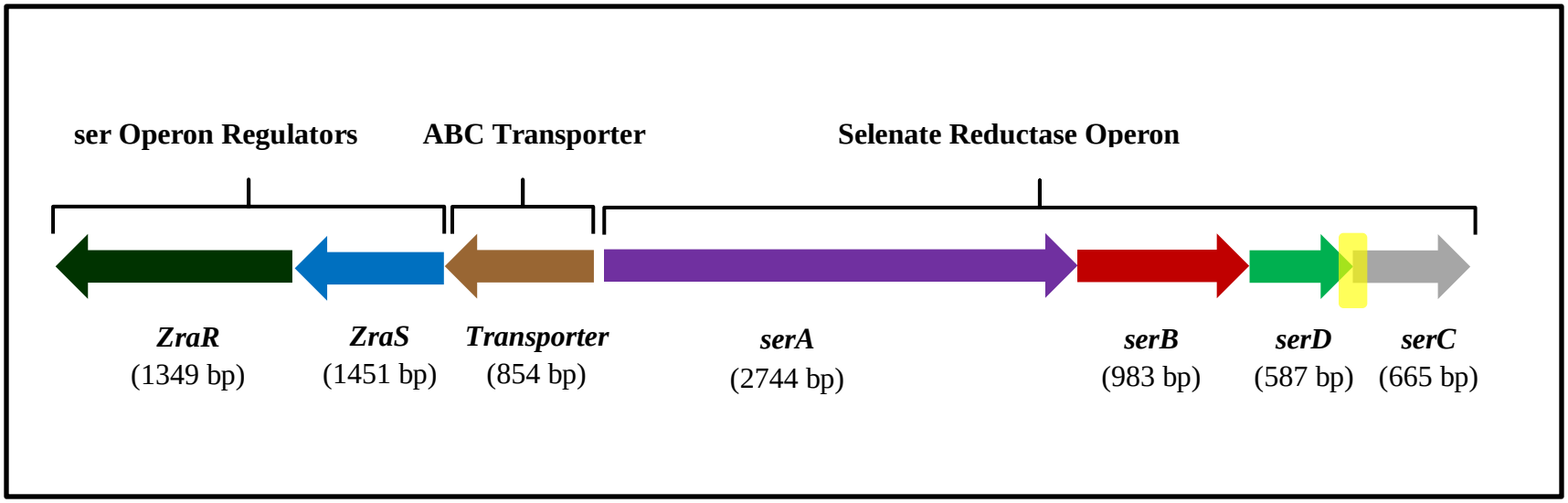

Figure 6.7. *Dechloromonas* sp. A34 selenate reductase operon. Full genome sequence showed the presence of ZraR and ZraS, a transcriptional regulator and sensor protein respectively, and a phosphate ABC transporter upstream of the *ser* operon. The yellow highlighted area in the *serD* and *serC* genes show where the substitution of 278 bp occurred in the *Dechloromonas* sp. A34 *ser* operon compared to *T. selenatis*.

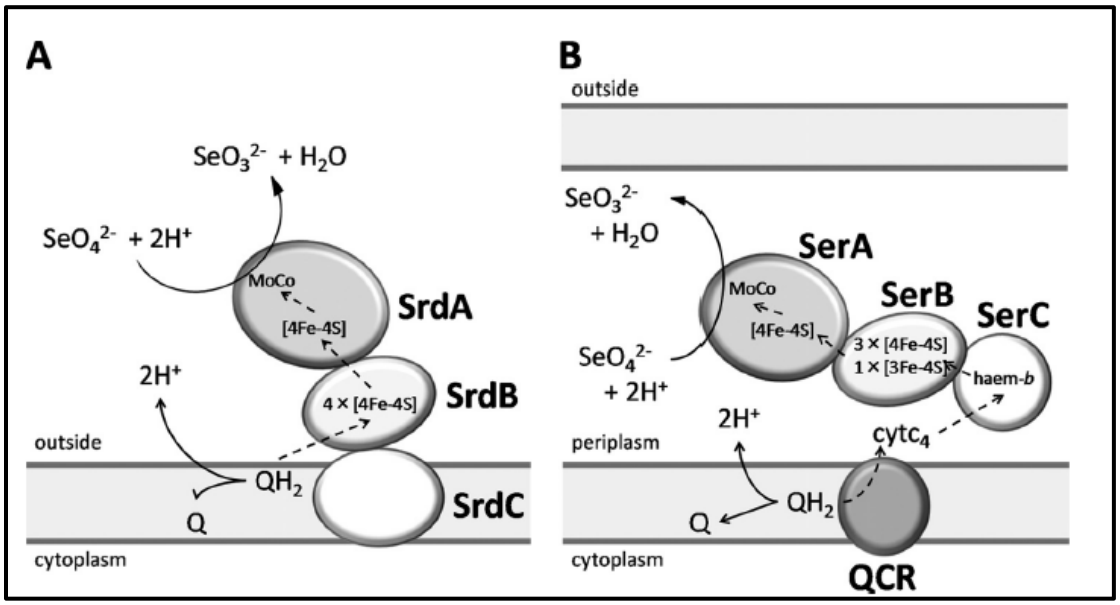

Figure 6.8. Conceptual model interactions of SrdBCA and SerABC. SrdBCA is from *Bacillus selenatarsenatis* and SerABC from *Thauera selenatis* (A) In *B. selenatarsenatis*, selenate reduction is coupled with quinol oxidation. SrdC mediates quinol oxidation, providing two electrons to SrdB. Electrons pass through the [4Fe-4S] clusters of SrdB and SrdA, and selenate is reduced after receiving electrons via a molybdenum cofactor. (B) In *T. selenatis*, SerABC is a periplasmic soluble enzyme. SerABC receives electrons from cytochrome *c*4, which is reduced by quinol-cytochrome *c* oxidoreductase coupled with quinol oxidation. The dashed arrows represent electron flow. QCR, quinol-cytochrome *c* oxidoreductase; Q, quinones; QH_2, quinols; $cytc_4$, cytochrome *c*4; [4Fe-4S], [4Fe-4S] iron-sulfur cluster; [3Fe-4S], [3Fe-4S] iron-sulfur cluster; MoCo, molybdenum cofactor; SeO_4^{2-}, selenate; SeO_3^{2-}, selenite (Kuroda 2011).

Chapter 7: Conclusions to Selenium Studies

Selenium is of great concern because, while an essential trace element required for cellular function, it can be toxic at higher doses, (Barceloux 1999, Knotek-Smith 2006). Ultimately, as much as 40% of all selenium is introduced into the environment by anthropogenic means, mostly from the use of fertilizers or from mining activities (Barceloux 1999, Lenz 2009). The Phosphoria Formation has been mined for over a century and stretches along portions of Idaho, Utah, Wyoming and Montana. In southeastern Idaho, excess selenium in the environment is primarily occurring as a result of phosphate mining activities. When waste rock is exposed to the environment, reduced selenium is oxidized to the more mobile forms, Se^{+6} and Se^{+4}, leading to the hyperaccumulation of Se in plants. Ultimately, livestock that grazes on the vegetation in the reclaimed areas are at risk for selenosis and some animals have had to be euthanized. Leaching of selenium from these soils as a result of precipitation runoff is another concern because the Se ends up in nearby streams and rivers posing a threat to aquatic animals due to overexposure (Hamilton 2004).

In other regions, the use of fertilizers in agriculture is the major contributor to selenium runoff and entry into watersheds and soils. One of the most infamous incidents of selenium contamination due to agricultural practices occurred at the Kesterson Reservoir in California. Selenium leached from soils entered the reservoir and the elevated levels of selenium proved toxic to the fish and waterfowl living in the nearby Kesterson Wildlife Refuge. Many fish and birds died or suffered deformities such as abnormal beaks, ectrodactyly, and anophthalmia (Barceloux 1999). Similar incidences occurred in nearby Tulare Basin, at Belews Lake in North Carolina, and in Martin Reservoir, Texas. In the cases of Belews Lake and Martin Reservoir, lakes were used to provide cooling water for coal-fired power plants. Exchange of the cooling water with basin water resulted in large concentrations of selenium being released into the water, and in Belews Lake, enough selenium was released into the water to eliminate most of the fish from the reservoir (Barceloux 1999).

Many treatment options are being considered for the attenuation and remediation of selenium in impacted areas. The major problem with attenuation and remediation is that there are

often multiple differences in conditions at different sites such as the total selenium concentration, the species of selenium present, biological differences and physio-chemical differences (Lenz 2009). These differences make it difficult to develop a one size fits all approach so many different methods are being considered. However, each method tends to have its drawbacks, as discussed in Ch. 5.

Natural attenuation would be an ideal process for selenium remediation as it does not require much in the way of resources (Stillings 2004). The use of plants to remediate selenium contaminated areas has been successful although plants would have to be harvested and either discarded safely, or utilized for feed or manufacturing processes thus increasing the costs. Plants which have accumulated selenium might pose a risk to wildlife that forage on lands within a contaminated site if they were not disposed of quickly.

Microbial transformation of selenium is another possibility since there are many microorganisms in soils that can interact with selenium species. Some microbes can reduce selenate to Se^0, and these organisms play a major role in affecting the fate of selenium species in aquatic environments (Lovely 1993). The efficacy of microbiological reduction of selenium is a favored and cost efficient option especially when compared to other processes. Studies by Knotek-Smith *et al*. (2006) showed that there were many indigenous microorganisms in soils surrounding a mine site in southeastern Idaho that could reduce selenate to elemental selenium, and additional studies have supported the use of indigenous microorganisms to immobilize selenium. For example, selenium reduction was reported using a consortium of *Bacillus* isolates from selenium enriched natural soils (Prakash 2010), and a selenate respiring microorganism, *Enterobacter hormaechei*, was isolated from a coal mine tailings pond sediment (Siddique 2007). Although nitrate can be inhibitory for effective selenium removal by microorganisms, studies have shown that some organisms, including *Sulfurospirillum barnesii*, can simultaneously reduce nitrate and selenate (Gadd 2000). Several other Se-reducing microorganisms have been identified and have been shown to be efficient at Se reduction (Baesman 2009,Yamamura 2007, Fisher 2008, Hockin 2005, He 2010, Antonioli 2007, Hunter 2009, Pearce 2009, Ikram 2010). These studies show that

organisms belonging to different genera can reduce selenium oxyanions to less toxic forms supporting the possibility that microbial selenium reduction is a viable remediation option.

Despite the studies reported thus far, there is still little known about the diversity of microorganisms that interact with Se in the environment and the metabolic pathways used during Se reduction. To date, only two selenium reducing organisms, *Thauera selenatis* and *Bacillus selenatarsenatis,* have been well studied (Schröder 1997, Kuroda 2011). Many organisms have been identified that are capable of reducing selenate but these are the only two organisms where the enzyme for selenate reduction has been characterized. There are still many questions to be answered as to what regulates Se reduction by these organisms and what is the function of certain genes identified as part of a Se reduction operon (Schröder 1997, Kuroda 2011). Putative selenate reductases have been identified in other organisms but more studies are needed to confirm the selenate reduction pathway in those isolates (Watts 2003, Bébien 2002).

Five selenate reducing *Dechloromonas* sp., collected from seleniferous shales, were previously identified and characterized in the laboratory. This was the first time that *Dechloromonas* sp. have been described as respiring selenate to conserve energy. Although it was known that these organisms could reduce selenate, it was not known if they contained a ser operon similar to that in *Thauera selenatis*, or if they used a different mechanism such as the one found in *Bacillus selenatarsenatis,* or a completely different one altogether. Previous attempts made to identify the selenate reductase in these organisms were unsuccessful however, primers to the serA operon in *T. selenatis* were successful and amplified an approximately 2.4 Kb fragment. Sequencing of the amplicon showed that we had indeed targeted the *serA* gene in these isolates. Using the sequence of *T. selenatis*, we were able to make a primer to *serB* and were able to amplify a 3.5 Kb fragment that included most of the *serA* and *serB* genes. Additional primers were used to complete sequencing of the fragments. As sequencing was completed additional primers targeting the end of those sequences could be manufactured.

As mentioned in the previous chapter, four of the *serA* amplicons from these isolates showed high sequence homology to the *serA* gene found in *T. selenatis* and four of the isolates showed high sequence homology to the *serB* gene also found in *T. selenatis*. These results indicated that all five of these organisms likely use the same pathway to reduce selenate to elemental selenium.

Efforts to identify *serC* and *serD* in the *Dechloromonas* sp. using both PCR and Tn-5 methods were unsuccessful after multiple attempts. Additionally, we constructed a serA::tet fragment in an attempt to replace the *serA* gene in A34 using crossover to further characterize the selenite reductase in A34. A34 was transformed with the serA::tet fragment using a method provided by J. Coates (unpublished; UC Berkeley, CA) but we have yet been able to obtain a mutant. Given our difficulty in further characterizing the ser operon we chose to perform full genome sequencing on A34 in collaboration with NBACC as a final option.

Analysis of the annotated genome sequence provided by NBACC showed that the *serABDC* operon was present in *Dechloromonas* sp. A34 and both serA and serB were closely related to that of *Thauera selenatis* indicating that that the ser operon is highly conserved between the two genera. A 278 bp replacement of sequence was found in the A34 operon and spanned approximately the last 20% of serD and first 23% of serC. However, these nucleotide replacements don't seem to impact the function of the ser operon in A34. It is currently unknown what this portion of the sequence does. BLAST searches against the NCBI database do not seem to match this fragment with anything in the database. Further sequencing of these genes will need to be performed to confirm that these nucleotides are not a match to those found in *Thauera*.

Research on *T.selenatis*' operon shows the presence of a histidine kinase immediately upstream of the ser operon. We wished to study this further because it was believed that this histidine kinase was involved in the regulation of the ser operon. Upon analysis of the A34 genome, the presence of the transcriptional regulatory and sensory proteins, ZraR and ZraS (hydH/G) respectively (Leonhartsberger 2001), were identified upstream of the A34 *ser* operon. It is likely that the ZraR and ZraS are equivalent to the histidine kinases identified in

T. selenatis and it is likely that these proteins regulate the ser operon in A34. As previously mentioned, studies have shown ZraR and ZraS are a two-component system which has been shown to regulate the zinc and lead response system as well as regulation of hydrogenase 3 (Leonhartsberger 2001). Given this, it would be likely that it would regulate the ser operon as well. A Phosphate ABC transporter immediately upstream of the ser operon in A34 was annotated based on the genome sequence that was obtained. Since this transporter lies between the ser operon and its likely regulators, we hypothesize that this transporter is responsible for transport of selenium in and out of the periplasm of the cell.

The presence of nitrate can be inhibitory for effective selenium reduction by microorganisms since reduction of nitrate tends to be favored over reduction of selenium since the organism can gain more energy reducing nitrate over that of selenium species. Additionally, it has been shown that nitrate might actually be involved in the oxidation of Se^0 to Se^{+6} (Wright 1999). However, work with *T. selenatis* showed that the ATP yield for nitrate respiration versus selenate respiration was 5.5 ATP/acetate oxidized and 5.7 ATP, respectively, so it appears that nitrate is not energetically favorable but nitrate just be more available (Macy 1993). In *Sulfurospirillum barnesii,* both nitrate and selenate are reduced simultaneously and it has been suggested that the enzymes for selenate and nitrate reduction may be constitutively expressed (Gadd 2000).

Based on the *Dechloromonas* A34 genome, genes for the periplasmic nitrate reductase, *napA* and *napB*, are located distant from the putative *ser* genes. This suggests that the periplasmic nitrate reductase in *Dechloromonas* A34 does not play a role in selenate reduction. However, it is unknown how the nitrate and selenate reductases operate in A34 and if they can co-reduce both molecules at the same time or if the operation of the nitrate reductase is inhibitory for selenate reduction. Preliminary results indicate that in the presence of nitrate and selenate, nitrate is used preferentially by *Dechloromonas* A34 (Childers, unpublished), but it is unclear if genes are constitutively expressed like they are in *Sulfurospirillum barnesii*. Similarly, a putative sulfate reductase is located approximately 1.8 Mb downstream of the *ser* operon in *Dechloromonas* A34, suggesting selenate and sulfate reduction are

performed by distinct enzymes. Future studies are necessary to confirm these predictions generated from analysis of the *Dechloromonas* A34 genome.

Based on our current understanding of microbial selenate redution, it is proposed that *Dechloromonas* sp. A34 contains a selenate reductase operon nearly identical to that of *Thauera* selenatis, both in form and function, rather than which has been described for *Bacillus selenatarsenatis* (Schröder 1997, Kuroda 2011). Kuroda et al. (2011) hypothesized that since Gram-positive bacteria do not have an outer membrane and periplasmic space, there are potential differences between the selenate reductases in Gram-positive versus Gram-negative bacteria, such as whether the proteins are soluble or membrane bound and whether they are cytoplasmic versus extracytoplasmic. Because dechloromonads are gram-negative, this explains why *Dechloromonas* sp. A34 produces a selenate reductase that functions more like the gram-negative *T. selenatis* selenate reductase rather than the *B. selenatarsenatis* selenate reductase. However, this is yet to be ascertained given that selenate reductases have only been studied in a handful of organisms. If the proposal by Kuroda et. al. (2011) holds true, then selenate reductase enzymes may show conservation across all selenate reducing organisms based solely on their Gram status but this is yet to be determined.

Although microbial reduction of selenate appears to be the most cost efficient method of remediation and has high efficacy, it would be beneficial to examine additional organisms that can reduce selenium oxyanions to better understand selenium cycling in nature. Many of the known selenate reducing organisms may be good candidates to manipulate for natural attenuation of Se, but they are so poorly understood that further studies are needed before selenium reduction strategies using microbes can be optimized in the field. It may be possible that a combination of organisms may prove to be the most efficient at selenate reduction, or that gram positive organisms may be more efficient at immobilizing selenium than gram negative organisms. Currently there are many unknowns regarding the microbial transformations of selenium and the only way to improve on these processes is to gain a better understanding of the microorganisms.

As mentioned in chapter 5, Applied Biosystems has developed an optimized mixture of bacterial enzymes from microorganisms isolated from selenium contaminated mining water and soil. These enzymes have the capability to reduce both selenite and selenate to elemental selenium thus making them ideal for use in remediation of contaminated soils and water. However, over time, there was a loss of stability in the enzyme preparations. Despite this process ending with a decision that it is unfeasible, I think the idea of using an enzymatic process could remain viable if more organisms were studied. It is likely that the stability of these enzymes could vary and some might prove to be more stable and efficient over others. Although the selenate reductase in *Dechloromonas* A34 appears to be nearly identical to that of *T. selenatis*, there are changes at the nucleotide level that may account for improved stability of one enzyme over that of the other.

Ultimately, it can be concluded that many more studies are needed, not only in understanding selenate reduction in *Dechloromonas* spp., but in all selenate respiring microorganisms. Until more information is available, it is difficult to draw any final conclusions based on what is currently known. Microorganisms appear to be very promising for use in remediating contaminated soils and water; however optimization of these processes cannot be done until more is known about the enzymes and their functions. The studies in Ch. 6 have provided some needed information to begin more detailed and in depth studies on one specific selenate reducer, *Dechloromonas* A34, but more information will be forthcoming as studies begin on the other selenate-respiring *Dechlromonas* species.

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Appendix A

Final Results for all MSL Isolates

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
1	*Bacillus*	*sp.*	10%	Neg	9	Pos	N/A	Neg
2.1	*Bacillus*	*subtilis subsp. subtilis*	10%	Neg	8	Pos	N/A	Ac+ClO_4^-
2.2	*Streptococcus*	*mitis*	10%	Neg	8	Neg	N/A	Neg
3.1	*Staphylococcus*	*epidermidis*	10%	Neg	8	Neg	N/A	Neg
3.2	*Streptococcus*	*sanguinis*	10%	Neg	8	Neg	N/A	Neg
3.3	*Streptococcus*	*sanguinis*	10%	Neg	8	Neg	N/A	Neg
4.1.1	*Neisseria*	*sp.*	10%	Neg	8	Neg	N/A	Neg
4.1.2	*Bacillus*	*pumilus*	10%	Neg	8	Neg	N/A	Neg
4.2	*Neisseria*	*sp.*	20%	Pos	9	Neg	Neg	Neg
4.3.1	*Staphylococcus*	*lugdunensis*	10%	Neg	8	Neg	N/A	Neg
4.3.2	*Bacillus*	*pumilus*	10%	Neg	8	Neg	N/A	Neg
6.1	*Bacillus*	*barbaricus*	10%	Neg	8	Neg	N/A	Neg
7.1A	*Brevibacterium*	*halotolerans*	20%	Neg	8	Neg	N/A	Neg
8.1	*Bacillus*	*niacini*	10%	Neg	8	Neg	N/A	Ac+ClO_4^-
9.1	*Bacillus*	*sp.*	10%	Pos	9	Neg	Pos	Neg
10.1	*Paenibacillus*	*sp.*	10%	Neg	9	Neg	N/A	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
11.1	*Bacillus*	*pumilus*	10%	Neg	9	Neg	N/A	Neg
12.1	*Staphylococcus*	*warneri*	10%	Neg	8	Neg	N/A	Neg
12.2	*Rothia*	*amarae*	10%	Neg	8	Neg	N/A	Neg
13.1	*Bacillus*	*megaterium*	10%	Neg	8	Neg	N/A	Neg
14.1	*Staphylococcus*	*sp.*	10%	Neg	8	Neg	N/A	Neg
15.1	*Bacillus*	*sp.*	10%	Neg	8	Neg	N/A	Neg
15.2	*Bacillus*	*oleronius*	10%	Pos	8	Neg	N/A	Neg
15.3	*Streptococcus*	*tigurinus*	10%	Neg	8	Neg	N/A	Neg
16.1	*Staphylococcus*	*epidermidis*	20%	Neg	8	Neg	N/A	Neg
17.1	*Staphylococcus*	*sp.*	20%	Neg	9	Neg	N/A	Neg
17.2	*Staphylococcus*	*sp.*	10%	Neg	8	Pos	N/A	Neg
18.1	*Acinetobacter*	*sp.*	10%	Pos	9	Neg	N/A	Neg
19.1	*Staphylococcus*	*epidermidis*	10%	Neg	8	Neg	N/A	Neg
20.1	*Acinetobacter*	*Iwofii*	10%	Pos	9	Neg	N/A	Neg
20.2	*Acinetobacter*	*sp.*	10%	Pos	9	Neg	N/A	Neg
21.1	*Bacillus*	*sp.*	20%	Neg	9	Neg	N/A	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
22.1	*Acinetobacter*	*sp.*	10%	Pos	9	Neg	N/A	Neg
23.1	*Bacillus*	*sp.*	10%	Pos	8	Neg	N/A	Neg
24.1	*Acinetobacter*	*sp.*	10%	Pos	9	Pos	N/A	Neg
26.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Neg	8	Neg	N/A	Neg
27.1	*Bacillus*	*sp.*	10%	Neg	10	Neg	N/A	Neg
27.2	*Bacillus*	*mojavensis*	10%	Pos	8	Neg	N/A	Neg
28.1	*Staphylococcus*	*epidermidis*	20%	Neg	9	Neg	N/A	Neg
28.2	*Staphylococcus*	*hominis subsp. hominis*	10%	Neg	8	Neg	N/A	Neg
29.1	*Bacillus*	*sp.*	10%	Neg	8	Neg	N/A	Neg
30.1	*Bacillus*	*sp.*	10%	Neg	8	Neg	N/A	Neg
30.2	*Bacillus*	*sp.*	10%	Neg	8	Neg	N/A	Neg
30.3.1	*Moraxella*	*osloensis*	10%	Neg	9	Neg	N/A	Neg
30.3.2	*Moraxella*	*osloensis*	10%	Neg	9	Neg	N/A	Neg
31.1	*Bacillus*	*sp*	10%	Pos	7	Neg	N/A	Neg
32.1	*Bacillus*	*sp.*	5%	Neg	8	Neg	N/A	Neg
34.1	*Bacillus*	*sp.*	5%	Neg	8	Pos	N/A	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
34.2	*Brevibacillus*	*invocatus*	10%	Neg	8	Neg	N/A	Neg
35.1	*Bacillus*	*sp.*	10%	Neg	8	Neg	N/A	Neg
36.1	*Brevibacillus*	*invocatus(97%)*	10%	Neg	9	Neg	N/A	Neg
37.1	*Bacillus*	*niabensis(89%)*	10%	Neg	8	Neg	N/A	Neg
38.1	*Brevibacillus*	*invocatus(93%)*	10%	Neg	8	Neg	N/A	Neg
39.1	*N/A*		10%	Neg	8	Neg	N/A	Neg
41.1	*N/A*		10%	Neg	8	Neg	N/A	Neg
42.1	*Staphylococcus*	*sp.*	10%	Neg	8	Neg	N/A	Neg
42.2	*Staphylococcus*	*warneri*	20%	Neg	9	Pos	N/A	Neg
43.1	*Bacillus*	*sp*	10%	Pos	8	Neg	N/A	Neg
44.1	*Sphingopyxis*	*alaskensis*	5%	Neg	8	Neg	N/A	Neg
44.2	*Acinetobacter*	*junii*	10%	Pos	8	Neg	N/A	Neg
45.1	*Staphylococcus*	*capitis*	20%	Neg	8	Neg	N/A	Neg
46.1	*Sphingopyxis*	*alaskensis*	20%	Pos	8	Neg	Neg	Neg
47.1	*Sphingopyxis*	*alaskensis*	20%	Pos	9	Neg	Neg	Neg
47.2	*Sphingomonas*	*paucimobilis*	10%	Neg	8	Neg	N/A	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
48.1	*Sphingomonas*	*sp.*	10%	Neg	8	Neg	N/A	Neg
48.2	*Micrococcus*	*luteus*	10%	Neg	9	Neg	N/A	Neg
48.3	*Bacillus*	*pumilus*	Neg	Neg	7	Pos	Pos	Neg
49.1	*Brevundimonas*	*sp.*	10%	Neg	8	Neg	N/A	Neg
50.1	*Bacillus*	*amyloliquefaciens*	5%	Neg	8	Neg	N/A	Neg
50.2	*Staphylococcus*	*epidermidis*	10%	Neg	8	Pos	N/A	Neg
50.3.1	*Staphylococcus*	*epidermidis*	20%	Neg	8	Neg	N/A	Neg
50.3.2	*Staphylococcus*	*epidermidis*	20%	Neg	9	Pos	N/A	Neg
51.1	*Bacillus*	*sp*	20%	Neg	7	Neg	N/A	Neg
52.1	*Sphingomonas*	*molluscorum*	10%	Neg	8	Neg	N/A	Neg
53.1.1	*Staphylococcus*	*luqdunensis*	10%	Neg	8	Neg	N/A	Neg
53.1.2	*Bacillus*	*sp.*	10%	Neg	7	Neg	N/A	Neg
54.1	*Bacillus*	*licheniformis*	20%	Neg	8	Neg	N/A	Neg
55.1.1	*Bacillus*	*megaterium*	10%	Neg	8	Neg	N/A	Neg
56.1	*Bacillus*	*anthracis/cereus*	20%	Neg	9	Neg	N/A	Neg
57.1.1	*Staphylococcus*	*epidermidis*	20%	Neg	8	Neg	N/A	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
57.1.2	*Staphylococcus*	*epidermidis*	20%	Neg	8	Neg	N/A	Neg
58.1.2	*Bacillus*	*niabensis*	10%	Neg	8	Neg	N/A	Neg
59.1.1	*Bacillus*	*anthracis*	10%	Neg	8	Neg	N/A	Neg
59.1.2	*Staphylococcus*	*luqdunensis*	10%	Neg	8	Neg	N/A	Neg
60.1.1	*Bacillus*	*atrophaeus*	10%	Neg	9	Neg	N/A	Neg
60.1.2	*Bacillus*	*sp.*	10%	Neg	9	Neg	N/A	Neg
61.1.1	*Pseudomonas*	*psychrotolerans*	Neg	Neg	7	Neg	Pos	Neg
61.1.2	*Bacillus*	*sp.*	10%	Neg	9	Pos	N/A	Neg
61.1.3	*Staphylococcus*	*aureus subsp. aureus*	20%	Neg	9	Neg	N/A	Neg
62.1.1	*Staphylococcus*	*epidermidis*	20%	Neg	9	Neg	N/A	Neg
63.2	*Bacillus*	*megaterium*	10%	Neg	8	Pos	N/A	Neg
64.1.1	*Bacillus*	*sp.*	10%	Neg	8	Neg	N/A	Neg
65.1.1	*Bacillus*	*pumilus*	10%	Neg	9	Neg	N/A	Neg
65.1.2	*Bacillus*	*pumilus*	10%	Neg	9	Neg	N/A	Neg
66.1.1	*Bacillus*	*pumilus*	20%	Pos	7	Neg	Neg	Neg
66.1.2	*Staphylococcus*	*sp.*	20%	Pos	9	Neg	Pos	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
66.1.3	*Staphylococcus*	*sp.*	20%	Pos	9	Neg	Neg	Neg
67.1	*Bacillus*	*sp*	10%	Pos	9	Neg	Neg	Neg
68.1	*Bacillus*	*pumilus*	20%	Pos	9	Pos	Pos	Neg
69.1.1	*Bacillus*	*safensis*	10%	Pos	9	Neg	Pos	Neg
70.1.1	*Bacillus*	*megaterium*	10%	Neg	9	Neg	Neg	Neg
71.1	*Streptomyces*	*albogriseolus*	10%	Pos	9	Neg	Pos	Neg
72.1.1	*Staphylococcus*	*epidermidis*	20%	Pos	7	Neg	Pos	Neg
73.1	*Stenotrophomonas*	*maltophilia*	20%	Pos	9	Neg	Neg	Neg
100	*Pseudomonas*	*putida*	20%	Pos	7	Neg	Neg	Neg
101.1	*Acinetobacter*	*sp*	10%	Pos	9	Neg	Pos	Neg
101.2	*Acinetobacter*	*sp.*	10%	Pos	9	Pos	Pos	Neg
102	*Bacillus*	*atrophaeus*	20%	Neg	7	Pos	Neg	Neg
103.1	*Leclercia*	*adecarboxylata*	10%	Pos	9	Neg	Pos	Neg
103.2.1	*Bacillus*	*anthracis/cereus*	10%	Pos	9	Neg	Neg	Neg
103.2.2	*Bacillus*	*cereus*	10%	Pos	7	Neg	Pos	Neg
103.3.1	*Leclercia*	*adecarboxylata*	10%	Pos	9	Neg	Pos	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
104.1.1	*Bacillus*	*cereus*	20%	Neg	9	Neg	Pos	Neg
104.1.2	*Staphylococcus*	*epidermidis*	20%	Pos	8	Pos	Pos	Neg
105.1	*Bacillus*	*cereus*	20%	Pos	7	Neg	Pos	Neg
105.2	*Bacillus*	*cereus*	20%	Pos	9	Neg	Pos	Neg
106	*Pseudomonas*	*putida*	10%	Pos	9	Neg	Neg	Neg
107	*Staphylococcus*	*epidermidis*	20%	Pos	7	Neg	Pos	Neg
108.1	*Bacillus*	*sp.*	10%	Pos	9	Neg	Pos	Neg
109.1	*N/A*		10%	Pos	9	Neg	Pos	Neg
110.1	*Bacillus*	*megaterium*	10%	Pos	9	Neg	Pos	Neg
111.1	*Staphylococcus*	*warneri*	10%	Pos	7	Neg	Pos	Neg
112.1	*Bacillus*	*pumilus*	10%	Pos	10	Neg	Pos	Neg
113.1	*Leclercia*	*adecarboxylata*	10%	Pos	9	Neg	Pos	Neg
114.1	*Bacillus*	*sp*	20%	Neg	8	Neg	Pos	Neg
116.1	*Sporosarcina*	*ginsengisoli*	20%	Pos	9	Neg	Neg	Neg
116.2	*Bacillus*	*licheniformis*	20%	Pos	9	Neg	Pos	Neg
117.1	*Paenibacillus*	*sp.*	20%	Pos	9	Neg	Pos	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
120.1A	*Bacillus*	*subtilis subsp. subtilis*	10%	Pos	7	Neg	Pos	Neg
125.1	*Leuconostoc*	*mesenteroides*	N/A	N/A	N/A	Neg	N/A	Neg
127.1.1A	*Bacillus*	*megaterium*	10%	Pos	9	Neg	Pos	Neg
127.1.1B	*Bacillus*	*megaterium*	20%	Pos	9	Neg	Pos	Neg
128.1.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Pos	9	Neg	Pos	Neg
128.1.2	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	5%	Pos	9	Neg	Pos	Neg
128.1.3	*Bacillus*	*jeotgali*	10%	Pos	7	Neg	Neg	Neg
129.1	*Bacillus*	*pumilus*	10%	Pos	9	Neg	Pos	Neg
130.1	*Bacillus*	*pumilus*	10%	Pos	10	Neg	Pos	Neg
131.1	*Bacillus*	*licheniformis*	20%	Neg	7	Neg	Neg	Neg
133.1	*Bacillus*	*subtilis subsp. subtilis*	20%	Pos	7	Neg	Pos	Neg
134.1	*Bacillus*	*weihenstephanensis*	5%	Pos	9	Neg	Pos	Neg
136.1	*Bacillus*	*amyloliquefaciens*	10%	Pos	7	Neg	Pos	Neg
137.1	*Bacillus*	*sp.*	20%	Pos	9	Pos	Pos	Neg
138.1	*Leclercia*	*adecarboxylata*	10%	Pos	9	Neg	Pos	Neg
139.1	*Brevibacillus*	*borstelensis(86%)*	10%	Pos	9	Pos	Neg	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
140.1	*Bacillus*	*subtilis*	10%	Pos	8	Neg	Neg	Neg
142.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	Neg	Neg	7	Pos	Pos	Neg
145.1	*Bacillus*	*subtilis*	10%	Pos	9	Neg	Pos	Neg
147.1A	*Bacillus*	*sp.*	10%	Pos	9	Neg	Pos	Neg
148.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Pos	9	Neg	Pos	Neg
149.1A	*Bacillus*	*mojavensis*	10%	Neg	9	Neg	Pos	Neg
149.1B	*N/A*		20%	Neg	8	Neg	Neg	Neg
149.2	*Bacillus*	*atrophaeus*	20%	Pos	9	Neg	Neg	Neg
150.1	*Brevibacterium*	*frigoritolerans T type*	10%	Pos	7	Neg	Neg	Neg
151.1.1	*Bacillus*	*pumilus*	Neg	Neg	7	Neg	Pos	Neg
151.1.2	*Bacillus*	*sp.*	Neg	Neg	7	Neg	Pos	Neg
151.2	*Bacillus*	*atrophaeus*	10%	Pos	9	Pos	Neg	Neg
152.1	*Oceanobacillus*	*sp.*	20%	Pos	9	Neg	Neg	Neg
153.1	*Bacillus*	*subterraneus*	10%	Pos	7	Neg	Pos	Neg
154.1	*Bacillus*	*subtilis*	10%	Pos	9	Pos	Pos	Neg
154.2	*Bacillus*	*subtilis subsp. subtilis*	10%	Pos	9	Pos	Pos	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
155.1.1	*Bacillus*	*sp.*	10%	Pos	9	Neg	Pos	Neg
155.1.2	*Bacillus*	*pumilus*	10%	Neg	9	Neg	Neg	Neg
156.1	*Paenibacillus*	*sp*	10%	Pos	7	Neg	Pos	Neg
157.1	*Bacillus*	*subtilis*	10%	Pos	7	Neg	Neg	Neg
159.1	*Bacillus*	*sp.*	10%	Pos	9	Pos	Pos	Neg
159.2	*Bacillus*	*amyloliquefaciens*	10%	Pos		Pos	Pos	Neg
160.1	*Bacillus*	*subtilis subsp. subtilis*	20%	Pos	8	Neg	Pos	Neg
160.2B	*Bacillus*	*sp.*	10%	Neg	9	Neg	Pos	Neg
160.2E	*Bacillus*	*amyloliquefaciens*	20%	Pos	9	Neg	Pos	Neg
161.1.1	*Williamsia*	*sp.*	10%	Pos	9	Neg	Neg	Neg
161.1.2	*Bacillus*	*subtilis*	10%	Neg	7	Neg	Pos	Neg
161.2A	*Bacillus*	*subterraneus*	10%	Pos	9	Neg	Pos	Neg
161.3	*Bacillus*	*humi*	10%	Pos	7	Neg	Pos	Neg
162.1A	*Bacillus*	*subtilis*	10%	Pos	7	Neg	Pos	Neg
162.1B	*N/A*		10%	Pos	7	Neg	Pos	Neg
163.1	*Bacillus*	*amyloliquefaciens*	10%	Pos	7	Neg	Pos	Neg

MSL#	ID		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
164.1.1	*Acinetobater*	*iwoffii*	10%	Pos	7	Neg	Pos	Neg
164.1.2B	*Bacillus*		20%	Pos	9	Pos	Pos	Neg
166.1	*Bacillus*	*licheniformis*	20%	Pos	9	Pos	Pos	Neg
166.1A	*Bacillus*	*amyloliquefaciens*	10%	Neg	9	Neg	Pos	Neg
167.1	*N/A*		10%	Pos	8	Neg	Pos	Neg
168.1	*Streptococcus*	*pneumoniae*	10%	Neg	7	Pos	Pos	Neg
169.1.1	*N/A*		10%	Pos	7	Neg	Neg	Neg
170.1.1	*Bacillus*	*sp.*	10%	Pos	9	Neg	Pos	Neg
170.2	*Bacillus*	*sp.*	20%	Pos	7	Neg	Neg	Neg
171.1.1A	*Bacillus*	*sp.*	10%	Neg	9	Neg	Pos	Neg
171.1.2	*Bacillus*	*pumilus*	10%	Neg	9	Neg	Pos	Neg
172.1.1	*Bacillus*	*pumilus*	10%	Neg	9	Pos	Neg	Neg
172.1.2	*Bacillus*	*sp.*	10%	Neg	9	Pos	Pos	Neg
173.1	*Bacillus*	*subtilis subsp. Inaquosorum*	20%	Pos	7	Neg	Pos	Neg
173.2.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Pos	9	Neg	Pos	Neg
173.2.2	*Bacillus*	*sp.*	10%	Neg	9	Neg	Neg	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
174.1	*Arthrobacter*	*sp.*	10%	Pos	9	Neg	Pos	Neg
179.1	*Bacillus*	*oleronius*	10%	Pos	8	Neg	Pos	Neg
181.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Pos	9	Pos	Pos	Neg
182.1	*Bacillus*	*sp.*	10%	Pos	9	Neg	Pos	Neg
183.1	*Bacillus*	*sp.*	10%	Neg	9	Neg	Pos	Neg
184.1	*Bacillus*	*sp.*	10%	Neg	9	Neg	Pos	Neg
185.1	*Bacillus*	*thermoamylovorans*	10%	Neg	9	Pos	Pos	Neg
186.1	*Bacillus*	*licheniformis*	10%	Neg	7	Neg	Pos	Neg
187.1	*Paenibacillus*	*lactis*	5%	Pos	9	Neg	Neg	Neg
188.1	*Bacillus*	*sp.*	10%	Neg	9	Pos	Pos	Neg
189.1	*Bacillus*	*subtilis subsp. subtilis(85%)*	Unk.	Neg	7	Neg	Pos, Neg	Neg
190.1	*Staphylococcus*	*hominis subsp. hominis*	10%	Neg	9	Neg	Pos	Neg
190.2A	*Staphylococcus*	*hominis subsp. hominis*	20%	Neg	8	Pos	Pos	Neg
194.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	20%	Neg	9	Pos	Pos	Neg
195.1A	*Paenibacillus*	*sp.*	20%	Pos	10	Pos	Pos	Neg
196.1.1	*Staphylococcus*	*sp.*	20%	Neg	9	Neg	Pos	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
196.1.2	*Staphylococcus*	*warneri*	20%	Neg	9	Neg	Pos	Neg
197.1	*Staphylococcus*	*warneri*	20%	Neg	9	Neg	Neg	Neg
197.2	*Staphylococcus*	*epidermidis*	20%	Neg	9	Neg	Neg	Neg
198.1.1A	*Enhydrobacter*	*sp.*	10%	Neg	8	Neg	Pos	Neg
198.1.1B	*Enhydrobacter*	*sp.(95%)*	5%	Neg	7	Neg	Pos	Neg
198.1.2	*Moraxella*	*sp.*	20%	Neg	7	Pos	Pos	Neg
200.1	*Bacillus*	*sp.*	10%	Neg	9	Pos	Pos	Neg
201.1	*Bacillus*	*megaterium*	10%	Neg	9	Pos	Neg	Neg
202.1	*Bacillus*	*megaterium*	5%	Neg	7	Neg	Pos	Neg
203.1	*Bacillus*	*pumilus*	20%	Neg	9	Pos	Neg	Neg
204.1	*Bacillus*	*clausii*	10%	Neg	7	Neg	Pos	Neg
204.2	*Bacillus*	*sp.*	20%	Neg	9	Pos	Pos	Neg
205.1.1B	*Bacillus*	*pumilus*	10%	Neg	9	Pos	Pos	Neg
205.1.2	*Bacillus*	*sp.*	10%	Neg	9	Neg	Pos	Neg
206.1	*Bacillus*	*pumilus*	20%	Neg	9	Neg	Pos	Neg
206.2.1	*Bacillus*	*subtilis subsp. subtilis*	20%	Neg	8	Neg	Pos	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
206.2.2	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Neg	8	Neg	Pos	Neg
207.1	*Bacillus*	*sp.*	20%	Pos	9	Neg	Pos	Neg
208.1	*Bacillus*	*sp.*	10%	Neg	9	Neg	Pos	Neg
209.1	*Bacillus*	*firmus*	10%	Neg	9	Pos	Neg	Neg
210.1	*Bacillus*	*clausii*	10%	Neg	11	Pos	Pos	Neg
211.1	*Bacillus*	*senegalensis*	20%	Neg	7	Pos	Pos	Neg
212.1	*Bacillus*	*pumilus*	10%	Neg	7	Neg	Neg	Neg
212.2	*Paenibacillus*	*sp.*	10%	Pos	7	Neg	Pos	Neg
213.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Neg	9	Pos	Pos	Neg
214.1.1	*Bacillus*	*sp.*	10%	Neg	9	Neg	Pos	Neg
214.1.2	*Bacillus*	*sp.*	10%	Neg	10	Neg	Pos	Neg
214.2	*Bacillus*	*sp.*	10%	Neg	10	Neg	Pos	Neg
215.1	*Bacillus*	*sp.*	20%	Pos	9	Neg	Pos	Neg
216.1.1	*Bacillus*	*pumilus*	20%	Unk.	7	Neg	Pos	Neg
216.1.2	*Bacillus*	*pumilus*	20%	Neg	9	Neg	Pos	Neg
218.1	*Bacillus*	*psychrodurans*	20%	Pos	7	Neg	Pos	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
220.1	*Bacillus*	*sp.*	10%	Neg	9	Neg	Pos	Neg
222.1.1	*Bacillus*	*sp.*	10%	Pos	9	Neg	Pos	For+SO4
222.1.2	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Neg	7	Neg	Pos	Neg
223.1	*Bacillus*	*licheniformis*	10%	Neg	7	Pos	Neg	Neg
224.1.1	*Bacillus*	*drentensis*	10%	Neg	9	Neg	Pos	Neg
225.1.1A	*Bacillus*	*sp.*	10%	Neg	9	Neg	Pos	Neg
225.1.2	*Bacillus*	*megaterium*	10%	Neg	9	Neg	Neg	Neg
225.2.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	20%	Pos	8	Neg	Pos	Neg
227.1.1	*Bacillus*	*cereus*	10%	Neg	9	Neg	Pos	Neg
227.1.2	*Bacillus*	*anthracis*	20%	Neg	9	Neg	Pos	Neg
228.1.1	*Bacillus*	*sp.*	10%	Neg	9	Neg	Neg	Neg
228.1.2	*Bacillus*	*cereus*	5%	Neg	9	Neg	N/A	Neg
233.1	*Staphylococcus*	*warneri*	20%	Neg	9	Neg	Neg	Neg
234.1	*Moraxella*	*osloensis*	10%	Neg	9	Pos	Pos	Neg
236.1.1	*Bacillus*	*pumilus*	10%	Neg	10	Pos	Pos	For+SO4
236.1.2	*Bacillus*	*pumilus*	5%	Neg	9	Pos	Pos	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
237.1.1A	*Bacillus*	*licheniformis*	10%	Pos	9	Neg	N/A	Neg
237.1.2A	*Bacillus*	*licheniformis*	10%	Neg	7	Neg	N/A	Neg
239.1.1A	*Bacillus*	*sp.*	10%	Neg	7	Neg	N/A	Neg
239.1.1B	*Bacillus*	*amyloliquefaciens*	10%	Neg	9	Neg	N/A	Neg
239.1.2	*Bacillus*	*pumilus*	10%	Neg	8	Neg	N/A	Neg
240.1.1	*Bacillus*	*pumilus*	20%	Unk.	9	Pos	Neg	Neg
240.1.2	*Bacillus*	*pumilus*	10%	Neg	9	Neg	Neg	Neg
241.1	*Paenibacillus*	*lautus*	10%	Neg	9	Pos	N/A	Neg
242.1.1	*Bacillus*	*pumilus*	10%	Neg	7	Pos	N/A	Neg
242.1.2	*Bacillus*	*pumilus*	10%	Neg	9	Neg	N/A	Neg
243.1	*Bacillus*	*sp.*	10%	Neg	7	Neg	Pos	Lac+As
244.1	*Bacillus*	*licheniformis*	10%	Neg	7	Neg	Pos	Neg
245.1	*Paenibacillus*	*polymyxa*	10%	Pos	9	Neg	Pos	Neg
246.1	*Bacillus*	*sp.*	10%	Neg	7	Neg	Neg	Neg
247.1	*Bacillus*	*pumilus*	10%	Neg	7	Neg	Pos	Neg
247.2.1	*Bacillus*	*pumilus*	10%	Pos	9	Pos	N/A	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
247.2.2	*Bacillus*	*sp.*	10%	Pos	9	Neg	N/A	Neg
248.1	*Bacillus*	*siralis (T)*	10%	Neg	9	Neg	Pos	Neg
249.1	*Bacillus*	*licheniformis*	10%	Neg	7	Neg	Neg	Lac+As
250.1	*Bacillus*	*licheniformis*	20%	Pos	7	Neg	Pos	Neg
251.1	*Bacillus*	*sp.*	20%	Neg	10	Neg	Pos	Neg
252.1	*Bacillus*	*licheniformis*	10%	Pos	7	Neg	Pos	Neg
253.1.1A	*Staphylococcus*	*pasteurii*	Unk.	Neg	9	Neg	N/A	Neg
253.1.2	*Bacillus*	*sp.*	10%	Neg	9	Neg	N/A	Neg
254.1	*Bacillus*	*pumilus*	10%	Neg	7	Neg	Pos	Neg
255.1.1	*Bacillus*	*megaterium*	10%	Pos	8	Neg	N/A	Neg
255.1.2A	*Bacillus*	*sp.*	10%	Neg	9	Pos	N/A	Neg
258.1A	*Bacillus*	*lentus*	10%	Neg	8	Pos	N/A	Neg
259.1A	*Bacillus*	*sp.*	10%	Neg	9	Neg	Pos	Neg
261.1.1	*Bacillus*	*pumilus*	10%	Neg	9	Neg	N/A	Neg
261.1.2	*Bacillus*	*pumilus*	10%	Neg	9	Pos	N/A	Neg
262.1.1	*Paenibacillus*	*mucilaginosus*	10%	Neg	7	Pos	N/A	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
262.1.2	*Staphylococcus*	*epidermidis*	10%	Neg	7	Pos	N/A	Neg
263.1	*Bacillus*	*sp.*	5%	Pos	7	Neg	N/A	Neg
264.1.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Pos	9	Neg	N/A	Neg
265.1	*Bacillus*	*sp.*	10%	Neg	10	Pos	N/A	Neg
266.1.1	*Bacillus*	*sp.*	10%	Pos	8	Neg	N/A	Neg
266.1.2	*Sporosarcina*	*sp.*	10%	Pos	9	Pos	N/A	Neg
266.2	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Pos	7	Pos	N/A	Neg
267.1A	*Paenibacillus*	*sp.*	10%	Neg	9	Pos	N/A	Neg
268.1	*Bacillus*	*licheniformis*	10%	Neg	9	Neg	N/A	Neg
269.1B	*Paenibacillus*	*favisporus*	10%	Neg	8	Pos	Pos	Neg
270.1	*Bacillus*	*megaterium*	10%	Neg	8	Neg	N/A	Neg
271.1A	*Bacillus*	*subtilis subsp. Subtilis*	10%	Pos	7	Neg	N/A	Neg
272.1.1	*Sporosarcina*	*sp.*	10%	Pos	9	Neg	N/A	Lac+As
272.1.2	*Sporosarcina*	*aquimarina*	10%	Pos	9	Neg	N/A	Lac+As
273.1.1	*Bacillus*	*megaterium*	10%	Pos	9	Neg	N/A	Lac+As
273.1.2	*Bacillus*	*megaterium*	10%	Neg	7	Neg	N/A	Lac+As

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
273.1.3	*Bacillus*	*megaterium*	10%	Neg	9	Pos	N/A	Lac+As
274.1B	*Bacillus*	*sp.*	10%	Neg	8	Pos	N/A	Neg
275.1	*Bacillus*	*sp.*	10%	Neg	7	Pos	N/A	Neg
276.1.1	*N/A*		10%	Neg	7	Neg	Pos	Neg
276.1.2	*Bacillus*	*sp.*	10%	Neg	7	Neg	N/A	Lac+ClO_4^-
277.1.1	*Bacillus*	*thuringiensis*	10%	Neg	9	Neg	N/A	Lac+As
277.1.2.1	*Bacillus*	*sp.*	10%	Neg	9	Neg	N/A	Lac+As
277.1.2.2	*Bacillus*	*sp.*	Neg	Neg	9	Neg	N/A	Neg
278.1.1B	*Bacillus*	*pumilus*	10%	Neg	9	Neg	N/A	Neg
278.1.2	*Bacillus*	*mojavensis*	10%	Neg	9	Neg	N/A	Neg
279.1.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Neg	7	Neg	N/A	Lac+ClO_4^-
279.1.2B	*Gracilibacillus*	*dipsosauri*	10%	Pos	9	Neg	Pos	Lac+ClO_4^-
280.1A	*Bacillus*	*sp.*	10%	Neg	9	Neg	N/A	Neg
281.1	*Sporosarcina*	*sp.*	10%	Neg	7	Neg	N/A	Neg
281.2	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Neg	9	Neg	N/A	Neg
282.1.1	*Bacillus*	*sp.*	10%	Neg	9	Pos	N/A	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
282.1.2	*Bacillus*	*pumilus*	10%	Neg	9	Neg	N/A	Neg
283.1	*Bacillus*	*licheniformis*	10%	Pos	9	Neg	N/A	Neg
284.1.1A	*Bacillus*	*sp.*	10%	Neg	7	Neg	N/A	Neg
284.1.1B	*Bacillus*	*sp.*	10%	Neg	7	Neg	N/A	Neg
284.1.2	*Bacillus*	*sp.*	10%	Neg	9	Neg	N/A	Lac+As
284.2.1	*N/A*		10%	Neg	9	Neg	N/A	Neg
284.2.2A	*Bacillus*	*sp.*	10%	Neg	10	Neg	N/A	Neg
284.2.2B	*Bacillus*	*sp.*	10%	Neg	9	Neg	N/A	Neg
285.1.1	*Bacillus*	*sp.*	10%	Neg	9	Neg	N/A	Neg
285.1.2A	*Bacillus*	*niacini*	5%	Neg	7	Neg	N/A	Lac+ClO_4^-
285.1.2B	*Bacillus*	*sp.*	Neg	Neg	7	Neg	N/A	Neg
286.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	5%	Neg	9	Neg	N/A	Lac+ClO_4
287.1.1	*Bacillus*	*megaterium*	10%	Neg	9	Pos	N/A	Neg
287.1.2	*Bacillus*	*megaterium*	10%	Neg	9	Neg	N/A	Neg
288.1.1	*Bacillus*	*sp.*	10%	Neg	7	Neg	N/A	Neg
288.1.2	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Neg	9	Neg	N/A	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
289.1	*Bacillus*	*sp.*	10%	Neg	9	Neg	N/A	Neg
290.1.1	*Bacillus*	*sp.*	10%	Pos	8	Neg	N/A	Neg
290.1.2	*Bacillus*	*sp.*	10%	Neg	8	Neg	N/A	Neg
291.1	*Paenibacillus*	*barcinonensis*	10%	Neg	9	Pos	Neg	Neg
292.1.1	*Bacillus*	*sp*	10%	Pos	9	Neg	N/A	Neg
292.1.2	*Bacillus*	*sp.*	10%	Neg	9	Neg	N/A	Neg
293.1	*Bacillus*	*safensis*	10%	Pos	9	Neg	N/A	Neg
294.2	*Bacillus*	*sp.*	10%	Neg	9	Neg	N/A	Neg
295.1	*Paenibacillus*	*sp.*	10%	Neg	9	Pos	N/A	Neg
296.1A	*Bacillus*	*sp.*	10%	Neg	7	Pos	N/A	Neg
297.1.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Pos	9	Neg	N/A	Neg
297.1.2	*Bacillus*	*sp.*	10%	Pos	9	Neg	N/A	Neg
298.1.1	*Bacillus*	*sp.*	10%	Neg	9	Neg	N/A	Neg
298.1.2	*Bacillus*	*sp.*	10%	Neg	7	Neg	N/A	Neg
298.2.1	*Bacillus*	*amyloliquefaciens subsp. amyloliquefaciens*	10%	Neg	8	Neg	N/A	Neg
298.2.2	*Bacillus*	*sp.*	10%	Neg	8	Neg	N/A	Neg

MSL#	*ID*		NaCl	4deg	pH	H_2O_2	Desiccation	Anaerobic Growth
104594	*Streptomyces*	*lanatus (T)*	Pos	Pos	9	Neg	Pos	Neg
104595	*Bacillus*	*pumilus*	10%	Pos	8	Neg	Pos	Neg
104596	*Bacillus*	*sp.*	10%	Pos	9	Neg	Neg	Neg
104597	*Bacillus*	*subtilis subsp. Subtilis*	10%	Pos	9	Neg	Pos	Neg
104598	*Bacillus*	*pumilus*	20%	Pos	9	Neg	Pos	Neg