Microbial implications for structural integrity during nuclear waste storage

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UMI
MICROBIAL IMPLICATIONS FOR STRUCTURAL INTEGRITY
DURING NUCLEAR WASTE STORAGE

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Entitled
Microbial Implications for Structural Integrity during Nuclear Waste Storage

is approved in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biology

Examination Committee Chair

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The study of microorganisms in oligotrophic environments is vital to understanding the geology and ecology of Nevada, and in particular, Yucca Mountain and the Nevada Test Site. Native to the Yucca Mountain tuff and to the Nevada Test Site soil are microorganisms, existing in biofilms, which are capable of corroding metals, and producing acids or enzymes that can degrade materials such as wood and cardboard. Studying the environmental conditions that could promote the growth of such microorganisms is essential for the modeling of the Yucca Mountain repository and the waste burial program on the Nevada Test Site. The first phase of this research is to determine boundary limits of temperature and humidity, as they relate to biofilm formation on candidate repository canister materials used in the Yucca Mountain Repository. It is necessary to model a stable, high-level nuclear waste repository that will not corrode within the institutionalized period of 300 years. The second phase involves a different approach from the Yucca Mountain repository modeling. The Nevada Test Site is the location for a proposed low-level nuclear waste repository. Breakdown of waste packaging material will cause a shifting and collapsing of waste
material and surrounding backfill which can collapse the closure cap. Water could percolate down into the waste material creating a leachate that could affect water reservoirs. This study of biofilms should allow the development of a microbially based process to accelerate degradation of waste packaging material prior to closure.

A separate investigation relating to the proposed, high-level nuclear waste repository deals with detecting thermophilic bacteria from calcite deposits within the fractures of Yucca Mountain. There is a possibility that the calcite deposits resulted from an upwelling of subsurface, thermal water containing dissolved calcium carbonate. As water cooled, the dissolved minerals precipitated forming calcite deposits. The thermal water might have selected for thermophilic bacteria; thus, their presence could lend support to the subsurface, thermal water origin of Yucca Mountain's calcite deposits. The possibility that subsurface, thermal water could rise inside Yucca Mountain may impact Yucca Mountain's suitability as the proposed site of a high-level nuclear waste repository.
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ACKNOWLEDGEMENTS

This research was funded by the Department of Energy, Yucca Mountain Project, DE-FC08-98NV12081, Department of Energy, Nevada Environmental Research Park, DE-FC08-98NV13499, and Department of Energy, Nevada TREC Program 097-013 and 098-020.

I gratefully acknowledge my advisor and mentor, Dr. Penny S. Amy, whose careful guidance, support, and encouragement sustained me. Dr. Amy believed in my capabilities as a research scientist and gave me the opportunity to work with her on these important projects. She took the time from her busy schedule to share her experiences with me so that I may learn the responsibilities and skills of a research scientist. I must also thank Dr. Beth Pitonzo who introduced me to Dr. Amy. Dr. Pitonzo was instrumental in giving me the desire to pursue a higher degree to further my career.

I would like to thank my committee members, Dr. Dawn Neuman, Dr. James Jay, Dr. Jacimaria Battista, and Dr. Ananda Malwane whose skills and expertise gave me great insight into what is to be expected as I go forth in my career. Their support and encouragements made even my competency exams an experience that will always be remembered in a positive way.

Two special individuals who must be recognized as instrumental in assisting me in my research are Dr. Curtis R. Pantle and Charles Neuwohner. Dr. Pantle and I have worked closely together on both the Yucca Mountain and Nevada Test Site projects. Working with Dr. Pantle has been a positive influence on my life and his thorough
approach to research has taught me the discipline required to conduct a scientific research study. Dr. Pantle did everything from organizing our QA to getting dusty and dirty in the Yucca Mountain tunnels. Charles Neuwohner has been more than just an undergraduate working in this laboratory. He has worked hard for us and was always there when we needed him. From organizing large assays and washing glassware to chiseling rock, Chuck never complained. Among his many talents, Chuck was our computer wizard and solved most of the technical problems that others and I ran into during our daily activities. It would not have been possible to get the work done without his assistance.

I must mention the many undergraduates who have helped us in one capacity or another. In particular, Chiaki Brown and Julpohng Vilai helped during various phases of this research. I would like to thank Chiaki, Julpohng and the many other undergraduates for their efforts.

I need to thank Dr. Clay Crowe and Leigh Justet for their assistance in making the SEM facility available to us and in good working order. A significant portion of my research involved scanning electron microscopy. Their instruction and guidance in the use of the SEM enabled me to collect the data needed for analyzing biofilms.

Finally, I would like to thank my family for their support and patience through my graduate studies. My mother, Mary Ann Nelson, and my sister, Kris Nelson have given me the peace of mind knowing that my children were well cared for when I could not be there for them. My children, Aimee, Kirstyn, and Sean have continued to work hard for their mother and to understand when I needed my quiet time to study. My beloved husband, Bob, always remained in the background supporting and loving me during the rough times as well as the good. I thank you all for your love and encouragement.
CHAPTER 1

INTRODUCTION

Designing stable storage facilities is critical for the safe, long-term storage of nuclear waste material. Considerations involve structural materials which can withstand variations in abiotic factors such as water availability, salinity, temperature, light, and radiation. Biotic factors, microorganisms, can have an influence on the structural integrity of substrates by their metabolic activities as they colonize the surfaces. Some microorganisms form biofilms which are exopolysaccharide matrices that protect bacteria from adverse environmental conditions such as dessication, extreme temperatures, poor nutrient availability, and predation.

Biofilms are supported by exopolysaccharide-producing bacteria and they allow diffusion of nutrients and metabolites between microorganisms. Bacteria within these biofilms form microenvironments which allow for a variety of microorganisms to survive due to chemical variations such as pH and the presence or absence of oxygen. Bacteria involved in microbially-influenced corrosion (MIC) are known to exist in biofilms and can form an extensive, corrosive layer over any surface. MIC can impact the integrity of repository packaging and structural support through corrosion and biodegradation activities.

Yucca Mountain is the proposed location for a high-level nuclear waste repository. The suitability of Yucca Mountain is being challenged by the hypothesis that calcite deposits found in fractures and cavities within the welded tuff were formed as a result of upwelling hot waters from Paleozoic limestones underlying present day tuff. The currently accepted hypothesis is that the calcite deposits precipitated from
rainwaters that percolated through overlying soil and down into rock fractures located within Yucca Mountain. Therefore, to properly design a high-level nuclear waste repository, isolating and characterizing thermophilic, calcium-precipitating bacteria will be necessary to refute or support the theory of upwelling, subsurface waters as the source of calcite deposits in Yucca Mountain.

Biofilms

Traditional “batch culturing” of bacteria in liquid medium has been used to study many aspects of bacterial activity. However, pure culture planktonic growth is not typically how bacteria exist in nature (Davey, et al. 2000). The majority of bacteria exist attached to surfaces within a biofilm and not as free-floating microorganisms. These assemblages of bacteria within the biofilm matrix act as an interactive consortium in a complex and coordinated manner. The bacterial interactions that establish microbial consortia within the biofilm are mediated in part by lectin-ligand interactions involving cell-surface polysaccharide molecules (Roberts, 1996). Research has shown that biofilms are more than organism-containing slime on surfaces but are biological systems where bacteria form structured and functional communities (Davey, et al. 2000).

Bacteria developed survival strategies early in their evolutionary development, and therefore, it is useful to consider the value of biofilm development as a survival strategy. Bacterial cells are attracted to nutrients that would naturally concentrate onto surfaces in aquatic environments. The exopolysaccharides facilitate their adhesion to surfaces and assist in adsorbing and concentrating organic nutrients and cations from the bulk fluid (Costerton and Lewandowski, 1995). Biofilms can also afford individual bacteria protection from phagocytic protozoans and infection by bacteriophages, as well as providing nutritional advantages (Roberts, 1996). Capsular polysaccharides have lubricating characteristics which, in contrast to adherence, allow bacteria such as
Proteus mirabilis to swarm over solid substrata. Photosynthetic bacteria would be favored by adhesion to a surface where light and carbon dioxide, produced by heterotrophs, would be in close juxtaposition. In turn, the heterotrophs would benefit from the primary production of the photosynthetic bacteria. Physical and chemical threats to aquatic bacteria would be minimized by the protective layers of biofilm which create microenvironments conducive to their survival. Biofilms are also resistant to dessication which would allow bacteria to adapt to fluctuations in water levels.

Studies have shown that planktonic bacteria have two possible mechanisms for obtaining needed energy. The first case is when some moribund bacteria leak their metabolites providing neighboring bacteria with nutrients. The second mechanism is through storage of endogenous reserves of poly-B-hydroxy alkanoate (PHA), a carbon reserve, which can be utilized during periods of limited nutrient availability.

Biofilms may provide another source of carbon storage, the exopolysaccharide (EPS) matrix (Freeman et al., 1995). EPS is a polyanionic substance which may allow nutrient entrapment through ion exchange processes. Exoenzymes, which allow microorganisms to utilize high molecular weight substrates, are released during the latent growth phase when EPS production is at its maximum. This suggests physiological optimization of the EPS-protein complex to maximize substrate usage (Wingender et al., 1999). The enzymes are able to cleave the high molecular weight compounds which are stored during periods of nutrient limitation. Therefore, the entrapment mechanism could permit storage of organic carbon in the biofilm providing for a third carbon storage option.

Biofilms in the environment are resistant to depletion of organic matter, thus, indicating an internal energy reservoir. For example, river biofilms are resistant to depletion of organic matter from overlying waters, indicating that a store of nutrients exists which supply the biofilm cells with food during periods of nutrient deprivation (Freeman et al., 1995). In a comparison of sessile bacterial community numbers to
planktonic community numbers, the sessile population in pristine alpine streams exceeded the planktonic numbers by 3-4 logarithmic units (Costerton et al., 1987).

Biofilm structure is affected by environmental parameters as well as by the microbial communities which inhabit the biofilm. Such parameters include surface and interface properties, nutrient availability, and hydrodynamics. Under high shear stresses, which typically occur on the surfaces of teeth, biofilms are usually compacted and stratified (Davey et al., 2000). Biofilms growing under laminar flow conditions are patchy and contain clusters of rough, round cells separated by interstitial voids. In upflow anaerobic sludge bed reactors, biofilms form in the shape of aggregates containing complex bacterial communities. By forming aggregates, these biofilm communities settle to the bottom of the reactor which readily exposes them to the nutrients which flow up from the bottom. In addition, bacterial flocs are formed due to the fact that degradation of complex organic compounds to methane and carbon dioxide requires close contact by multiple species of bacteria interacting in a food web (Davey et al., 2000).

Important features of biofilm structure are the interstitial voids and channels. Particle tracking techniques have shown that water flows through these channels. This indicates that these voids may provide a means of circulating nutrients and exchanging metabolites with the bulk fluid layer (Costerton et al., 1994). If oxygen is available at the substratum:aqueous interface; the interstitial voids may be able to transport oxygen from the bulk fluid, throughout the biofilm to the attachment surface. Likewise, in a situation involving toluene biodegradation by multispecies biofilms, toluene was available to cells deep in the biofilm by this mechanism (Davey et al., 2000). Therefore, these interstitial channels are important not only in the structures of biofilms, but in their maintenance as well.

DNA transfer may be facilitated by biofilms. Studies have shown increased transformation rates with adherent bacterial cells compared with planktonic cells. The
hydrodynamic conditions within biofilms may increase the possibility of cell-to-cell contact and facilitate DNA transfer (Wingender et al., 1999). A flow chamber biofilm community which could degrade benzyl alcohol was tested for the ability to transfer the TOL plasmid from a donor strain of *Pseudomonas putida* to a *Pseudomonas putida* recipient strain indigenous to the biofilm community (Christensen et al., 1998). Although, plasmid transfer rates were low, the transconjugates were able to thrive and form new colonies. This technique may be useful in improving the biodegradation of wastes in wastewater treatment and other types of bioremediation.

**Biofilm Matrix**

The biofilm matrix or exopolysaccharide (EPS) matrix is formed from a variety of extracellular polymeric substances of biological origin that are involved in the formation of microbial aggregates (Wingender, et al. 1999). Another definition is a group of organic polymers of microbial origin which biofilm systems utilize to bind cells and other particulate materials together and to a substratum. The term, glycocalyx, has been used to describe EPS and implies that carbohydrates are involved (Christensen, 1989).

The most common sugar residues in microbial polysaccharides are either neutral or negatively charged. Therefore, the absence of hydrophobic groups, such as long hydrocarbon chains or aromatic rings and the presence of hydrophilicity and zero or negative charge is characteristic for microbial polysaccharides (Christensen, 1989). The shapes of the biofilm molecules are closely linked to the hydrodynamic and viscoelasticity of the biofilm. Polysaccharide molecules are usually stiff and extended like xanthan, an exopolysaccharide from *Xanthomonas* strains. There are some random coil type polysaccharides, like dextran, produced by several oral bacterial strains which are flexible. However, EPS also contains proteins, nucleic acids, amphiphilic compounds such as phospholipids, humic substances, and particles such as clay.
Death and lysis of cells contributes to the release of glycogen, peptidoglycan, and phospholipids which become part of the EPS. Thus, a biofilm can function to recycle intracellular components.

Colanic acid, Type I EPS, is a highly viscous capsular polysaccharide that is produced by microorganisms such as *Klebsiella* spp., *Salmonella* spp., and mucoid variants of *Escherichia coli*. This biopolymer may function as a means to protect cells under stress conditions such as exposure to toxic materials or dessication (Ophir et al., 1994). EPS can absorb significant amounts of water when fixed onto sand particles and may function in the same capacity on the surface of a bacterial cell. Researchers have shown that colanic acid can bind many times its weight in water and may play a role in maintaining the needed humid environment required by bacterial cells. EPS can provide a way for bacterial cells to resist dessication.

The exopolysaccharide matrix may be actively secreted through discrete export machineries involving translocation of EPS across bacterial membranes to the cell surface (Wingender, et al. 1999). Another possible mechanism is the release of extracellular polymers in the form of cellular components like lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria. Gram-negative bacteria form outer membrane-derived vesicles called blebs. This surface blebbing occurs during normal growth and represents a process by which cellular macromolecules such as nucleic acids, enzymes, LPS, and phospholipids are shed into the extracellular space possibly as a result of metabolic turnover (Beveridge, et al. 1997). These membrane vesicles into which hydrolytic enzymes are packaged could serve to degrade surrounding cells in the biofilm. Predatory vesicles could be capable of liberating nutrients for the vesicle-forming bacteria.
Genetic Control of EPS

Phenotypic changes occur in bacteria that adhere to a surface and in the production of their EPS (Costerton and Lewandowski, 1995). To understand the genetic basis for this, Davies, et al. (1993) used *Pseudomonas aeruginosa* which produces an exopolymer composed of alginate upon attachment. Alginate is a biofilm matrix polymer which plays a role in diseases such as cystic fibrosis and urinary tract infections. Three alginate structural genes algC, algD, algA encode enzymes involved in the biosynthetic pathway of alginate (Gacesa, P., 1998, Watnick and Kolter 2000, Costerton 1995). Activation of the algC promoter is a result of the cell’s attachment and it has been shown to be activated by environmental signals such as high osmolarity (Davies, et al. 1993). Additional studies revealed that another promoter, algD, is also upregulated at the time of adhesion (Costerton and Lewandowski, 1995) as well as, during nitrogen limitation, membrane perturbation by ethanol, and high osmolarity. Experiments involving the effects of ethanol and increased salt concentrations demonstrate increased alginate production similar to the response seen in the cystic fibrosis lung. Another study demonstrated that dessication of *Pseudomonas aeruginosa* growing in a sand matrix resulted in more EPS being produced as compared to an environment with a higher water potential (Davies, et al. 1993).

*Escherichia coli, K-12,* is a strain which produces a thick biofilm on inert surfaces such as glass and polystyrene, and exhibits increased adhesion by the overproduction of curli, a class of pili. In identifying the genes involved in biofilm formation, mutations in the cpxA gene resulted in decreased transcription of the curlin encoding gene, csgA, which decreased biofilm formation by affecting microbial adherence to solid surfaces (Dorel et al. 1999).

Biofilms composed of mixed bacterial communities as well as single species such as *Pseudomonas aeruginosa* form layers of mushroom and pillar-like structures separated by water-filled spaces. Findings show that there are two extracellular signals,
produced by *Pseudomonas aeruginosa*, involved in cell-to-cell communication and cell density-dependent expression of many secreted virulence factors (Davies, et al., 1998). At sufficient population densities, the two signaling systems of *Pseudomonas aeruginosa*, *lasR-lasI* and *rhlR-rhII*, reach the necessary concentrations required for gene activation. This type of gene regulation, quorum sensing, would indicate that these two signaling systems would not be involved in the initial stages of attachment, biofilm formation, and proliferation because a cell “quorum” is not yet present. Cell-to-cell signaling could be involved in biofilm differentiation similar to the cell-to-cell signaling involved in the production of specialized structures by *Myxococcus*. When a *lasI-rhII* double mutant was compared to the wild-type for biofilm formation and differentiation, the double mutant formed only 20% of the thickness of the wild-type biofilm. Furthermore, it was densely packed and lacking water channels. The wild-type had the characteristic clusters of microcolonies separated by water channels (Davies et al., 1998). To determine if *lasI* and/or *rhII* are involved in biofilm differentiation, mutants defective in one or the other were tested. The *rhII*-deficient mutant resembled physical characteristics similar to the wild-type; whereas, the *lasI*-deficient mutant developed characteristics similar to the double mutant. The *lasI* gene encodes the synthesis of N-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL). When 3OC12-HSL was added to medium flowing through a reaction chamber containing the *lasI*-deficient mutant, the biofilm formed had a thickness and cell density similar to the wild-type biofilms (Davies et al., 1998). Thus, 3OC12-HSL is involved in the cell-cell signaling that leads to biofilm differentiation and its architecture.

Quorum sensing not only governs the architecture of biofilms but, there is evidence to show that control of genes by quorum sensing may be necessary for relieving oxidative stress and may explain biofilm resistance to antimicrobial agents. In addition to the hypothesis that the EPS matrix serves as a barrier to the inherent diffusive mobility of antimicrobial agents, biofilm resistance can also be attributed to
the fact that cells in the biofilm are metabolically and physiologically different from planktonic cells. Alginate production in *Pseudomonas aeruginosa* is controlled by the sigma factor, AlgT. AlgT is involved in the positive regulation of the mucoid phenotype and it has been suggested that AlgT could be a factor in converting *Pseudomonas aeruginosa* to the biofilm mode of growth (Cochran, et al., 2000). AlgT is functionally similar to RpoE, an extreme stress sigma factor found in the Enterobacteriaceae. Therefore, up-regulation of AlgT may induce biofilm formation in response to extracellular stress. A second sigma factor, RpoS, controls a number of genes involved in the protection of the cell against hydrogen peroxide. RpoS has been shown to be up-regulated in *Pseudomonas aeruginosa* biofilms and is highly expressed in the sputum of cystic fibrosis patients.

Attachment - The First Step in Biofilm Formation

Most bacteria in nature, disease, and industrial applications exist attached to surfaces in biofilms (Lappin-Scott and Costerton, 1995). Bacterial attachment occurs continuously in natural environments and on most surfaces. Bacteria which reside in biofilms, termed sessile bacteria, are inherently different from planktonic bacteria. Biochemical analysis shows that planktonic and biofilm cells have different cellular protein expression (Costerton and Lewandowski, 1995). Studies indicate that this planktonic-biofilm transformation is controlled by a sigma factor similar to that which controls sporulation in Gram-positive bacteria and similar to the sigma factor that controls the rough-smooth transformation in Gram-negative bacteria. If biofilm bacteria are the product of sigma factor-directed phenotypic changes, then the biofilm bacteria and expression of EPS may constitute a phenotypically distinct expression of the bacterial genome.

The first step that takes place in the attachment process is the development of a conditioning film on a solid surface after it is immersed in an aqueous environment.
The basis for bacterial attachment to a surface may be in the balance of dispersion and electrostatic forces leading to a minimization of repulsive forces between a cell and a potential substratum (Fletcher and Loeb, 1979) rather than attractive forces. It is difficult to predict the adherence of bacterial populations in aquatic systems because of the variable and undefined dissolved components which may alter substratum and bacterial attachment through adsorption. The presence of different bacteria possessing different surface characteristics also make such predictions difficult.

To better understand the types of interactions which can occur between bacterial and substratum surfaces during attachment, work has been done to determine the correlation between the wettability of a substrate's surface and the attachment of bacteria (Pringle and Fletcher, 1983). This was accomplished by reducing the adsorption of macromolecules onto substrates by using artificial seawater with low organic content. From this study, substratum properties were found to be important in the initial stages of bacterial attachment. A number of bacterial strains preferred hydrophobic surfaces, even when isolated from a hydrophilic surface such as glass.

Aqueous environments often contain only dilute concentrations of substances which can be used for metabolism and growth. Such oligotrophic environments have a nutrient flux from near zero to a fraction of a milligram of carbon per liter per day (Marshall, 1988). Substrate surfaces tend to concentrate these nutrients by charge-charge or hydrophobic interactions (Beveridge et al. 1997). This conditioning film provides bacteria with an opportunity to take advantage of concentrated nutrients. Nutrients such as hydrophobic molecules and humic substances accumulate onto surfaces which are then metabolized by bacteria for growth and reproduction (Marshall, 1988). It has been demonstrated that glucose utilization by bacteria attached to surfaces exceeded that of planktonic cells. Possible explanations for this surface-enhanced uptake are (i) an increase in nutrient concentration at the surface through adsorption or
mass transfer by fluid movement and (ii) modification of cell surface physiological processes such as substrate transport (Fletcher, 1986).

Bacteria in natural environments are often confronted with low nutrient concentrations and, as a result, exhibit small size and reduced metabolic activity. This starvation-survival phase is associated with an increase in adhesiveness to surfaces possessing conditioning films (Geesey et al., 1994). It is postulated that the increase in adhesiveness of starved bacteria provides an opportunity for growth under conditions of low-nutrient availability in the aqueous phase.

Nutrient availability also plays a key role in the detachment of bacteria from biofilms, an advantage for the planktonic state. In a study done by Delaquis, et al. (1989), detachment of *Pseudomonas fluorescens* from glass surfaces was observed in response to nutrient stress. Deprivation of either glucose or nitrogen led to active detachment of cells from the biofilm. When glucose or nitrogen compounds were available in excess, detachment of bacterial cells was not evident (Delaquis et al., 1989). This detachment response may be a strategy for bacterial cells to be released into the aqueous phase in order to seek alternative sources of nutrition (Geesey et al., 1994).

The range of organic molecules which can rapidly adsorb onto solid surfaces, i.e., the conditioning film, include macromolecules such as proteins, polysaccharides, nucleic acids, humic acids, and smaller hydrophobic molecules such as fatty acids, lipids, and pollutants such as DDT, polyaromatic hydrocarbons, and polychlorinated biphenyls (Geesey et al., 1994). Therefore, an abundance of reactive binding sites would be available for reactions with additional solute molecules or with chemical groups expressed on bacterial surfaces. The organic molecules move from the bulk liquid phase to the surface by molecular diffusion. This diffusion process is rapid, resulting in significant deposits, 0.8-1.5 mg organic matter/ m² surface, after only 15 minutes (Lappin-Scott and Costerton, 1995). The effects of conditioning films may be
to alter the numbers of adherent bacteria, relative strength of bacterial adhesion, the nature of the adherent populations, as well as to provide a concentration of nutrients for subsequent growth of adherent bacteria (Geesey et al., 1994).

The macromolecular components of bacterial surfaces vary in quantity and composition depending upon their metabolic states and they affect the cell surface chemical and physical properties involved in attachment (McEldowney and Fletcher, 1986). Varying nutrient conditions and growth rates results in changes in the physicochemistry of bacterial surfaces. The attachment of a given strain of bacterium can be influenced by the presence of other attaching strains and depended upon the combination of species, the surface composition, and the sequence of attachment (McEldowney and Fletcher, 1987).

The initial attachment of bacteria occurs in two stages: reversible attachment phase and irreversible attachment phase. The reversible phase usually involves the attachment to the surface by a portion of the bacterial cell or flagellum. The cell may even continue to revolve on its flagellum during this initial phase. Reversible attachment involves a period of instability. Cells revolve around the axis of attachment, detach and emigrate from the attachment site to another site where the bacterial cell will again reversibly attach to the surface with a portion of its cell surface or flagellum. An explanation for the spinning behavior might be that it functions as a chemosensory mechanism whereby the cells determine the suitability of a potential colonization site by binding, rotating, and sensing ambient conditions through chemoreceptors (Lappin-Scott and Costerton, 1995). Spinning behavior is then followed by irreversible cell attachment (Lappin-Scott and Costerton, 1995), assuming the bacterial cell finds the site suitable. Irreversible attachment involves the use of exopolysaccharide glycocalyx polymers (Costerton et al., 1987). These extracellular polymeric substances are viewed as important mediators in the adhesion of bacteria and other microorganisms to surfaces.
and may be involved during the initial stages of attachment as well (Wingender et al., 1999).

An interesting question that should be asked is whether bacteria adhere to a substratum spontaneously or whether a physiological response is required. Work done by Fletcher (1977) addressed this by investigating the effects of culture concentration, age of the culture, time allowed for attachment, and temperature upon the attachment of a marine pseudomonad to polystyrene. There was a time dependence for attachment of pseudomonads resulting in an increase in attachment with increased bacterial concentration and time of incubation. An increase in both factors probably led to more bacterial collisions with the substratum, and therefore, more opportunities for attachment. The growth phase of the pseudomonads also affected attachment. The largest number of attached cells were in log-phase. An explanation may be that log-phase cultures had the largest number of motile cells and that there was a decline in motility with onset of stationary phase (Fletcher, 1977). However, there is evidence that production of adhesive polymers decreases with age of the bacterial culture. Adhesive polymers play a role in attachment which could explain the decline in attachment with age. Decreasing temperatures correlated with a decline in bacterial attachment. This might be explained by increased viscosity of the liquid medium. Higher temperatures favor adsorption of solutes from the liquid phase onto the substratum surface. These results seem to suggest that bacterial attachment may be controlled to a significant extent by non-biological factors.

An advantage to attachment onto surfaces is demonstrated by a wide range of bacteria which rely on adherent behavior to position themselves where the supply of organic nutrients is optimal, the pH allows utilization of specific growth factors, oxygen requirements are met, and temperatures are favorable. The colonization of sulfur springs by *Thermothrix thiopara* demonstrates positioning behavior using adherent mechanisms (Lappin-Scott and Costerton, 1995). *Thermothrix thiopara* attaches itself
to the sulfide-oxygen interfaces in hot, sulfur springs, permitting it to use reduced sulfur as a source of energy in the presence of oxygen. Adherence of heterotrophic bacteria to cyanobacterial filaments may optimize oxygen uptake by the heterotrophs who in turn produce carbon dioxide for cyanobacterial photosynthesis. Positioning behavior is common in streams, at aerobic-anaerobic interfaces of iron rich waters, surfaces of pipes, water supply equipment, and biological reactors.

Microbial Energetics/ Physiology of Microbially-Influenced Corrosion (MIC)

Microbial metabolism involves two types of reactions. They are catabolism and anabolism. Degradation, dissimilation, or catabolism comprises the oxidation-reduction reactions that provide energy to the organism. Assimilation, synthesis, or anabolism corresponds to those reactions that utilize the energy produced during catabolism to synthesize new cellular material (Videla, 1996). Bioenergetics is based on redox reactions requiring the participation of electron donors that become oxidized while part of the released energy is stored by the bacteria in some usable form such as ATP. The routes of oxidation of inorganic compounds can also take place when oxygen acts as the electron acceptor (aerobic respiration) and in the absence of oxygen, when a different electron acceptor such as nitrate or sulfate are used (anaerobic respiration). The electron transport chain located in the cytoplasmic membrane links catabolism with ATP synthesis. The electron transport chain is a series of oxidation-reduction electron carriers which create a membrane potential (Videla, 1996). The membrane potential consists of a charge differential and proton concentration gradient across the membrane. Therefore, the metabolic processes of microorganisms are electrochemical in nature, involving electron transfer reactions. The corrosion reaction, too, is electrochemical and can be a consequence of the metabolic activity of microorganisms (Videla, 1996).

Corrosion is viewed as a series of electrochemical reactions at a metal surface in contact with an electrolyte-containing aqueous phase (Geesey, 1991). Biofilms have
structural features that are important in microbially-influenced corrosion. First, the substratum on which the biofilm is built may become the source of metabolic energy for corrosion (Lappin-Scott and Costerton, 1995). Surface heterogeneities, e.g., patchiness of microbial growth, can establish localized electrochemical corrosion cells. When a clean surface is submerged in an aqueous environment microorganisms colonize the surface, often due to the chemoattraction of a conditioning film formed by organic compounds in the bulk fluid. As microbes replicate, microcolonies form and are usually distributed in an uneven manner. Some areas of the substratum can be heavily colonized, and in an oxygenated environment, the oxygen-consuming activities of surface-associated bacteria can create oxygen gradients (Geesey, 1991). The oxygen concentration gradient results in low oxygen levels at or near the surface and higher oxygen levels near the bulk aqueous phase. If the bulk aqueous phase is oxygenated, an oxygen concentration cell, or differential aeration cell, is likely to develop (Geesey, 1991).

The region of the substratum below active microbial growth becomes anaerobic, and therefore anodic, relative to the surrounding metallic surface exposed to air or an oxygenated aqueous phase. Thus, metal dissolution occurs (Lappin-Scott and Costerton, 1995). As the metal is oxidized, electrons migrate to a region exposed to significant levels of oxygen and react with oxygen and water molecules to form hydroxyl ions (Geesey, 1991). The hydroxyl ions can react with the metal ions to form a complex of metal hydroxides and metal oxides. In the case of iron rusting, Fe⁰ is oxidized to ferric ion (Fe³⁺) at the anode which reacts with hydroxyl ions at the cathode forming ferric oxides. Essentially, a battery is produced with metal dissolution occurring at the anode and reduction of oxygen and water occurring at the cathode.

Microorganisms also facilitate the formation of differential aeration cells on metal surfaces that contain an uneven distribution of corrosion products due to abiological reactions. Precipitated metal salts provide sites for microbial attachment.
and colonization (Geesey, 1991). Respiration further decreases oxygen concentrations at the metal surface which is already oxygen-compromised by the presence of the metal salt deposits. These localized areas of low redox potential at the metal surface allow for facultative and obligate anaerobic bacteria to grow. This explains how anaerobic sulfur-reducing and acid-producing bacteria, both types implicated in corrosion, can exist in aerobic environments.

Microorganisms Implicated in MIC

There are a number of microorganisms involved in microbially-influenced corrosion; the group most commonly implicated is the sulfate-reducing bacteria, SRBs (Lappin-Scott and Costerton, 1995). These bacteria are strict anaerobes with the capacity to reduce sulfate as the terminal electron acceptor in a respiratory mode of metabolism. The energy yield from substrate oxidation using sulfate is low, \( E_0 = -60 \text{mV} \). This effect is even more marked due to the fact that sulfate cannot act directly as an electron acceptor; At the expense of 2 ATP, it has to be activated to adenosine phosphosulfate. SRBs are dependent on the metabolic activities of other members in the microbial biofilm community. As obligate anaerobes, SRBs depend on the oxygen respiring heterotrophs to reduce oxygen tension, and fermentative facultative anaerobes to supply organic electron donors for energy production (Geesey, 1991). Sulfate-reducing bacteria can utilize a range of organic electron donors including fumarate, acetate, propionate, and fatty acids.

Aerobic, metal-oxidizing bacteria such as *Gallionella* sp. are associated with aerobic-induced metal corrosion (Geesey, 1993). *Gallionella* sp. is autotrophic and it obtains its energy from electrons produced by the oxidation of metals such as iron. At the anode *Gallionella* oxidizes iron, producing soluble ferrous hydroxide which reacts with oxygen to produce ferric hydroxide and eventually iron oxide. Iron oxides are corrosion by-products which form deposits on the metal known as tubercles. These
tubercles are hemispheric in shape and can be as large as 5.1-7.6 cm in diameter. The tubercles increase in size as bacteria continue to metabolize, setting down layers of ferrous hydroxide on the surface of the tubercles. The interface between the tubercle and the metal surface often becomes anaerobic, allowing obligate anaerobes such as sulfate-reducing bacteria to grow, creating metal dissolution and pitting.

Acid-producing bacteria, such as *Thiobacillus*, *Nitrosomonas*, and *Nitrobacter*, are aerobic and can produce copious amounts of acid as by-products of their metabolism (Geesey, 1991). When acid production exceeds the utilization rate by other microorganisms in the biofilm, the acids will accumulate and create localized pH gradients at the metal surface. Consequently, both organic and inorganic acid-production can promote electron removal from the cathode by hydrogen or dissolution of protective calcareous film from metal surfaces. Acid-induced corrosion of concrete and iron-reinforcing bars used in the construction of sewage pipes can occur through the activities of these autotrophic bacteria. *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* can produce sulfuric acid during metabolism of reduced sulfur compounds present in sewage effluent (Geesey, 1991).

Iron-reducing bacteria can modify the protective oxide film that forms over steel surfaces causing the surface to depolarize. Members of the genus *Pseudomonas* are capable of using ferric ions and sulfite as terminal electron acceptors and low-molecular weight compounds, such as lactate, as their carbon source (Geesey, 1991). Some of these bacteria can attach to the surface of steel coupons and remove the protective iron-oxide film by reducing ferric to ferrous ions, and replacing the iron-oxide coating with a pitting biofilm.

**Thermophilic, Calcium-Precipitating Bacteria**

Carbon dioxide generated by biological respiration can be fixed into insoluble carbonates (Ehrlich, 1996) and, actually, a significant portion of the insoluble carbonate...
at the earth’s surface is biogenic in origin. Biological fixation of carbon in carbonates is carried out by bacteria, fungi, and algae. Soils, freshwater, and saline habitats are natural environments where calcium carbonate precipitation has been observed (Rivadeneyra et al., 1994). The ability to precipitate CaCO₃ has been related to the formation of marine calcareous skeletons, carbonate sediments, soil carbonates, and carbonate rocks.

Aerobic and anaerobic oxidation of carbon compounds, e.g., carbohydrates, organic acids, and hydrocarbons, generate carbon dioxide. Carbon dioxide, in a buffered neutral or alkaline environment containing adequate amounts of calcium, will precipitate as calcium carbonate because of the insolubility of carbonate, $K_{\text{sol}}$ of CaCO₃ = $10^{-8.32}$ (Ehrlich, 1996). Microbial carbonate is also precipitated in the aerobic or anaerobic oxidation of organic nitrogen compounds with release of ammonia and carbon dioxide (Stocks-Fischer et al., 1999). In unbuffered environments containing sufficient levels of calcium, magnesium, or other cations, the ammonia hydrolyzes to ammonium hydroxide and further disassociates into ammonium and hydroxyl ions. The pH increases to a point where some of the carbon dioxide produced can precipitate as calcium carbonate (Ehrlich, 1996).

Bacteria can bind cations, including calcium ion. In extracellular microbial calcium-carbonate precipitation, evidence points to the possible initiation of calcium carbonate crystal formation on the surface of the bacterial cells (Morita, 1980). Calcium is loosely bound to the cell surface, which under the correct conditions, will react with carbonate in solution. Once the initial calcium carbonate crystal is formed, it acts as a nucleation site for the deposition of more calcium carbonate. Studies of marine isolates from the Great Barrier Reef showed that, with time, cultured cells decreased in number, but the amount of calcium carbonate precipitation increased (Morita, 1980). This observation suggests that the precipitation process is not
microbial, but may be aided by microbial processes such as an increase in pH, carbon dioxide production, and binding of calcium ions to the cell surface.

The role of thermophilic bacteria in precipitating calcium carbonate is not clear. It has been suggested that temperature influences calcium carbonate precipitation only when conditions are not favorable for its precipitation (Rivadeneyra et al., 1991). A number of studies on microbial calcium carbonate precipitation have been done primarily with cyanobacteria and other eubacteria at temperatures below $75^\circ$ C. At temperatures greater than $75^\circ$ C, the influence of thermophilic bacteria on calcite (calcium carbonate), precipitation has been investigated by Jones and Renaut (1996) where they looked at the effect of thermophiles in hot springs. At one location, the hot spring was saturated with calcite, yet there was no evidence of direct calcification by the thermophilic bacteria. In another hot spring, the mucous produced by the bacteria appeared to have served as a template for calcite precipitation (Jones and Renaut 1996). These results suggested that microbial precipitation of calcite may be incidental at temperatures near boiling.

Overview

This dissertation describes research focused on biofilms, environmental conditions affecting biofilm formation, and the relationship of biofilms to microbially-influenced corrosion and biodegradation of substrates other than metals. Emphasis was placed on conditions that enhance, as well as inhibit, biofilm formation so that the suitability of structural materials under consideration for use in the radioactive waste repositories at Yucca Mountain and the Nevada Test Site could be determined.

An additional study was undertaken to detect thermophilic, calcium-precipitating bacteria in calcite deposits within Yucca Mountain. These deposits suggest that the calcite deposits may have resulted from upwelling of subsurface,
thermal waters which could impact Yucca Mountain’s suitability as a site for a high-level nuclear waste repository.

Humidity and Temperature Boundaries for Biofilm Formation in Yucca Mountain - Chapter 2

This research investigation concentrated on the boundary limits for biofilm formation and addressed a variety of temperature and relative humidity levels as they relate to biofilm formation on candidate repository packaging materials for the proposed Yucca Mt. high-level nuclear waste repository. Biofilm production by exopolysaccharide-producing bacteria can directly or indirectly cause microbially-influenced corrosion (MIC). The data collected on biofilm boundary limits will be essential for engineering canisters and near field structures.

Three different metal alloys, candidates for the repository spent fuel canisters, were buried in crushed Yucca Mt., muckpile rock and allowed to incubate under varying levels of temperature and humidity. At designated timepoints, the metal coupons were harvested and the biofilm removed and quantitated. Means of determining the extent of biofilm formation and microbially-influenced corrosion included direct bacterial counts, culturable counts, confocal laser microscopy, and scanning electron microscopy.

Isolation and Characterization of Thermophilic, Calcium-Precipitating Bacteria from Calcite Deposits at Yucca Mountain - Chapter 3

Calcite deposits, composed of calcium carbonate and silicon dioxide, are found in fractures and small cavities within the welded tuff of Yucca Mountain. This investigation involves determining the presence of thermophilic, calcium-precipitating bacteria within these deposits. The possible existence of thermophilic bacteria may help to resolve the issue of whether these calcite deposits formed from precipitation of

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dissolved calcium carbonate rain water transported from the overlying soil environment or as a result of upwelling hot waters transported from geothermal activity below the mountain. Resolving the origin of the calcite deposits will be essential in determining the suitability of Yucca Mountain as a high-level nuclear waste repository.

Evaluation of Biological Factors that Influence and Enhance Degradation of NTS Landfill Waste Containers - Chapter 4

Subsidence is a problem in the storage of low-level nuclear waste because of the potential for the closure cap to shift and collapse, allowing water to penetrate the waste material and travel to the groundwater. To address the problem of controlling disposable waste, microorganisms were used to evaluate enhanced degradation of container materials. This investigation encompassed the effects of bacterial colonization and biofilm development on proposed waste cell support and packaging materials to determine what conditions promoted the degradation of these materials.

In the first phase, three major groups of microorganisms, a *Streptomyces* sp. (cellulose-degrader), a *Paracoccus* sp. (EPS-producer), and a consortium of iron oxidizing bacteria, were tested on cardboard, wood, and metal coupons. Measurements involving culturable microbial counts, confocal laser microscopy, and scanning electron microscopy were used to monitor the extent of biofilm formation and biodégradation.

In the second phase, additional treatments to accelerate biodégradation of NTS landfill waste containers were used to determine the optimal conditions for compromising structural integrity. The use of fungi, which can degrade cellulose and lignin, has been added to the degradation regimens. Fungi can tolerate water activity levels as low as $a_w = 0.6$ and are more suited for survival in an arid environment. The use of sulfate-reducing bacteria, a group of microorganisms implicated in microbially-influenced corrosion, has been added along with the iron-oxidizing bacteria to accelerate corrosion on metal surfaces.
To achieve maximum attachment of microbes to waste container surfaces, a water-filled matrix has been mixed with the microbes before application. One such matrix is microcrystalline cellulose gel which thickens upon contact with liquids. Mixing microbes, water, and nutrients should theoretically keep the microbes viable long enough to allow colonization and development of a biofilm. The stickiness of the cellulose gel allowed the microbial mixture to attach to the surface of the waste container materials long enough for the microbes themselves to attach and form a biofilm.

As in the first phase, measurements to monitor biofilm formation, biodegradation, and microbially-influenced corrosion included culturable counts, confocal laser microscopy, and scanning electron microscopy.

Biofilm Formation and Microbial Effects on Polyethylene Encapsulating Material for Low-Level Nuclear Waste Storage - Chapter 5

Prior to the need to accelerate biodegradation of NTS landfill waste containers, a portion of low-level nuclear waste were encapsulated in polyethylene to reduce the risk of shifting contents which could cause waste items to protrude and puncture waste containers. The concern at the time was to determine if microbes would have any effect on compromising the integrity of the polyethylene encapsulating material. As described in the previous chapter reviews, microorganisms are capable of forming biofilms to protect themselves from dessication, predation, fluctuations in temperature, and to conserve nutrients. Biofilms have also been associated with biodegradation and microbially-influenced corrosion.

Polyethylene coupons were buried in Nevada Test Site soil and treated with water, nutrients, and several different groups of microorganisms. Biofilm formation, over the course of three years, was monitored using the same techniques as described previously: direct bacterial counts, culturable counts, confocal laser microscopy, and
scanning electron microscopy. In addition, the Department of Mechanical Engineering, UNLV, assisted with stress test measurements to determine if biofilms had any effect on the structural integrity of polyethylene.

Discussion - Chapter 6

This chapter will summarize results on the effects of environmental conditions such as temperature and relative humidity on biofilm formation and the treatments to accelerate biodegradation. Discussion of these results has and continues to impact the designing of the high-level nuclear waste repository in Yucca Mountain and the low-level nuclear waste repository at the Nevada Test Site.

Results of investigating the presence of thermophiles in calcite deposits within Yucca Mountain will be discussed in addition to the implications this may have in designing a high-level nuclear waste repository in Yucca Mountain.

References


Figure 1-1. Location of Nevada Test Site and Yucca Mountain.
CHAPTER 2

HUMIDITY AND TEMPERATURE BOUNDARIES FOR BIOFILM FORMATION IN YUCCA MOUNTAIN

This chapter has been prepared for submission to Applied and Environmental Microbiology and is presented in the style of that journal. The complete citation is:

Abstract

To determine the long-term success of the proposed Yucca Mountain high-level nuclear waste repository, studies of bacterial colonization and biofilm development are needed. Bacteria involved in microbially-influenced corrosion and degradation are known to form biofilms with the potential to impact the integrity of repository packaging and structural materials. Temperature and humidity are environmental factors that can greatly affect biofilm formation. Therefore, it is necessary to determine the temperature and humidity conditions that bound biofilm formation.

Microcosms, which simulated the repository environment of Yucca Mountain, were placed at temperatures ranging from 30° C to 70° C and in relative humidities ranging from 100% to 32%. The microcosms contained titanium, C22 carbon steel, and N316 stainless steel coupons buried in crushed Yucca Mountain muckpile rock. The uniform-sized metal coupons were sacrificed at the following timepoints: day 0, 1 day, 1 month, 6 months, 1 year, and 18 months. The average number of culturable bacteria harvested from the entire surface of each of the three coupon types, incubated at 100% relative humidity and 30° C, increased from 1x10^4 CFU at day 0 to a range of 4-7x10^4 CFU at 5 months, followed by a decrease to 5-8x10^2 CFU after 18 months of incubation. The average number of culturable bacteria harvested from the surfaces of the three metals, incubated at 84%, 70.5%, and 32% relative humidity and 30° C, were unchanged at 10^2 CFU from day 0 to 18 months or decreased to numbers below the level of detection. Culturable bacterial counts, from the three candidate metals incubated at 60 and 70° C and 100% relative humidity, showed a decrease from 10^2 CFU at day 0 to numbers below the level of detection at 18 months. Confocal laser microscopy showed minimal differences in the extent of microbial colonization on the three metal surfaces in all but optimum conditions, 100% RH and 30° C, at each timepoint after day zero. These data indicate that a decrease in relative humidity level or an increase in temperature severely affects biofilm formation on the three candidate...
metals being considered for repository packaging at the proposed Yucca Mountain nuclear waste repository.

Introduction

Yucca Mountain is the proposed site for our nation’s first permanent high-level nuclear waste repository. To accurately determine the long-term success of a repository, information concerning the long-term success of the repository structure is needed. This investigation will encompass the effects of bacterial colonization and biofilm development on proposed repository support and packaging materials.

Microbiologically-influenced corrosion (MIC) is used to designate corrosion due to the presence and activities of microorganisms within biofilms on metal surfaces. MIC also includes microbial metabolites that may be produced at one location and diffuse to a corrosion site (Little and Wagner, 1996). MIC is localized corrosion resulting in pitting, crevice corrosion, underdeposit corrosion and selective leaching. The microbial metabolic activities within biofilms can accelerate rates of reactions in a number of corrosion processes.

Previous studies have shown that certain bacterial species are involved in the degradation and corrosion of structural materials (Geesey, 1991). Corrosion can occur due to the action of heterotrophic bacteria and fungi as well as iron-oxidizing, sulfate-reducing, and exopolysaccharide (EPS)-producing bacteria. One such action can take the form of acid production. *Thiobacillus ferrooxidans* is an iron-oxidizing bacterium which is also capable of oxidizing reduced sulfur species, producing sulfuric acid and creating rust on a variety of metal substrates (Pronk et al., 1992, Hallmann et al., 1992). This can be accomplished with the use of iron or sulfur as a sole energy source and carbon dioxide as a sole carbon source. Such iron-oxidizing bacteria have been isolated from the welded tuff of Yucca Mountain. Other acid-producing bacteria, e.g., nitrifying bacteria, have also been recovered from Yucca Mountain subsurface environments.

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Another group, sulfate-reducing bacteria, is implicated in microbially-influenced corrosion of metals (Hamilton, 1985). Metal-sulfides are produced from sulfate respiration and these may form the basis of corrosive capabilities of sulfate-reducing bacteria. For example, iron sulfides may promote the accumulation of atomic hydrogen at the metal surface, causing hydrogen embrittlement. Microorganisms involved in microbially-influenced corrosion can occur in and are facilitated by biofilms (Costerton et al., 1987, Ford and Mitchell, 1990, Costerton and Boivin, 1991).

ATP generation by chemoautotrophs is similar to that of chemoheterotrophs except that the electron donor is inorganic instead of organic (Madigan et al., 2000). Inorganic compounds that can serve as raw materials for chemoautotrophic metabolism include molecular hydrogen, reduced sulfur compounds such as hydrogen sulfide, reduced iron compounds such as iron sulfide, and nitrogen-containing compounds such as ammonium and nitrite ions (Atlas, 1997). Metal redox reactions influenced by microbial metabolic activities, e.g., microbially-influenced corrosion (MIC), relate to the energetics and physiology of these chemoautotrophs (Jones and Amy, 2000).

The redox potential, $E_{m}$, of the environment is critical because many enzymatic reactions to the surrounding metallic surface exposed to air or an oxygenated aqueous phase; thus, metal dissolution occurs (Lappin-Scott and Costerton, 1995). As the metal is oxidized, electrons migrate to a region exposed to significant levels of oxygen and react with oxygen and water molecules to form hydroxyl ions (Geesey, 1991). Hydroxyl ions can react with the metal ions to form a complex of metal hydroxides and metal oxides. For example, sulfate-reducing bacteria (SRB) can cause the oxidation of steel when removing the protective hydrogen layer by linking the electron flow from the metal to the respiratory reduction of sulfate to sulfide. In this process, hydrogen serves as an electron carrier (Cord-Ruwisch, 2000). The metal is the source of energy for the SRB which feed on metallic iron by using the electrons released during the corrosion process for respiration. SRB, known to be present in Yucca Mountain, are of interest in
the study of MIC of nuclear fuel waste containers (King and Stroes-Gascoyne, 1997) because of their involvement in the biocorrosion process.

Factors that influence microbial activity and microorganisms' ability to form biofilms include the presence of water, tolerance to high temperatures, availability of nutrients or energy sources, and ionizing radiation (Little and Wagner, 1996). Microorganisms have been found to exist and thrive in unusual environments such as radioactive and nutrient-deficient waters and subsurface environments. For example, algae, fungi, and bacteria were found in water that covered the damaged reactor core at Three Mile Island nuclear power plant.

The first objective of this study was to collect data on the boundary limits of biofilm formation by determining the relative humidity requirements for biofilm establishment. This was done with the expectation that relative humidity and available nutrients may be the primary determinant of microbial activity. The second objective was to determine the boundary limits of biofilm formation based on temperature. Individual as well as bacterial groups exhibit diverse optimal growth temperatures. The range of temperatures that a particular microorganism can tolerate will determine its ability to survive in a given ecosystem (Atlas et al., 1998).

Materials and Methods

Collection and Preparation of Yucca Mountain Muckpile Rock

Mined Yucca Mountain tuff is stored in large mounds which surround the north portal entrance to the mountain. This muckpile rock was collected by carefully removing top layers of loose rock with alcohol-flamed sterilized shovels and collecting the rock below these layers. Muckpile rock was placed into sterile, plastic bags and placed on ice. The rock was then transported, within 6 hours, to the laboratory and stored at -20°C (Kieft et al., 1997).
In preparation for microcosm assembly, the muckpile was crushed into fine grains using alcohol-flamed sterilized mortar and pestles.

**Coupons and Microcosms**

One cm$^2$ coupons were constructed from the following metals: C-22 nickel alloy, N-316 stainless steel, and titanium (Metal Samples, Mumford, Alabama). Aseptically crushed muckpile rock was transferred to sterile glass Petri plates. The metal coupons were placed inside the muckpile rock. Microcosms were then placed in chambers held to specific relative humidities and temperatures.

**Relative Humidity**

To achieve different relative humidity (RH) levels, saturated salt solutions filled the bottom reservoirs of the microcosm chambers (Nalgene Autoclavable Dessicators, Rochester, NY). The saturated salt solutions included KCl (83.6% RH), KI (67.9% RH), and MgCl$_2$ (32.4% RH). Distilled water was used to create 100% RH. The saturated salt solutions were prepared by adding 50 g aliquots of each salt to approximately 600-700 mL of distilled water while stirring continuously on a magnetic stir plate. Salt was added until crystals no longer dissolved. The total volume of each salt solution was 1 liter. The Petri plate microcosms were placed on a grid above the salt solution. After chambers were assembled, the lid was sealed with high vacuum grease (Dow Corning, Midland, MI). Relative humidity levels were checked using a digital thermohygrometer (Cole-Palmer P-37450-52, Vernon Hills, ILL).

**Temperature**

Three temperatures were chosen to bound the limits of biofilm formation. Temperatures were controlled using a Revco (Asheville, NC) RA50-1060-ABA (30° C), a Napco (Winchester, PA) Model 303 Incubator (60° C), and a Precision Scientific (Winchester, PA) Thelco Laboratory Oven Model 160 (70° C). NIST-traceable thermometers (H-B Instruments, Trappe, PA) were used to monitor temperature in each
of the incubators. Each controlled humidity chamber was placed at the appropriate
temperature.

Treatment Groups and Timepoints

At 30°C the following relative humidities were used: 100%, 84%, 68%, and 32%. At 100% relative humidity the following temperatures were used: 30, 60, and 70°C. For each treatment, 3 coupons were used for heterotrophic plate counts, one for confocal laser microscopy, and one for scanning electron microscopy.

The coupons were sacrificed at day 0, 1 day, 1 month, 5 months, and 1 year. With the exception of Day 0, coupons were exposed to a set temperature and relative humidity values prior to sacrificing. At day 0, the three metal coupons were placed into the crushed rock, and removed immediately.

Heterotrophic Plate Counts

Adherent material and biofilm was scraped off coupons at each timepoint and vortexed in 1.0 mL R2B broth (yeast extract, 0.5 g; proteose peptone, 0.5 g; casamino acids, 0.5 g; glucose, 0.5 g; sodium pyruvate, 0.3 g; K2PO4, 0.3 g; MgSO4.7H2O, 0.05 g per liter distilled H2O, pH = 7.2) (Difco). Homogenized biofilm (100 mL) was spread-plated onto R2A agar (Difco/BD Diagnostics Systems, Sparks, MD), and incubated at room temperature for 2 weeks. Colony forming units were counted, and microbial diversity observed and calculated using Shannon-Weaver diversity indices (Atlas and Bartha, 1998).

Scanning Electron (SEM) and Confocal Laser Microscopy (CLSM)

Coupons were fixed in 4% glutaraldehyde (Sigma) for scanning electron and confocal laser microscopic analysis (See APPENDIX I). Scanning electron microscopy (Jeol JSM - 5600, Peabody, MA, Fig. 2-22) was done at the EPMA/ SEM Facility, Department of Geosciences, and confocal laser microscopy (Zeiss LSM 5, Fig. 2-21) was done at the Center for Biological Imaging, Department of Biological Sciences, University of Nevada, Las Vegas.
Results

At 100% relative humidity (RH), 30°C, the heterotrophic plate counts (HPC) from day 0 to 1 day on C22, N-316, and titanium coupons increased from less than 1x10^3 CFU to 2-4x10^4 CFU per coupon (Figs. 1, 2, 3). HPCs increased at 1 month on N-316 and titanium coupons to 4-6x10^4 CFU per coupon (Figs. 1, 3), and decreased on C-22 to 3x10^3 CFU per coupon (Fig. 2). All three metal types increased HPC values by 5 months to 4-7x10^4 CFU. After 18 months of incubation, N-316 and titanium coupons showed decreased HPC values to 8x10^2 CFU, and 5x10^2 CFU for C22.

HPCs at 84% RH, 30°C, were lower than 100% RH for all three metal types. There was attachment of microorganisms, 1-2x10^2 CFU per coupon, at 24 hours for all three metal coupon types followed by a gradual decrease to undetectable values after 18 months of incubation (Tables 1, 2, 3). At 70.5% RH, 30°C, HPCs did not increase from 1 x10^2 at Day 0 throughout the test for all three metal types (Tables 1, 2, 3).

For the 32% RH, 30°C treatment group, N-316 stainless steel and C22 carbon steel coupons demonstrated microbial attachment, less than 1x10^2 CFU per coupon, at Day 0 which gradually increased to 2-3x10^2 CFU after 5 months of incubation (Tables 2, 3). After 18 months of incubation, the HPC values on N-316 stainless steel and C22 carbon steel coupons did not change significantly, 2.51x10^2 CFU per coupon (Tables 2, 3). Titanium coupons showed microbial attachment, less than 10^2 CFU per coupon, at day 0 and 1 day with an increase in CFU values to almost 1x10^3 CFU per coupon after 5 months of incubation (Table 1). After 18 months of incubation, CFU values per titanium coupon decreased by 10-fold.

When temperatures were increased to 60 and 70°C at 100% RH, HPC values decreased for all three types of metal coupons from less than 1x10^2 CFU to undetectable levels at 1 day (Tables 4, 5, 6) and remained at undetectable levels for the duration of the test. This decrease in HPC values is significant when compared to the HPC values at 30°C, 100% RH (Figs. 4, 5, 6).
Confocal laser and scanning electron microscopy revealed that under 100% RH and 30°C conditions significant biofilm formed on the surfaces of the three types of metal coupons. The titanium coupons showed increased biofilm development on the surface from day 0 to 5 months (Figs. 7 - 14) which typifies the biofilm development on the C22 and N-316 surfaces. After one year of incubation confocal laser and scanning electron microscopy showed a decrease in the amount of biofilm covering the titanium surface (Figs. 15, 16) which correlates with HPC data of the C22 and N-316 coupons as well.

Only small patches of biofilm formed on C22, N-316, and titanium coupon surfaces under lower than 100% relative humidity conditions and 30°C. At 84% and 70.5% RH, small patches of biofilm and rock particles were seen attached to the C22 carbon steel surface after one year of incubation using scanning electron microscopy (Figs. 17, 18). Only small clumps of rock and biofilm patches were attached to the surfaces of titanium coupons after incubating at 32%RH for one year (Fig. 19).

At 60 or 70°C and 100% RH, biofilm development was negligible as evidenced by scanning electron microscopy compared to biofilm development observed at 30°C. On the surfaces of N-316 stainless steel, biofilm development was negligible after one year of incubation at 60°C, 100% RH (Fig. 20). Similar results were observed on C22 carbon steel and titanium surfaces (data not shown).

Discussion

HPC data suggested that a significant decrease in biofilm development occurred with relative humidity values less than 100% and increased temperature. After 5 months of microcosm incubation, total culturable counts ranged from 2-4x10⁴ CFU per coupon at 100% RH and 30°C. At relative humidities ranging from 86% to 32% at 30°C, culturable counts were negligible for the three metals tested (Tables 1, 2, 3).
Temperatures ranging from 60 to 70°C at 100% RH inhibited biofilm formation resulting in negligible culturable counts after 18 months of incubation (Tables 4, 5, 6). Therefore, increased temperature to 60 or 70°C or decreased relative humidity values of 86%, 68%, or 32% impeded biofilm formation on metal surfaces used in this study. The microorganisms able to grow at these temperatures would need to be thermophilic with optimal growth temperatures greater than 40°C (Atlas and Bartha, 1998). The crushed muckpile rock used to bury the metal coupons was exposed to human and mechanical perturbation (Haldeman, et al., 1995) which likely increased the number and types of microorganisms present; however, it did not guarantee the introduction of thermophilic microbes.

Temperature could have an effect on the production of extracellular polymeric substances which can enhance the adherence capability of bacterial cells. SEM studies have shown that *Listeria monocytogenes* cells produced EPS at 21°C, but not at 10°C or 35°C (Norwood and Gilmour, 2001). At increased temperatures of 60 and 70°C, microorganisms would not only have to be thermophilic but capable of producing EPS as well. EPS is critical not only for initial adhesion, but in the firm anchorage of bacteria to solid surfaces (Norwood and Gilmour, 2001) and in the ability of biofilm microorganisms to deal with environmental stresses such as nutrient limitations, solar radiation, and variations in temperature (Perrot et al, 1998).

Optimal conditions for activity of aerobic soil microorganisms require a water activity value, a_w, of 0.98 - 0.99 (Atlas and Bartha, 1998). However, some microorganisms can exhibit a greater tolerance of desiccation than others (Potts, 1994). Such dessication tolerance was not exhibited in this study.

The culturable counts from C22 carbon steel coupons decreased from approximately 3x10^4 to 2x10^3 CFU at 100% RH, 30°C between 1 day and 1 month (Fig. 2). A possible explanation is that as the C22 carbon steel coupons began to corrode,
some products may have been mildly toxic to the early bacterial community. However, as the biofilm developed, the succeeding bacterial communities appeared to better tolerate the C22 carbon steel because culturable counts increased from $2 \times 10^3$ CFU at 1 month to approximately $6 \times 10^4$ CFU at 5 months of incubation.

The increased tolerance of the succeeding bacterial communities may be as a result of the developing biofilm structure which helps to protect microorganisms from environmental stresses such as toxic metals and organic pollutants (Cowan, et al, 2000). The organization and morphology of a biofilm can change due to the selective pressure of toxic compounds. This is illustrated in a study (Cowan et al, 2000) using biofilms composed of two species of *Pseudomonas* (GJ1 and DMP1) where the distribution of the microorganisms within the biofilm matrix was affected by the presence of toxic wastes. A commensal relationship was observed using confocal laser microscopy where *Pseudomonas* GJ1 benefitted from the ability of *Pseudomonas* DMP1 to detoxify these toxic wastes. The EPS of the mixed culture biofilm was concentrated in mushroom-shaped clusters and its distribution unaffected by the presence of toxic wastes as opposed to the single species biofilm comprising *Pseudomonas* GJ1 which exhibited a uniform distribution of EPS in the presence of toxic wastes and whose biofilm development was inhibited (Cowan et al, 2000).

Culturable counts from C22, N-316, and titanium decreased from $4-7 \times 10^4$ CFU per coupon after 5 months of incubation ($30^0$ C, 100% RH) to approximately $5-8 \times 10^2$ CFU per coupon after 18 months of incubation. The culturable count graphed data for all three metal Viable bacteria can be found in most subterranean environments. Typical culturable counts range from $10^2$ to $10^6$ bacteria per cubic centimeter of solid surface (Pedersen, 1996). Subsurface bacteria are found alive and active; although, their metabolism proceeds at slower rates than in surface environments (Pedersen, 1996). However, subsurface environments have demonstrated high proportions of heterotrophic bacteria (Balkwill, 1989), especially in studies of oxygenated volcanic
tuff at the Nevada Test Site (Amy 1992, Haldeman and Amy, 1993). With increased awareness of viable subsurface and endolithic microorganisms, signature lipid biomarkers have been used to quantitatively assess viable biomass, community composition and the nutritional/physiological status of subsurface microbiota (White and Ringelberg, 1996) because metabolic rates are below the level of detection (Kieft et al., 1997). Determining whether these microorganisms can colonize repository structures and containers, and affect physical and chemical processes is of paramount importance. These processes could include corrosion of metals or concrete, gas formation from organic materials or mobility of elements in ground water and rocks (Bachofen, 1991). Microbial metabolism on repository structures could promote accelerated reactions, accumulation of degradation products, or changes in their physical integrity.

SEM did not indicate corrosion on the metal surfaces; however EDS (Electron Dispersion Spectroscopy) analyses may provide information on whether corrosion processes began to take place at the biofilm-substrate interface by the detection of corrosion byproducts. Previous studies by Castro 1997, have demonstrated metal coupon corrosion with Yucca Mountain rock and isolated MIC cultures. Bacteria of the genus *Thiobacillus* and sulfate-reducing bacteria (SRB) are known to exist in the rock and soil of the Nevada Test Site and are capable of metal corrosion (McCabe, 1990, Pitonzo, 1996, Castro, 1997). *Thiobacillus* and other iron-oxidizers have aerobic metabolism. Aerobes predominate unsaturated subsurface regions, but anaerobes, such as sulfate-reducers, can be found in anaerobic microenvironments in the subsurface (Kaiser and Bollag, 1990, Pitonzo, 1996). For example, sulfate-reducing bacteria are the most abundant class of chemoautotrophs in the subsurface layers of the Savannah River Plant site (Kaiser and Bollag, 1990).

This study shows that muckpile rock excavated from a subterranean environment harbors microorganisms that can colonize metal surfaces, given the
appropriate conditions. Initially, high-level nuclear waste will create high temperatures and low water activity that will slow biofilm formation. With time, temperatures will decrease and water activity will increase as water migrates through the natural vadose zone and backfill. These conditions will be favorable for biofilm formation by microorganisms. Some of these may be responsible for MIC. Therefore, the bounding limits of temperature and relative humidity for biofilm formation need to be determined so that the stability of the high-level nuclear waste packaging and structures can be predicted.

References


Table 2-1
Titanium: Relative Humidity, Colony Forming Units at 30°C

<table>
<thead>
<tr>
<th>Incubation</th>
<th>100% RH</th>
<th>84% RH</th>
<th>70.5% RH</th>
<th>32% RH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>56 ± 12*</td>
<td>56 ± 12</td>
<td>56 ± 12</td>
<td>56 ± 12</td>
</tr>
<tr>
<td>24 hr</td>
<td>3.65x10⁴ ± 4.51x10⁴</td>
<td>75 ± 21</td>
<td>20 ± 11</td>
<td>1.06x10⁵ ± 2.3x10⁵</td>
</tr>
<tr>
<td>1 Mo</td>
<td>5.54x10⁴ ± 1.31x10⁴</td>
<td>88 ± 36</td>
<td>1 ± 1</td>
<td>87 ± 8</td>
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<tr>
<td>5 Mo</td>
<td>4.86x10⁴ ± 1.90x10⁴</td>
<td>0</td>
<td>17 ± 6</td>
<td>9.28x10⁴ ± 1.70x10⁴</td>
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<td>1 Yr.</td>
<td>1.22x10⁴ ± 1.37x10⁴</td>
<td>26 ± 15</td>
<td>0</td>
<td>1.33x10⁴ ± 2.9x10⁴</td>
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<tr>
<td>18 Mo</td>
<td>7.56x10⁴ ± 8.60x10⁴</td>
<td>3.37x10⁴ ± 5.40x10⁴</td>
<td>0</td>
<td>2.46x10⁴ ± 2.20x10⁴</td>
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*Mean ± S.E.
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<thead>
<tr>
<th>C22</th>
<th>100% RH</th>
<th>84% RH</th>
<th>70.5% RH</th>
<th>32% RH</th>
</tr>
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<td>Day 0</td>
<td>53 ± 12</td>
<td>53 ± 12</td>
<td>53 ± 12</td>
<td>53 ± 12</td>
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<tr>
<td>24 Hrs</td>
<td>2.98x10^4 ± 1.80x10^4</td>
<td>2.10x10^4 ± 7.3x10^4</td>
<td>22 ± 4</td>
<td>52 ± 11</td>
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<tr>
<td>1 Mo</td>
<td>3.22x10^4 ± 8.30x10^4</td>
<td>32 ± 10</td>
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<td>92 ± 14</td>
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<tr>
<td>5 Mo</td>
<td>6.73x10^4 ± 1.23x10^4</td>
<td>17 ± 8</td>
<td>30 ± 11</td>
<td>2.88x10^4 ± 3.10x10^4</td>
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<td>1 Yr</td>
<td>4.88x10^3 ± 1.47x10^3</td>
<td>8 ± 3</td>
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<td>43 ± 6</td>
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<td>18 Mo</td>
<td>5.04x10^6 ± 1.00x10^6</td>
<td>3.16x10^4 ± 4.70x10^4</td>
<td>0</td>
<td>2.51x10^6 ± 2.60x10^6</td>
</tr>
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</table>

* Mean ± S.E.
### Table 2-3
**N316 Stainless Steel: Relative Humidity, Colony Forming Units at 30 °C**

<table>
<thead>
<tr>
<th>N316</th>
<th>100% RH</th>
<th>84% RH</th>
<th>70.5% RH</th>
<th>32% RH</th>
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<tr>
<td>Day 0</td>
<td>1.06X10^2 ± 2.7x10^1</td>
<td>1.06X10^2 ± 2.7x10^1</td>
<td>1.06X10^2 ± 2.7x10^1</td>
<td>1.06X10^2 ± 2.7x10^1</td>
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<tr>
<td>24 Hrs</td>
<td>1.88x10^4 ± 2.18x10^3</td>
<td>84 ± 20</td>
<td>32 ± 8</td>
<td>1.90x10^2 ± 1.15x10^2</td>
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<tr>
<td>1 Mo</td>
<td>3.88x10^4 ± 5.91x10^7</td>
<td>97 ± 28</td>
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<td>38 ± 6</td>
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<td>5 Mo</td>
<td>4.25x10^4 ± 3.30x10^7</td>
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<td>7 ± 3</td>
<td>3.52x10^2 ± 7.20x10^1</td>
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<td>1 Yr</td>
<td>8.78x10^3 ± 1.89x10^3</td>
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<td>1 ± 1</td>
<td>58 ± 12</td>
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<td>18 Mo</td>
<td>7.61x10^2 ± 1.57x10^2</td>
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<td>1.11 ± 1.11</td>
<td>2.51x10^2 ± 2.60x10^1</td>
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* Mean ± S.E.
Table 2-4
Titanium: Temperature, Colony Forming Units at 100% Relative Humidity

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<th>30 °C</th>
<th>60 °C</th>
<th>70 °C</th>
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<tr>
<td>Day 0</td>
<td>56 ± 12*</td>
<td>56 ± 12</td>
<td>56 ± 12</td>
</tr>
<tr>
<td>24 Hrs</td>
<td>3.65x10^4 ± 4.51x10^3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 Mo</td>
<td>5.54x10^4 ± 1.31x10^4</td>
<td>36 ± 34</td>
<td>0</td>
</tr>
<tr>
<td>5 Mo</td>
<td>4.86x10^4 ± 1.90x10^4</td>
<td>12 ± 4</td>
<td>1.44x10^2 ± 1.39x10^2</td>
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<tr>
<td>1 Yr</td>
<td>1.22x10^4 ± 1.37x10^3</td>
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<tr>
<td>18 Mo</td>
<td>7.56x10^2 ± 8.60x10^1</td>
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* Mean ± S.E.
Table 2-5
C22 Nickel Alloy: Temperature, Colony Forming Units at 100% Relative Humidity

<table>
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<tr>
<th>C22 Nickel</th>
<th>30 °C</th>
<th>60 °C</th>
<th>70 °C</th>
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<tbody>
<tr>
<td>Day 0</td>
<td>53 ± 12*</td>
<td>53 ± 12</td>
<td>53 ± 12</td>
</tr>
<tr>
<td>24 Hours</td>
<td>2.98x10^4 ± 1.80x10^3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 Month</td>
<td>3.22x10^3 ± 8.30x10^2</td>
<td>0</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>5 Months</td>
<td>6.73x10^4 ± 1.23x10^4</td>
<td>44 ± 25</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>1 Year</td>
<td>4.88x10^3 ± 1.47x10^3</td>
<td>2 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>18 Months</td>
<td>5.04x10^2 ± 1.00x10^2</td>
<td>21 ± 21</td>
<td>9 ± 7</td>
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* Mean ± S.E.
Table 2-6
N316 Stainless Steel: Temperature, Colony Forming Units at 100% Relative Humidity

<table>
<thead>
<tr>
<th>N316</th>
<th>30 °C</th>
<th>60 °C</th>
<th>70 °C</th>
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<tbody>
<tr>
<td>Day 0</td>
<td>1.06x10^2 ± 2.7x10^1*</td>
<td>1.06x10^2 ± 2.7x10^1</td>
<td>1.06x10^2 ± 2.7x10^1</td>
</tr>
<tr>
<td>24 Hr</td>
<td>1.88x10^3 ± 2.18x10^3</td>
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<td>0</td>
</tr>
<tr>
<td>1 Mo</td>
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</tr>
<tr>
<td>5 Mo</td>
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<td>78 ± 78</td>
<td>17 ± 11</td>
</tr>
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<td>1 Yr</td>
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<td>0</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>18 Mo</td>
<td>7.61x10^2 ± 1.57x10^2</td>
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* Mean ± S.E.
Figure 2-1. N316 Stainless Steel - Relative Humidity. Heterotrophic Plate Counts from Day 0 to 18 months.
316 Stainless Steel

Heterotrophic Plate Counts (x 10^4 CFU) per Coupon

Day 0 1 day 1 Month 5 Months 1 year 18 Months

- 100% RH
- 84% RH
- 70.5% RH
- 32% RH

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Figure 2-2. C22 Nickel Alloy - Relative Humidity. Heterotrophic Plate Counts from Day 0 to 18 months.
Heterotrophic Plate Counts (× 10^4 CFU) per Coupon

Day 0  1 Day  1 Month  5 Months  1 year  18 Months

C22 Nickel Alloy

- 100% RH
- 84% RH
- 70.5% RH
- 32% RH

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Figure 2-3. Titanium - Relative Humidity. Heterotrophic Plate Counts from Day 0 to 18 months.
Figure 2-4.  N316 Stainless Steel - Temperature. Heterotrophic Plate Counts from Day 0 to 18 months.
Figure 2-5.  C22 Nickel Alloy - Temperature. Heterotrophic Plate Counts from Day 0 to 18 months.
C22 Nickel Alloy

Heterotrophic Plate Counts (x 10^4 CFU) per Coupon

- 30 degC
- 60 degC
- 70 degC

Day 0  1 Day  1 Month  5 Months  1 year  18 Months
Figure 2-6. Titanium - Temperature. Heterotrophic Plate Counts from Day 0 to 18 months.
Figure 2-7. Titanium, Day 0, incubated at 30°C, 100% RH. Green areas indicate metal surface. Orange-red areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 630x magnification.
Figure 2-8. Titanium, Day 0, incubated at 30° C, 100% RH. Scanning Electron Microscopy, 500x magnification.
Figure 2-9. Titanium after 1 day incubated at $30^\circ$ C, 100% RH. Green areas indicate metal surface. Orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 2-10. Titanium after 1 day incubated at $30^\circ C$, 100% RH. Scanning Electron Microscopy, 500x magnification.
Figure 2-11. Titanium after 1 month incubated at $30^\circ$ C, 100% RH. Green areas indicate metal surface. Orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 630x magnification.
Figure 2-12. Titanium after 1 month incubated at $30^\circ$ C, 100% RH. Scanning Electron Microscopy, 500x magnification.
Figure 2-13. Titanium after 5 months incubated at $30^\circ$ C, 100% RH. Green areas indicate metal surface. Orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 2-14. Titanium after 5 months incubated at 30° C, 100% RH. Scanning Electron Microscopy, 1000x magnification.
Figure 2-15. Titanium after 1 year incubated at 30° C, 100% RH. Green areas indicate metal surface. Orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 2-16.  Titanium after 1 year incubated at 30°C, 100% RH. Scanning Electron Microscopy, 500x magnification.
Figure 2-17. C22 Nickel Alloy after 1 year incubated at 30\(^0\) C, 84% RH. Scanning Electron Microscopy, 500x magnification.
Figure 2-18. C22 Nickel Alloy after 1 year incubated at 30°C, 70.5% RH. Scanning Electron Microscopy, 500x magnification.
Figure 2-19. Titanium after 1 year incubated at 30\(^0\)C, 32\% RH. Scanning Electron Microscopy, 1000x magnification.
Figure 2-20. N316 Stainless Steel after 1 year incubated at 60° C, 100% RH. Scanning Electron Microscopy, 500x magnification.
Figure 2-21. Confocal Laser Scanning Microscope, Zeiss LSM 5.
Figure 2-22. Scanning Electron Microscope, Jeol JSM - 5600
CHAPTER 3

ISOLATION AND CHARACTERIZATION OF THERMOPHILIC, CALCIUM-PRECIPITATING BACTERIA FROM CALCITE DEPOSITS AT YUCCA MOUNTAIN

This chapter has been prepared for submission to Applied and Environmental Microbiology and is presented in the style of that journal. The complete citation is:

Abstract

Calcite deposits, composed of a mixture of calcium carbonate and silicon dioxide, were found in fractures and small cavities within the welded tuff of Yucca Mountain. This research investigation involves determining the presence of thermophilic, calcium-precipitating bacteria within these deposits. The possible existence of thermophilic bacteria may help to resolve the issue of whether these calcite deposits formed from precipitation of dissolved calcium carbonate in rain water transported from the overlying soil environment or as a result of upwelling of geothermally-heated waters transported from below the mountain. Evidence for microbially-influenced calcite precipitation in these deposits is indicated by the presence of moderately-thermophilic, calcium-precipitating bacteria.

Growth of bacteria enriched from crushed calcite and calcite/tuff mixed samples collected from tunnels within Yucca Mountain indicate a tendency for thermophiles to be found in calcite deposits and mixed rock samples compared to tuff samples (no calcite) which lacked bacterial growth at temperatures of 50°C and higher. Calcite isolates growing at 60 and 70°C were identified as thermophiles, the most common identification being Bacillus stearothermophilus. SEM and electron dispersion spectroscopy (EDS) results showed that bacteria, isolated from Yucca Mountain calcite and calcite/tuff, produced calcite (CaCO₃) when grown on calcium-enriched medium. This evidence indicates a possible warm water influence in the history of Yucca Mountain.
Introduction

Yucca Mountain, NV is the proposed site for a high-level nuclear waste repository. The suitability of Yucca Mountain has been challenged by the hypothesis that calcite deposits found in fractures and cavities within the welded tuff were formed as a result of upwelling hot waters from Paleozoic limestones underlying present day tuff. The currently accepted hypothesis is that the calcite deposits precipitated from rainwater recharge that percolated through overlying soil and into rock fractures located within the mountain.

Studies of fluid inclusions within the calcite indicate that a number of the calcite samples collected from Yucca Mountain were formed at temperatures ranging from 35°C to as high as 75°C (Dublyansky, et al. 1998, Dublyansky, 1998). Data have already been collected on the characterization of microbial communities indigenous to Yucca Mountain as part of a general site characterization (Kieft et al., 1997) and from Rainier Mesa at the Nevada Test Site (Haldeman and Amy, 1993, Haldeman, et al. 1994b, Haldeman, et al. 1995). Further characterization of the effects these microbial communities might have on the corrosion of structural materials such as metal, concrete, grout, and wood are also under investigation (Castro et al., 1996, See Chaps. 2 and 4). What has not been analyzed is the presence of thermophilic bacteria within or in close proximity to the calcite deposits. Concerns over the possibility that these calcite deposits are a result of hydrothermal activity make it necessary to determine if thermophiles can be isolated from these calcite deposits in comparison to the welded volcanic tuff that comprises Yucca Mountain. Upwelling of hot waters from the subsurface may have deposited thermophiles, therefore, the presence of thermophilic
bacteria in the calcite minerals may lend support for the hydrothermal origin of the calcite deposits within Yucca Mountain.

Individual bacteria, as well as larger bacterial groups, exhibit wide growth temperature ranges. In addition, they exhibit diverse optimal growth temperatures. The range of temperatures that a particular microorganism can tolerate will determine its ability to survive in a given ecosystem (Atlas and Bartha, 1998). Psychrophiles exhibit optimal growth temperatures from less than 0°C to approximately 15°C. Mesophiles grow optimally from about 20 to 40°C. Optimal growth temperatures for thermophiles range from 40 to 80°C with extreme thermophiles typically growing from above 80°C to as high as 110°C (Atlas and Bartha, 1998). The ability for thermophiles to grow at elevated temperatures lies in the presence of high proportions of saturated lipids in their membranes which prevent melting and the production of enzymes that are not readily denatured by high temperatures.

Calcium plays a major role in many biological processes of both prokaryotes and eukaryotes. Intracellular calcium is intricately regulated due to the fact that its concentration mediates diverse physiological activities (Appanna et al. 1997). The cytoplasm content of calcium returns to basal level, through different calcium transport systems, after the biochemical responses have been elicited (Norris et al., 1991). Elevated levels of free calcium can cause irreversible damage to cells by inhibiting these physiological responses. Therefore, many organisms have devised strategies to maintain cytoplasmic levels of calcium. One strategy, as demonstrated by *Pseudomonas fluorescens*, is to precipitate calcite, a crystalline calcium carbonate, in order to avoid the negative effects of excess free calcium. This is the first example of
microbial calcite precipitation as a detoxification strategy (Anderson and Appanna, 1994). In addition, strontianite, the crystalline SrCO₃, is also precipitated by Pseudomonas fluorescens when challenged with excess levels of strontium (Anderson and Appanna, 1994).

In order for calcite to precipitate, the mobilization of calcium and carbonate is necessary along with the presence of a matrix where nucleation of crystals can occur. Carbonate could be provided by the fixation of carbon dioxide into bicarbonate ion, HCO₃⁻, which requires the use of carbonic anhydrase by the microorganisms. Under normal conditions, the regulation of HCO₃⁻ allows for the decomposition of toxic cyanate into ammonia. It is also possible that this CO₂-fixing system may be involved in calcite precipitation. Similarly, in photosynthetic organisms, the fixation of CO₂ during photosynthesis can precipitate calcite as long as sufficient calcium levels exist and a matrix, e.g., the S-layer, is present for nucleation (Thompson et al., 1997, Schultze-Lam, 1992).

A second focus of this study will be to determine if calcium-precipitating bacteria isolated from the calcite deposits, including thermophiles, might be responsible, in part, for the calcite deposition. Mineralization of calcite by marine microorganisms is widely recognized as well by bacteria in lacustrine environments (Monger et al., 1991, Thompson and Ferris, 1990, Buczynski and Chafetz, 1991, Schultze-Lam et al., 1992, Robbins and Blackwelder, 1992, Hodell et al., 1998). Precipitation of calcium carbonate by microorganisms is significant in the development of a variety of geological formations, however, calcite deposition in arid soils has been viewed as an inorganic process. Recent evidence suggests that soil microorganisms
may play a key role in the formation of calcite precipitation in modern soils as well as paleosols (Monger et al., 1991). The presence of calcite has become increasingly important in determining the paleoclimate and age of desert soils (Monger et al., 1991). The presence of calcium-precipitating bacteria in the calcite deposits of Yucca Mountain may provide additional evidence for the origin of these calcite deposits.

Bacteria, cyanobacteria, and some fungi deposit calcium carbonate extracellularly (Thompson and Ferris, 1990, Robbins and Blackwelder, 1992, Schultze-Lam et al., 1992, Thompson et al., 1997). One exception is *Achromatium oxaliferum* which has been reported to deposit calcium carbonate intracellularly (Ehrlich, 1996). Extracellular precipitation of calcium carbonate can occur during the removal of carbon dioxide from photosynthesis (Thompson et al., 1997). *Synechococcus* can alkalinize its surrounding microenvironment due to the exchange of $\text{HCO}_3^-$ into and $\text{OH}^-$ out of the cell. This exchange process results from fixation of $\text{HCO}_3^-$ during photosynthesis (Thompson and Ferris, 1990). Increased $\text{OH}^-$ levels will drive the bicarbonate/carbonate equilibrium reaction towards higher carbonate ($\text{CO}_3^{2-}$) levels. If calcium ion concentrations are high enough, calcium carbonate precipitation will occur. Filamentous cyanobacteria associated with stromatolites, cyanobacteria, and algae can precipitate calcium carbonate as a result of their photosynthetic activities (Ehrlich, 1996, Thompson et al., 1997).

To further investigate the origin of the calcite deposits in Yucca Mountain, microbial analysis of subsurface water from monitoring wells and warm springs located in neighboring locations around Yucca Mountain were conducted to see if these water sources might be connected to subsurface thermal waters located below paleozoic
limestone layers beneath Yucca Mountain. Microbial analysis of water sampled from these sources may provide additional evidence for a connection between Yucca Mountain calcite deposits if similar populations of thermophilic bacteria are found.

Materials and Methods

Calcite/ Tuff Collection and Preparation

Samples of calcite, tuff adjacent to calcite, and welded tuff from areas not associated with calcite deposition were removed from natural rock formations using tools cleaned with 10% bleach followed by 95% ethanol and air drying. The calcite and tuff specimens were placed in sterile, plastic sample bags and stored on ice. The samples were transported within 6 hours of collection and stored at -20°C (Kieft et al., 1997).

Rock surfaces were sterilized using serial washes of 10% bleach, sterile water, and 95% ethanol. After air drying, the calcite and tuff were then crushed aseptically using an alcohol flame-sterilized mortar and pestle.

Preparation of Bacterial Cultures from Calcite/ Tuff

Slurries composed of a 1:10 dilution of crushed calcite or tuff and 0.1% sodium-pyrophosphate were prepared and shaken at 100 rpm for 1 hr. Portions of the slurry preparations (3 mL) were filtered through 0.45 mm filters (Gelman, Ann Arbor, MI) and then the filters were placed onto culture plates containing either R2A minimal nutrient agar (Difco/ BD Diagnostics Systems, Sparks, MD) or B4-calcium enriched agar (Monger et al., 1991). The cultures were incubated at 23, 45, 60, and 70°C for 2 wk. Bacterial growth was then semiquantitatively scored using a scale of 0, 1, 2, 3, and
4 (0 - no growth, 4 - heaviest growth). Isolates growing at 45, 60, and 70°C were cultured and identified.

Water Sample Collection and Preparation

Water collection from the monitoring wells began with QA-controlled sampling of water pumped from various depths in these wells (Fig. 4). Collection of water began after open flow for 16 hours. Water was aseptically collected using autoclaved Nalgene (Rochester, NY) polypropylene bottles. Water samples from warm springs (Ash Meadows, northwest of Yucca Mountain and Ash Springs, northeast of Yucca Mountain, see Fig. 5) were collected using sterile 500 mL Nalgene polypropylene bottles attached to a 12-foot pole (Fig. 6). Samples were collected from the center of the springs as deep and as close to the source of the springs as possible. The water samples were maintained at ambient air temperature and transported to the lab within 6 hr of collection.

The samples were processed on the same day by filtering the water through 0.2 mm Gelman filters and placed onto culture plates containing R2A minimal nutrient agar. Cultures were grown at 23, 45, 60, and 70°C for two weeks. Bacterial growth was semiquantitatively scored and those microorganisms growing at 45, 60, and 70°C were cultured and identified.

Bacterial Identification

Isolates were identified using the polymerase chain reaction (PCR) to amplify the 16S rDNA region of each bacterial genome using universal primers 16S-27f and 16S-1492r (Bandi et al., 1994, Integrated DNA Technologies Inc., Coralville, IA). The PCR procedure was performed using a GeneAmp 2400 PCR System thermocycler.
(Perkin Elmer Biosystems, Wellesley, MA). The amplified 16S rDNA from each bacterial isolate was sequenced and, using the BLAST search database, identified by matching the sequence to the 16S rDNA sequence of a known bacterium (See APPENDIX I).

SEM Microscopy

Bacterial isolates were grown on B4 calcium-enriched medium (Monger et al., 1991). Scanning electron microscopy was performed to visualize calcite crystals formed by calcite isolates (Electron Microscope Facility, Northern Arizona University, and the EPMA/SEM Laboratory, Dept. of Geosciences, University of Nevada, Las Vegas - See APPENDIX I).

Results

Bacterial growth data showed that thermophilic bacteria were found in the calcite and calcite/tuff samples (Tables 1-3) collected in the ESF tunnel. Mesophilic bacteria were also isolated at 25 and 45°C from calcite and calcite/tuff in the ESF and ECRB tunnels, as well as, the control tuff (no calcite) sampled from Alcove 5 (north end of ESF tunnel). Bacterial growth at 25°C was heavier and occurred with more frequency than bacterial growth at 45°C for most samples from all Yucca Mountain sites (Tables 1-3).

Bacteria were not isolated at temperatures 50°C and higher from the control Alcove 5 tuff (Fig. 2). Also, no bacteria were isolated at temperatures 60°C and higher from the ECRB tunnel specimens (Table 3).
Isolate identification results are listed in Table 4. Thermophilic isolates from the ESF tunnel (60 - 70°C) were primarily Bacillus sp. and included: Bacillus thermoruber, Bacillus stearothermophilus, a thermal soil bacterium, and Bacillus thermoleovorans. Thermophilic bacteria isolated from Nye County monitoring well NC-EWDP-1DX (west of Yucca Mountain) and growing at 60 and 70°C were identified as Bacillus stearothermophilus, a thermal soil bacterium, and Bacillus flavothermus. Thermophiles growing at 60 and 70°C were not isolated from any of the tuff/calcite samples collected from the ECRB tunnel in Yucca Mountain. Isolates have been obtained at 60°C from the warm springs water samples from Ash Meadows Wildlife Refuge (90 miles northwest of Yucca Mountain), but have yet to be identified.

EDS analysis revealed that the crystals formed on and near the bacterial colonies on B4 medium (See APPENDIX I) were calcium carbonate (Fig. 1). SEM microscopy illustrated the shapes of calcite crystals formed by thermophilic calcite isolates on B4 medium (Figs. 2, 3).

Discussion

The potential for subsurface, thermal waters to rise within Yucca Mountain is an important factor in assessing the suitability of Yucca Mountain as a high-level, nuclear waste repository and in the materials/design selected for the repository. From a microbial ecology perspective, entombed endolithic bacteria are of interest. Thermal water exposure has recently been determined to have last been present approximately 2 million years ago (Wilson and Cline, in press). The survival implications of these
findings are interesting and in line with other subsurface findings (Amy et al., 1993, Amy, 1997, Lamber et al., 1998, Morita, 2000).

Compared to bacterial growth at 25° and 45°C, bacteria which grew at 60 and 70°C were found with less frequency and in smaller quantity (Tables 1-3). To date, only calcite and calcite/tuff samples obtained from the ESF tunnel have been shown to contain thermophilic bacteria. Bacteria growing at 25 and 45°C were found in the ESF and ECRB tunnel samples as well as in the control tuff (no calcite) samples of Alcove 5.

The calcite and calcite/tuff samples from the ESF were categorized by location; samples taken from locations at the northern half of the tunnel and samples from the southern half of the tunnel. Samples from the northern half supported more bacterial growth at 25 and 45°C compared to samples from the southern half (Tables 1, 2). In contrast, there was a tendency to find more thermophilic bacteria in the southern half of the ESF compared with the northern half of the ESF.

The Alcove 5 tuff was chosen as a control due to the lack of calcite present in the welded tuff. It is important to note that there was no bacterial growth from this sample at temperatures 50°C and higher. Thermophiles growing at 60°C and higher were isolated only in samples containing calcite (Tables 1-3). Such a finding indicated that there was warm water influence in specific regions of Yucca Mountain.

Identification of the thermophilic bacteria isolated from the Yucca Mountain again suggest a warm water influence. *Bacillus thermoleovorans* was originally isolated in soil near hot water effluent in Pennsylvania and in hot spring mud in
Arkansas (American Type Culture Collection, Manassas, VA). *Bacillus flavothermus* is a thermophile originally isolated from hot springs in New Zealand (Pikuta, et.al. 2000).

A connection between outlying monitoring wells and warm springs and subsurface thermal waters beneath Yucca Mountain has yet to be determined. Thermophilic bacteria have been isolated from water collected at the Nye County monitoring Well NC-EWDP-1DX but have not been identified. Water samples collected from warm springs in Ash Meadows (Fig. 5), and hot springs located in Ash Springs, NV, northeast of Yucca Mountain, are being processed (Figs. 7, 8). If similar species of thermophilic bacteria found in Yucca Mountain calcite deposits are isolated in the surrounding warm and hot springs as well as monitoring wells, a physical connection between subsurface thermal waters beneath Yucca Mountain and these outlying thermal water sources would be suggested.

The biochemical evidence of microbial precipitation of calcite indicate that soil microorganisms may have played a significant role in pedogenic calcite precipitation in modern soils and paleosols (Monger et al., 1991). Further evidence that microorganisms are able to precipitate calcite is found in laboratory experiments which demonstrate bacterial cultures able to precipitate calcite in media containing calcium acetate. Biomineralization of calcite has important implications, such as in microfossils, that may reflect paleoclimatic conditions and in the origin of calcite deposits in Yucca Mountain.

EDS analysis has shown that the thermophilic bacteria isolated from Yucca Mountain calcite deposits precipitate calcite crystals when cultured on calcium-enriched B4 medium (Fig. 1). The morphologies of the calcite crystals formed (Figs. 2, 3) are
similar to the crystal morphologies of calcite precipitated by *Bacillus pastueri* during a study of microbially-mediated calcite precipitation (Warren, et.al. 2001). Although the thermophiles isolated from Yucca Mountain, to date, have been capable of precipitating calcium carbonate, there is little evidence to suggest that these thermophiles may have had a significant influence on the formation of calcite deposits in Yucca Mountain. The thermophilic microorganisms isolated so far are too few in number to study *in situ*.

Based on bacterial growth data and identification of thermophilic bacterial isolates, there is evidence to suggest a warm water influence occurred in Yucca Mountain which led to bacterial deposition. Data continues to be collected regarding groundwater connections between outlying thermal springs and subsurface thermal waters beneath Yucca Mountain. The possibility of warm/hot water rising from the subsurface could compromise the structural integrity of the repository if it had been demonstrated in the last 65,000 years.

Whether bacterial isolates found in calcite deposits within Yucca Mountain had a significant influence on calcite formation is yet to be determined. There are numerous examples of microbially-mediated calcite precipitation in the environment: the whiting events of Fayetteville Green Lake, New York (Thompson, et. al. 1997) and in the Bahama Banks (Robbins and Blackwelder, 1992); a saltern pond (Rivadeneyra, et. al. 1994); the marine environment (Morita, 1980); hot springs (Jones and Renaut, 1996); and caves (Contos, et. al. 2001). It is possible that thermophiles isolated from Yucca Mountain calcite deposits had some influence on calcite formation within Yucca Mountain. Direct study of the Yucca Mountain calcite deposits to locate and examine
bacteria and isotopic analysis of the calcite crystals may provide evidence needed to
determine the origin of the Yucca Mountain calcite deposits.

References


Amy, P. S. 1997. Microbial Dormancy and Survival in the Subsurface, pp. 185-203, In
P. S. Amy and D. L. Haldeman (ed.), The Microbiology of the Terrestrial Deep


Flavobacteria as Intracellular Symbionts in Cockroaches. Proc. R. Soc. London 257:43-
48.

Buczynski, C. and H. S. Chafetz. 1991. Habit of Bacterially Induced Precipitates of
Calcium Carbonate and the Influence of Medium Viscosity on Mineralogy. J. Sed.
Petrol. 61:226-233.

Castro, P., Pitonzo, B. J., Bergman, D., and D. Jones. 1996. The Microbially-
Influenced Corrosion Capability of Yucca Mountain Isolates. Arizona Branch,
American Society for Microbiology Meetings, Tucson, Az. Feb. 10.

Contos, A. K., James, J. M., Heywood, B., Pitt, K., and P. Rogers. Morphoanalysis of
Bacterially Precipitated Subaqueous Calcium Carbonate from Weebubbie Cave,

Dublyansky, Y. V. 1998. Fluid Inclusions in Calcite Samples from the ESF, Nevada
Test Site, Nevada. Report Assembled by Yuri Dublyansky for the Office of the
Attorney General of the State of Nevada.


Table 3-1  Bacterial Growth Data of Calcite/Tuff Samples Collected from the Northern Half of the ESF (Exploratory Studies Facility) Tunnel, Yucca Mountain

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Table 3-2  Bacterial Growth Data of Calcite/Tuff Samples Collected from the Southern Half of the ESF (Exploratory Studies Facility) Tunnel, and Tuff Samples from Alcove 5, Yucca Mountain

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</tr>
<tr>
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<td>0,0,0,0,3</td>
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<td></td>
</tr>
<tr>
<td>555078 calcite/tuff</td>
<td>0,0,0,0,0,0</td>
<td>0,0,0,0,0,0,0</td>
<td>0,0,0,0,2,1</td>
<td>0,0,0,3,0,0</td>
<td>0,0</td>
</tr>
<tr>
<td>555079 calcite/tuff</td>
<td>0,0,0,0,0,0</td>
<td>0,0,0,0,0,0</td>
<td>2,0,0,0,0,2,2</td>
<td>2,0,0,0,0</td>
<td>0,0,3</td>
</tr>
<tr>
<td>Control Alcove 5 tuff</td>
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<td>4,3,3, 1,1,0, 3,2,1, 0,0,0, 0,0,0, 0,0,0, 0,1,1, 0,0</td>
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</tr>
</tbody>
</table>

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Table 3-3  Bacteria Growth Data of Calcite/Tuff Samples Collected from the ECRB (Enhanced Characterization of the Repository Block) Tunnel, Yucca Mountain

| Sample Identification | Growth | | | | | |
|-----------------------|--------|--------|--------|--------|--------|
| ECRB                  |        | RT     | 45 °C  | 60 °C  | 70 °C  |
| 566334 calcite/tuff   | 2,1,3,0,1,0 | 4,4,3,2,1,0 | 1,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566335 calcite/tuff   | 2,0,1,0,1,1 | 3,2,1,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566336 calcite/tuff   | 2,1,3,1 | 2,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566337 calcite/tuff   | 3,3,0,0 | 2,1,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566338 calcite/tuff   | 1,0,2,2 | 0,0,0,3,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566329 calcite/tuff   | 3,1,4,2 | 3,2,0,1,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566330 calcite/tuff   | 1,0,4,3,1,1 | 0,0,0,4,4,3 | 1,1,1 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566326 calcite/tuff   | 3,0,0,0,1,0 | 2,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566327 calcite/tuff   | 1,0,3,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566331 calcite/tuff   | 1,0,3,1 | 2,1,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566332 calcite/tuff   | 2,0,2,1 | 4,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566333 calcite/tuff   | 2,0,1,1,2,1 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |
| 566328 calcite/tuff   | 0,1,4,1 | 0,0,0,1,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 | 0,0,0,0,0,0 |

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Table 3-4  Identification of Thermophilic Bacteria Isolated from the ESF (Exploratory Studies Facility) Tunnel, Yucca Mountain and Water Collected from Nye County Monitoring Well NC-EWDP

<table>
<thead>
<tr>
<th>Sample Identification</th>
<th>Microbial Identification</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESF north 566343 calcite/tuff</td>
<td>Bacillus stearothermophilus</td>
<td>60 °C</td>
</tr>
<tr>
<td></td>
<td>Unidentified low G+C</td>
<td>60 °C</td>
</tr>
<tr>
<td>566341 calcite/tuff</td>
<td>Bacillus stearothermophilus</td>
<td>60 °C</td>
</tr>
<tr>
<td></td>
<td>Thermal soil bacterium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 °C</td>
<td></td>
</tr>
<tr>
<td>ESF south 555076 calcite</td>
<td>Bacillus thermoruber</td>
<td>60 °C</td>
</tr>
<tr>
<td></td>
<td>Thermal soil bacterium</td>
<td>70 °C</td>
</tr>
<tr>
<td></td>
<td>Bacillus stearothermophilus</td>
<td>70 °C</td>
</tr>
<tr>
<td>555077 calcite/tuff</td>
<td>Bacillus stearothermophilus</td>
<td>70 °C</td>
</tr>
<tr>
<td></td>
<td>Bacillus sp.</td>
<td>70 °C</td>
</tr>
<tr>
<td></td>
<td>Thermal soil bacterium</td>
<td>70 °C</td>
</tr>
<tr>
<td></td>
<td>Bacillus thermoleovorans</td>
<td>70 °C</td>
</tr>
<tr>
<td>555078 calcite/tuff</td>
<td>Bacillus stearothermophilus</td>
<td>70 °C</td>
</tr>
<tr>
<td></td>
<td>Thermal soil bacterium</td>
<td>70 °C</td>
</tr>
<tr>
<td>555079 calcite/tuff</td>
<td>Bacillus sp.</td>
<td>70 °C</td>
</tr>
<tr>
<td></td>
<td>Bacillus stearothermophilus</td>
<td>70 °C</td>
</tr>
<tr>
<td>Nye Country Monitoring Well NC-EWDP-IDX 555079 calcite/tuff</td>
<td>Bacillus stearothermophilus</td>
<td>60 °C</td>
</tr>
<tr>
<td></td>
<td>Bacillus stearothermophilus</td>
<td>70 °C</td>
</tr>
<tr>
<td></td>
<td>Bacillus flavothermus</td>
<td>60 °C</td>
</tr>
<tr>
<td>Sample Depth-2160</td>
<td>Bacillus flavothermus</td>
<td>60 °C</td>
</tr>
</tbody>
</table>
Figure 3-1. EDS analysis of crystals precipitated by thermophilic bacteria on B4 medium.
Figure 3-2. Calcite crystals precipitated by thermophilic bacteria on B4 medium.

Scanning Electron Microscopy, 330x magnification.
Figure 3-3. Calcite crystals precipitated by thermophilic bacteria on B4 medium.

Scanning Electron Microscopy, 330x magnification.
Figure 3-4. Nye County Monitoring Well NC-EWDP-1DX.
Figure 3-5.  Point of Rock warm spring (32\textdegree C) - Ash Meadows, NV.
Figure 3-6. Water sampling apparatus.
Figure 3-7. Warm spring (60° C) - Ash Springs, NV.
Figure 3-8.  Warm spring (60° C) - Ash Springs, NV.
Figure 3-9. Map - Location of Monitoring Well NC-EWDP-1DX.
Isolation of Thermophiles from Nye County Monitoring Well

Off-site Monitoring Well
NC-EWDP-1DX
Sample Depth 2160 feet

Thermophiles Isolated:

60° C
*Bacillus stearothermophilus*
*Bacillus flavothermus*

70° C
*Bacillus stearothermophilus*
Figure 3-10. Map - Location of Yucca Mountain Calcite/Tuff Samples.
Isolation of Thermophiles from Yucca Mountain Rock

- Isolates at 60degC
- Isolates at 70degC
- Isolates at 60degC and 70degC

Yucca Mountain

Thermophiles Isolated at 60deg C

1. 00566343 Calcite/Tuff
   Bacillus steatothermophilus
   Unidentified low G+ C

2. 00566341 Calcite/Tuff
   Bacillus steatothermophilus
   Thermal soil bacterium

3. 00555079 Calcite/Tuff
   Bacillus steatothermophilus
   Bacillus sp.

Thermophiles Isolated at 70deg C

4. 00555078 Calcite/Tuff
   Bacillus steatothermophilus
   Thermal soil bacterium

5. 00555076 Calcite
   Bacillus steatothermophilus
   Bacillus thermoeovorans
   Bacillus sp.
   Thermal soil Bacterium

6. 00555076 Calcite
   Bacillus steatothermophilus
   Thermal soil bacterium
CHAPTER 4

EVALUATION OF BIOLOGICAL FACTORS THAT INFLUENCE DEGRADATION OF LANDFILL WASTE CONTAINERS AT AN ARID SITE

This chapter has been prepared for submission to Applied and Environmental Microbiology and is presented in the style of that journal. The complete citation is:


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Abstract

To adequately determine the success of a low-level, radioactive, shallow trench disposal system, information concerning the long-term stability of container structures is needed. Permanent cap closures over these trenches often subside unevenly creating places where water can collect and subsequently transport to ground water. Subsidence will occur over a number of years as void space collapses and container materials degrade. Accelerated degradation of the container materials may increase the rate of subsidence and thereby provide an opportunity to control the rate. This could lead to lower maintenance costs and a reduced possibility of closure cap failure. Microbial degradation is one method currently under investigation that may be used to accelerate container collapse. The focus of this study was to determine whether bacterial colonization and biofilm development could enhance degradation of waste cell support and packaging materials, and to demonstrate degradation rates. A second objective was to quantify the effect of microbial degradation on the mechanical strength and stiffness of several different container forms.

Storage container materials used on the Nevada Test Site (NTS) were subjected to biological attack over a period of time ranging from 0 to 2 years. Materials tested were corrugated cardboard, wood, and steel. Microbial treatments included sulfate-reducing and iron-oxidizing bacteria isolated from the native test site soil and key fungal cultures capable of plant polymer biodegradation. The sulfate-reducing and iron-oxidizing bacteria are known for their ability to corrode metals (Pronk et al., 1992, Hamilton, 1985). The use of fungi, which can degrade cellulose and lignin, is another important treatment option for accelerating wood and cardboard degradation. Fungi can also tolerate water activity levels as low as 0.6 and are therefore better suited for survival in an arid environment.
Analysis of microbial degradative actions included quantitation of microbial colonization through cell enumeration, quantitative and qualitative measurements of biofilm production, and pitting or other degradative processes using scanning electron and confocal laser microscopy.

Results indicated that wood, cardboard, and metal materials were all substantially colonized by microorganisms. Heterotrophic plate counts increased from $1 \times 10^2$-1x$10^7$ CFU at day 0 to $1 \times 10^4$-$1 \times 10^8$ CFU after 8 months of incubation. Scanning electron microscopy showed an increase in colonization of bacteria and fungi from day 0 to 8 months for all treatment regimes. The wood and cardboard surfaces were colonized primarily by fungi. The metal surfaces were colonized primarily by bacteria. The experimental systems can now be used in an effort to make long term, qualitative predictions for trench disposal integrity.

Introduction

The Nevada Test Site (NTS) is one of several currently operating locations for low-level radioactive waste, shallow trench disposal. To adequately determine the long-term success of a shallow trench, information concerning its long-term stability is needed. One of the needs of the Department of Energy (DOE) is to develop methods to monitor breakdown of contained waste materials in long-term disposal sites located in arid desert environments. Breakdown of container materials could result in shifting and uneven collapsing, potentially causing the shallow trench closure cap to subside. If the closure cap is allowed to shift and collapse, rain collected on the uneven surface of the cap closure could come into contact with waste material, forming a leachate that could impact local water reservoirs. The timing and extent of this subsidence is a priority of the DOE so that models for the waste cell closure caps can be developed.

Microorganisms residing in oligotrophic environments such as at the NTS may have a significant impact on the low-level radioactive waste disposal program. The
ability of these native microorganisms to form biofilms enhances their survivability in these low nutrient environments by enabling different microbial species to develop syntrophic relationships made possible by the juxtapositioning of various microbial species within the biofilm matrix. Native to the NTS soil are sulfate-reducing and iron-oxidizing bacteria which are associated with microbially-influenced corrosion (MIC). MIC has been documented to exist in concert with biofilms; therefore, conditions which would promote biofilm formation must be studied so that suitable models can be developed for the waste burial program at the NTS.

Biofilms consist of cells immobilized on a substrate and frequently embedded in an organic polymer matrix of microbial origin (Characklis and Marshall, 1990). Bacteria obtain a degree of shelter and homeostasis while residing in a biofilm. One of the key components which allows for this stability is the exopolysaccharide (EPS) matrix, composed of anionic uronic acids and sugars. Biofilm cells have a greater capacity to convert glutamate to cell components and CO$_2$ than cells in the planktonic phase. This increased metabolic activity of biofilm cells may be the result of phenotypic changes in response to sessile growth (Costerton et al., 1987). The increased metabolic activity of biofilm cells may also be the result of nutrient trapping which occurs from the absorption of nutrients by the EPS matrix. High rates of biofilm development occur in oligotrophic environments and in distilled water systems, thus, indicating the significance of this nutrient trapping strategy (Costerton et al., 1987, Kim and Frank, 1995, Wirtanen et al., 1993).

When biofilms form on surfaces of insoluble nutrients, i.e., cellulose, the initial events of adhesion will favor bacteria capable of digesting that substrate, i.e., cellulolytic bacteria. These bacteria are able to produce digestive enzymes that can breakdown the insoluble substrate into soluble nutrients, which can then be utilized by adjacent heterotrophic microorganisms. Such associations result in the formation of a digestive consortium which is maintained by the biofilm mode of growth.
The proximity of metabolically cooperative microorganisms in biofilms allows for the degradation of other complex organic matter into methane and carbon dioxide (Davey and O'Toole, 2000). Such a degradation procedure requires the interaction of several species of bacteria and anaerobic conditions which are facilitated by biofilms. Fermentative bacteria initiate the catabolism, producing acids and alcohols that are utilized by acetogenic bacteria. The methanogens are then able to convert acetate, carbon dioxide, and hydrogen to methane if conditions are such that they meet the strict anaerobic requirements of these microbes.

Therefore, a general model for microbial consortia within biofilms is a nutritionally-linked group of aerobic, facultative, and anaerobic bacterial species. The initial heterotrophic species are determined by the nature and availability of the substrates.

Biodegradation usually involves the focused enzymatic attack at a particular site on a material surface. The focused attack produces pitting characteristic of the biodegradation of substrates ranging from the digestion of cellulose to the corrosion of stainless steel (Costerton et al., 1987). Physical attachment of microorganisms is necessary for active biodegradation, such as cellulose digestion. The enzymes involved remain in close association with bacterial cells growing in biofilms on the surface of cellulose fibers. Monocultures of cellulose-degrading bacteria have been shown in the laboratory to strongly adhere to cellulose fibers, but degrade at a much slower rate than found in nature, e.g., rumen (Costerton et al., 1987). However, the development of a microbial consortium involving several species of cellulose-degrading bacteria, and/or with fungi, results in an accelerated rate of degradation.

The products of cellulose degradation are available not only to the cellulolytic bacteria, but to other heterotrophic microorganisms as well. These products can create a chemotactic response as well as stimulate other heterotrophs to divide and form structured consortia. Noncellulolytic bacteria can accelerate the degradation of
cellulose by decreasing the levels of degradative byproducts. Other heterotrophic bacteria can, therefore, subsist on the soluble products of cellulose digestion.

Anaerobic fungi and other anaerobic microorganisms located in the animal rumen form a consortium capable of increased cellulose fermentation as allowed through the juxtapositioning of these microorganisms in biofilms. The fermentation products produced by fungi include $\text{H}_2$ which can be utilized by methanogens (Marvin-Sikkema et al., 1990). This interspecies transfer of $\text{H}_2$ results in increased $\text{CO}_2$ and acetate formation and is similar to the metabolic relationship between methanogens and $\text{H}_2$-producing bacteria in sewage sludge (Conrad et al., 1985). As much as 94-95% of methane produced in sewage sludge digestors results from juxtapositioned syntrophic partners within biofilm flocs (Conrad et al., 1985) which reinforces the importance of biofilm structure for the metabolic activities of environmental microorganisms.

The choice of microorganisms used to inoculate wood and cardboard in this research was based on the fact that lignin, an aromatic polymer covalently linked to cellulosic polysaccharides, is resistant to degradation by most microorganisms. This recalcitrant polymer can only be degraded by a few species of fungi and filamentous bacteria (Pometto and Crawford, 1986). One species of filamentous bacteria, *Streptomyces viridosporus*, degrades lignin to a water-soluble lignin polymer, acid-precipitable polymeric lignin (APPL), to gain access to the plant polysaccharides. APPL, however, is only slowly degraded by other lignin-degrading microorganisms.

Where biofilms facilitate microbially-influenced corrosion, SRBs benefit from the metabolic activities of respiring heterotrophs, which reduce oxygen tension, and the fermentation activities of facultative anaerobes, which supply organic electron donors for energy production (Geesey, 1991). Sulfate-reducing bacteria can utilize a range of organic electron donors to include fumarate, acetate, propionate, and fatty acids. SRBs, like the methanogens, use hydrogen and acetate for their energy and carbon sources,
which implies that the acetogenesis and interspecies hydrogen transfer are important processes in biofilms (Lappin-Scott and Costerton, 1995).

SRBs produce iron sulfide which promotes the ionization of metals, and therefore, induces pitting as well as allowing the accumulation and absorption of atomic hydrogen onto the metal causing hydrogen embrittlement (Lappin-Scott and Costerton, 1995). Metal corrosion can also occur as a result of a corrosion potential generated between an uncolonized metal surface and a metal surface colonized by bacteria. Biofilm activity can cause corrosion of metals by creating physicochemical differences between adjacent loci on a metallic surface until corrosion pits have been formed (Costerton et al., 1987).

The first objective of this study was to demonstrate biofilm formation on wood, cardboard, and metal materials by soil isolates from the NTS and seeded cellulolytic microorganisms. Enhancement of the application of microbes to container surfaces is the second important phase to this project. To achieve maximum attachment of microbes to waste container surfaces and to keep them viable long enough to allow colonization and development of substantial biofilm, a water-filled, nutrient-rich matrix was mixed with the microbes before application. The matrix used was a microcrystalline, cellulose gel which provides microbes with enough water and nutrient resources to colonize and form a protective biofilm (Potera, 2000).

In a third phase, the extent of biological colonization of the waste packaging materials and subsequent mechanical damage is correlated. In this way, a model to describe the processes of void space collapse, collapse of cap materials, and degradation and collapse of waste forms can be developed.
Materials and Methods

Soil Collection

Samples were collected from large mounds of soil excavated from the waste storage pits in Area 5, NTS. The top soil layers were removed using alcohol flame-sterilized tools and discarded. Soil below the discarded layer was removed and transferred to sterile plastic bags. The collected soil was placed on ice and transported to the laboratory within 6 hours of collection and subsequently stored at \(-20^\circ C\) (Kieft et al., 1997).

Isolation of Microorganisms

Microbial characterization of NTS backfill soil was accomplished using most probable number (MPN) assays (Alexander, 1965) and bacterial enumeration on R2A agar (Difco/ BD Diagnostics Systems, Sparks, MD). MPN assays revealed the presence of iron-oxidizing and sulfate-reducing bacteria in the backfill soil of Area 5, Nevada Test Site. A *Paracoccus* sp. was isolated from NTS soil which produced EPS (see Chapter 5). A cellulose-degrading ATCC bacterial strain of *Streptomyces* sp. was obtained. In addition, two species of fungi, known to degrade cellulose and lignin, *Scopulariopsis koningii* (ATCC 16280) and *Acremonium kiliense* (ATCC 16292) were used.

Microcosm Assembly

In the first phase of this biodegradation study, approximately 200 microcosms were assembled. Wood, cardboard, or metal coupons were cut to dimensions of 2.0 cm x 2.0 cm and autoclaved at \(121^\circ C\) for 20 minutes (Amsco Scientific SG-120 Sterilizer, Mentor, OH). The coupons were selected based on the packaging types used to transport and store low-level radioactive waste at the NTS. Coupons were buried in the backfill soil under aseptic conditions in 125 mL sterilized, glass jars with Teflon-lined screw caps (VWR Scientific, West Chester, PA). The microcosms were sealed and incubated at \(30^\circ C\) (Revco Scientific Inc. Model RI-50-1060-ABA, Asheville, NC).
Treatments for the wood and cardboard included: untreated, addition of water, *Streptomyces* sp. (cellulose-degrading bacterium), and *Paracoccus* sp. (EPS-producing bacterium). The treatments for the metal coupons included: untreated, addition of water, iron-oxidizing bacteria, or *Paracoccus* sp. The iron-oxidizing bacteria were enriched from NTS soil using a medium formulated according to Manning (1975). Each treatment group was sacrificed at the following timepoints: day 0, 1 month, 6 months, 1 year, and 2 years.

In the second phase of this study, backfill NTS soil was distributed into sterile glass Petri plates (20 minutes at 121°C, Amsco Scientific SG-120 Sterilizer). Coupons composed of wood, cardboard, and metal (approximately 1 cm x 1 cm) were left untreated or treated with a combination of microorganisms, culture media, cellulose gel, and backfill NTS soil (Fig. 1). The cellulose gel was prepared by mixing 7.5 g of microcrystalline cellulose with 100 mL of distilled water and autoclaving the preparation for 20 minutes at 121°C.

After treatment, the coupons were buried in the backfill NTS soil. The bottom reservoir in an autoclavable dessicator (Nalgene, Rochester, NY) was filled with sterile, distilled water to simulate 100% relative humidity or left dry to simulate ambient relative humidity (RH level that exists in a desert environment). Complete Petri plate microcosms were transferred to the dessicators and placed in incubators at 30°C (Revco Scientific Inc., Model RI-50-1060-ABA, Asheville, NC).

The wood and cardboard coupons were treated as follows: cellulose gel in water, cellulose gel in R2B broth, and cellulose gel in R2B broth inoculated with *Scopulariopsis koningii* (ATCC 16280) or *Acremonium kiliense* (ATCC 16292). Each of the cellulose gel treatment groups were duplicated (+/-) 10% w/v backfill NTS soil (Table 1).

C22 metal coupons, one cm², were treated as follows: coated with cellulose gel/water, or sulfate-reducing bacteria (SRB) medium (+/-) sulfate-reducing bacteria, or...
iron-oxidizing bacteria medium (+/-) iron-oxidizing bacteria (See APPENDIX I). Again, in the cellulose gel treatment groups, there was the presence or absence of 10% (w/v) backfill NTS soil mixed into the cellulose gel matrix (Table 1). All treatment groups were sacrificed at the following timepoints: day 0, 1 month, and 8 months.

Heterotrophic Plate Counts

At the time of analysis biofilms were scraped off coupons using into a 1.0 mL volume of R2B broth using sterile cell scrapers, vortexed, spread-plated onto R2A agar plates and incubated at room temperature (approximately 25°C) for two weeks. Colony forming units were counted and microbial equitability observed and calculated (Atlas and Bartha, 1998).

Scanning Electron Microscopy

Coupons were fixed in 4% glutaraldehyde (Sigma, St. Louis, MO) for scanning electron and confocal laser microscopic analyses (See APPENDIX I). SEM was performed at the EPMA/SEM Lab, University of Nevada, Las Vegas.

Results

Heterotrophic plate counts decreased from day 0 to 2 years for all treatments used in Phase I of the biodegradation study. Culturable counts ranged from $1 \times 10^4-1 \times 10^7$ CFU at day 0 to $1 \times 10^1-5 \times 10^5$ CFU after two years of incubation (See APPENDIX II). Wood coupons inoculated with Streptomyces sp. or Paracoccus sp. showed a decline in culturable counts from day 0 to one year followed by no further reduction in culturable cell number from one year to two years (See APPENDIX II). Cardboard coupons inoculated with Streptomyces sp. provided culturable counts which remained approximately $1 \times 10^7$ CFU from day 0 to 1 year followed by a decline in culturable counts to $1 \times 10^5$ CFU (See APPENDIX II). However, Paracoccus sp.-inoculated cardboard showed only a slight decline from $1 \times 10^6$ at day 0 to $5 \times 10^5$ at 2 years.
Treatments on the metal coupons resulted in culturable counts which declined from $1 \times 10^4$-$1 \times 10^6$ CFU at day 0 to $1 \times 10^3$-$5 \times 10^3$ at 2 years (See APPENDIX II). There was, however, a sharp increase in culturable counts from $5 \times 10^3$ at day 0 to $1 \times 10^5$ after 1 month of incubation on metal coupons incubated in native soil with no amendments (See APPENDIX II).

Equitability values remained the same or increased slightly from 0.4-0.8 at day 0 to 0.5-0.8 after 2 years for most treatment groups (See APPENDIX II). The one exception to the general pattern of static or slightly increased equitability was seen with cardboard coupons treated with sterile water only, where equitability decreased from 0.6 at day 0 to 0.2 by 2 years (See APPENDIX II).

Scanning electron microscopy demonstrated little colonization of microbes on wood and metal coupons from day 0 to 2 years (Figs. 1 - 4). One exception was the metal coupon group incubated in backfill soil with no further amendments, which showed an increase in colonization from the 1 year to 2 year timepoint (Figs. 5-6). Cardboard treatment groups showed an increase in colonization of bacteria and fungi from day 0 to 6 months (Figs. 7 - 9), followed by a decrease in colonization from 6 months to 2 years (Figs. 10-11).

In contrast to the first phase of the biodegradation study, heterotrophic plate counts in the second phase of the biodegradation study increased from $1 \times 10^3$-$1 \times 10^7$ CFU at day 0 to $1 \times 10^4$-$1 \times 10^8$ CFU after 8 months of incubation (See APPENDIX III). The culturable counts from treatment groups with cellulose gel were approximately the same as the culturable counts from treatment groups using cellulose gel mixed with backfill soil. The wood, cardboard, and metal coupons incubated in backfill soil alone provided culturable counts ranging from $5 \times 10^2$-$1 \times 10^3$ CFU at day 0 to $1 \times 10^4$-$5 \times 10^6$ CFU at 8 months (See APPENDIX III). Wood, cardboard, and metal coupons incubated with cellulose gel in backfill soil provided culturable counts ranging from $1 \times 10^5$ CFU at day 0 to $1 \times 10^5$-$1 \times 10^8$ CFU after 8 months (See APPENDIX III).
The addition of soil to the cellulose gel matrix reduced the rate of colonization on wood (Fig. 13) compared to the rate of colonization on wood exposed to backfill soil only (Fig. 15) or on wood inoculated with cellulose gel only (Fig. 16). The rate of colonization of wood treated with cellulose/ gel/ soil was similar to the colonization of wood resulting from cellulose/ gel/ soil incubated at ambient RH (Fig. 14).

Heterotrophic plate counts for the fungal-inoculated wood and cardboard coupons ranged from approximately $1 \times 10^5$ CFU at day 0 to $1 \times 10^7$ CFU after 8 months of incubation (See APPENDIX III). Compared to wood inoculated with a mixture of R2B medium, cellulose gel, and soil (Fig. 17), wood coupons inoculated with a mixture of *Acremonium kiliense*, cellulose gel, and soil demonstrate a smaller net increase in heterotrophic plate counts from day 0 to 8 months (Fig. 19). Wood coupons inoculated with *Scopulariopsis koningii*, cellulose gel, and soil provided an increase in heterotrophic plate counts from day 0 to 8 months (Fig. 18) that was similar to the wood coupons inoculated with R2B medium, cellulose gel, and soil (Fig. 17).

Equitability values for most treatment groups in the second phase increased from 0.2-0.8 at day 0 to 0.7-0.9 after 8 months of incubation (See APPENDIX III). Treatment groups that showed a decrease in equitability were the wood, cardboard and metal coupons incubated in backfill soil alone; the cellulose gel-treated wood, cardboard, and metal coupons incubated at ambient relative humidity; and the metal coupons treated with cellulose gel and iron-oxidizing microbes and medium (See APPENDIX III). These equitability values ranged from 0.7-0.8 at day 0 to 0.2-0.6 after 8 months of incubation.

On the wood coupons inoculated with fungi, cellulose gel, and soil, the trend in diversity values was similar from day 0 to 8 months (Figs. 21-22) with initial equity values of 0.6 at day 0, increasing to approximately 0.9 at 2 months, and decreasing to approximately 0.8 at 8 months. At the 8 month timepoint, the control group consisting of wood coupons inoculated with a mixture of R2B medium, cellulose gel, and soil
(Fig. 20) had diversity values equal to those at 8 months for the *Scopulariopsis
*konigii*-inoculated and *Acremonium kiliense*-inoculated wood coupons (Figs. 21-22).

The equitability values ranged from approximately 0.7 at day 0 to approximately
0.6 at 8 months on wood coupons incubated with soil only and wood coupons
inoculated with cellulose gel and soil (Figs. 23-24). Wood coupons inoculated with
cellulose gel and soil incubated at low RH also had equitability values which ranged
from 0.8 at day 0 to 0.6 at 8 months (Fig. 25). This is in contrast to the equitability
range on wood coupons inoculated with cellulose gel only which showed a significant
decrease in equitability values from 0.7 at day 0 to 0.3 at 8 months (Fig. 26).

Scanning electron microscopic analyses corroborate the culturable count results
for this phase of the biodegradation study. Under most treatment regimes, there was an
increase in colonization of microbes from day 0 to 8 months (Figs. 27-36). Wood and
cardboard treatment groups were colonized primarily with fungi (Figs. 27-33) as
opposed to the metal treatment groups which were primarily colonized by bacteria
(Figs. 34-36). One exception was the metal coupons treated with cellulose gel only and
with cellulose gel mixed with native soil. SEM results showed colonization of these
metal surfaces with fungi (Figs. 37-38).

Discussion

The first phase of the biodegradation study was to demonstrate microbial
colonization and biofilm formation in order to accelerate degradation of the waste
packaging material. Treatments were selected based on microorganisms known to
degrade cellulose and lignin, major components of wood and cardboard (Costerton et
al., 1987, Pometto and Crawford, 1986), and to corrode metals (Geesey, 1991, Pronk et
al., 1996).

Current treatment includes backfill with native soil, therefore, acceleration of the
degradative process must be compared to native soil treatment alone. Microbial
colonization on the wood and metal coupons decreased over time and SEM analyses revealed no discernible microbial attachment on either the untreated or treated wood and metal surfaces. The wood and metal coupons were subjected to inocula of sterile water, or suspensions in sterile water with *Streptomyces* sp., *Paracoccus* sp., or an iron-oxidizing microbial consortium enriched from the native soil. It is possible that the sterile water preparations did not adhere to the wood and metal surfaces effectively and dried before the microorganisms could adhere. Adherence is necessary for active biodegradation, such as cellulose digestion (Costerton et al., 1987), and the water activity level for most microorganisms needs to be 0.96 or greater for active metabolism (Atlas and Bartha, 1998).

Monocultures, as used in this study, degrade cellulose at a much slower rate than found in nature (Costerton et al., 1987). In addition, lignin, a recalcitrant polymer, can only be degraded by a few species of fungi and filamentous bacteria (Pometto and Crawford, 1986). It is not known if the necessary components needed for a successful degradation process are present in the surrounding NTS soil.

The untreated and treated cardboard coupons showed increased fungal and bacterial colonization on the surfaces from day 0 to 6 months (Figs. 7-9). Upon inoculation with sterile water or bacterial suspensions, the cardboard coupons absorbed water thus providing a possible reservoir of water for microbial use. Buried in the backfill soil, the cardboard could provide a source of water for the indigenous microbes as well as for the added bacterial inocula used. Water allowed a conditioning film of nutrients to concentrate on the cardboard surface which was available for cell replication and biofilm formation. Initial water activity levels for the native soil were measured at greater than 0.92 which would be sufficient for fungi and some bacteria (Atlas and Bartha, 1998).

EPS material in matrix of the biofilm can continue to absorb nutrients and increase metabolic activity of the biofilm cells (Costerton et al., 1987, Davey and
As fungi colonized the cardboard surfaces, the products of cellulose degradation could have been made available to other heterotrophic bacteria thus forming a consortium of microbes which could effectively degrade the cellulose fibers of the cardboard (Costerton et al., 1987). SEM images of the cardboard coupons from day 0 to 6 months showed colonization of fungi along with biofilm formation on the cardboard surface (Figs. 7-9). Evidence of biodegradation of the cardboard surfaces can be seen in the form of embedded fungal hyphae within the cardboard fibers.

Following initial growth, fungal and bacterial colonization decreased from 6 months to 2 years on the cardboard surfaces (Figs. 10-11). It is possible that decreased water and nutrient availability could have inhibited further biofilm formation (See Chapter 2). The microcosms used were closed systems; there was no influx of water or nutrients, and the reservoir provided by the biofilm and cardboard surfaces could have been exhausted.

Equitability for most treatment groups in the first phase biodegradation study, remained at approximately the same value or they increased from day 0 to 2 years (See APPENDIX II). Although, the culturable counts decreased, the species equitability either did not change significantly or became more evenly distributed. The cardboard coupons inoculated with sterile water were an exception to this because they showed a sharp decline in equitability from 6 months to 2 years (Fig. 12). This decline indicated that as the number of culturable counts declined, the species diversity became less evenly distributed with fewer species dominating (Atlas and Bartha, 1998).

Based on the initial findings, the second phase of the biodegradation study focused on ensuring adherence of microorganisms to the coupon surfaces, providing a water and nutrient reservoir, and selecting a more effective group of microorganisms to degrade lignin and corrode metal.

A water-filled, nutrient-rich matrix was needed to mix with the microbial inocula in order to achieve maximum attachment onto the coupon surfaces and keep the
microbes viable for colonization and biofilm formation. The matrix chosen was a microcrystalline, cellulose gel (Opta Food Ingredients, Inc., Bedford, MA) which absorbs liquids and became a very sticky, paste-like substance (Potera, 2000).

Two fungal species were chosen from ATCC for their cellulose and lignin-degrading characteristics, *Scopulariopsis koningii* (ATCC 16280) and *Acremonium kiliense* (ATCC 16292). In addition to an iron-oxidizing consortium enriched from the native soil, a SRB consortium enriched from the backfill soil was also used to determine effectiveness in colonizing and forming biofilms to effect microbially-influenced corrosion on metal.

Compared to native soil controls with no cellulose gel and to the results obtained in the first phase of the biodegradation study, initial adherence of microorganisms to the coupons increased with use of the cellulose gel. During the course of the study, the cellulose gel appeared to provide the necessary nutrients and water availability for adherence and colonization. Heterotrophic plate counts were higher at day 0, as compared to the phase 1 study, and increased over the 8 month period (See APPENDIX III). SEM images showed extensive colonization of fungi on wood and cardboard with fungal hyphae embedded in the wood and cardboard (Figs. 27-33). In fact, there was difficulty seeing the actual wood and cardboard surfaces.

In phase 2 of the biodegradation study there was extensive colonization of bacteria on metal surfaces as well (Figs. 34-36). Cellular morphologies varied and included cocci and bacilli in chains, and small diameter, filamentous bacteria (Figs. 39-41). One exception was the metal coupons treated using cellulose gel with no nutrient or microbial addition. In this case, colonization of the metal by fungi (Figs. 37-38) was observed by SEM, however, the significance of this finding is not completely understood. Microbially-influenced corrosion was not evident from the SEM images, however, electron dispersion spectroscopy (EDS) analysis at the 1 year timepoint will be used to find evidence of corrosion by-products at a later date.
Equitability indices increased from day 0 to 8 months for most of the treatment groups in Phase 2 (See APPENDIX III). As colonization increased, the species diversity became more evenly distributed (Atlas and Bartha, 1998). However, some treatments demonstrated decreased equitability, these included: metal coupons with iron-oxidizing medium, microbes and cellulose gel; and wood, cardboard, and metal coupons treated with cellulose gel at low relative humidity (See APPENDIX III). These treatment conditions, along with untreated wood, cardboard, and metal coupons in backfill soil, showed increased colonization but with species diversity less evenly distributed. Apparently the iron-oxidizing medium and the low relative humidity treatments favor a smaller variety of microorganisms.

The equitability values of wood coupons inoculated with cellulose gel only showed a significant decrease from 0.7 at day 0 to 0.3 at 8 months (Fig. 26) indicating a shift towards an unevenness in species diversity. This is in contrast to wood coupons inoculated with cellulose gel and soil, cellulose gel and soil at low RH, and wood coupons incubated in native soil only. These treatments resulted in equity values ranging from 0.8 at day 0 to 0.6 at 8 months (Figs. 23-25). The presence of soil in the cellulose gel and native soil treatments resulted in stability of species distribution and higher evenness in species distribution present in the biofilm, possibly due to maintenance of water activity.

As noted earlier, the addition of soil to the cellulose gel matrix either reduced the rate of colonization on wood (Fig. 13) or showed no significant change in the rate of colonization compared to wood surfaces exposed to backfill soil alone (Fig. 15), wood inoculated with cellulose gel alone (Fig. 16), and wood inoculated with cellulose gel and soil incubated at ambient RH (Fig. 14). It is possible that the presence of soil in the cellulose gel matrix introduced microorganisms that were antagonistic, thus slowing down the progress of adhesion (Norwood and Gilmour, 2001, Ichida et al., 2001).

Wood coupons inoculated with *Acremonium kiliense* in cellulose gel with soil had a
lower net increase in heterotrophic plate counts than *Scopulariopsis koningii* in cellulose gel and soil (Figs. 18-19). It is possible that *Acremonium kiliense* produced antibacterial compounds which inhibited some of the native soil microorganisms.

Bacteria and fungi were seen coexisting in the biofilms formed on wood surfaces (Fig. 42). The added fungi, as well as fungi from the native soil, may have provided heterotrophic bacteria with metabolic products from cellulose degradation (Costerton et al., 1987).

Microbial communication needed to form the typical three dimensional structure of biofilms with mushroom towers and water channels was not evident. However, biofilms in soil systems often appear as patchy films or dense microcolonies (Auerbach, et al., 2000) and these could be seen on the wood, cardboard, and metal surfaces in this study (Figs. 27-36). These unsaturated biofilms do not exhibit the mushroom-like structures and presence of channels for fluid flow typical of aquatic biofilms.

Findings on accelerated biodegradation will be combined with mechanical stress testing to determine if microbial colonization and apparent degradation of the coupon surfaces are sufficient to compromise the structural integrity of these materials. The UNLV Department of Mechanical Engineering will provide stress testing at appropriate timepoints. The results will indicate which treatments are the most effective in accelerating biodegradation and therefore, reducing the risk associated with subsidence in the shallow trench, low-level radioactive waste disposal cells.

References


Table 4-1  Biodegradation Phase 2 Treatment Groups

<table>
<thead>
<tr>
<th>Metal</th>
<th>100% RH</th>
<th>Low RH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Cellulose</td>
<td>- Cellulose</td>
</tr>
<tr>
<td></td>
<td>+ Dirt</td>
<td>- Dirt</td>
</tr>
<tr>
<td>SRBm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRBb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeOxm</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>FeOxb</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Dirt</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Wood/Cardboard</th>
<th>100% RH</th>
<th>Low RH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Cellulose</td>
<td>- Cellulose</td>
</tr>
<tr>
<td></td>
<td>- Dirt</td>
<td>- Dirt</td>
</tr>
<tr>
<td>F1b</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>F2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Dirt</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F1B; Scopulariopsis koningli  
F2b; Acremonium killiense  
Fm; R2B medium  
SRBb; sulfate-reducing bacteria  
SRBm; SRM medium  
FeOxb; Iron oxidizing bacteria  
FeOxm; FeOx medium
Figure 4-1. Biodegradation Phase 1 - Wood at Day 0 treated with *Streptomyces* sp.

Scanning Electron Microscopy, 5000x magnification.
Figure 4-2. Biodegradation Phase 1 - Wood after 2 years treated with *Streptomyces* sp. Scanning Electron Microscopy, 500x magnification
Figure 4-3. Biodegradation Phase 1 - Metal at Day 0 treated with *Paracoccus* sp.

Scanning Electron Microscopy, 5000x magnification.
Figure 4-4. Biodegradation Phase 1 - Metal after 2 years treated with Paracoccus sp. Scanning Electron Microscopy. 500x magnification.
Figure 4-5. Biodegradation Phase 1 - Metal after 2 years incubated in backfill soil unamended. Scanning Electron Microscopy, 1000x magnification
Figure 4-6. Biodegradation Phase 1 - Metal after 2 years incubated in backfill soil unamended. Scanning Electron Microscopy, 2000x magnification.
Figure 4-7. Biodegradation Phase 1 - Cardboard at Day 0 treated with

*Streptomyces* sp. Scanning Electron Microscopy. 5000x magnification.
Figure 4-8. Biodegradation Phase 1 - Cardboard after 1 month treated with *Streptomyces* sp. Scanning Electron Microscopy, 5000x magnification.
Figure 4-9. Biodegradation Phase 1 - Cardboard with 6 months treated with

*Streptomyces* sp. Scanning Electron Microscopy. 5000x magnification.
Figure 4-10. Biodegradation Phase 1 - Cardboard after 1 year treated with

*Streptomyces* sp. Scanning Electron Microscopy, 1500x magnification.
Figure 4-11. Biodegradation Phase 1 - Cardboard after 2 years treated with

*Streptomyces* sp. Scanning Electron Microscopy, 1000x magnification.
Figure 4-12. Biodegradation Phase 1 - Cardboard treated with sterile water.

Equitability values from Day 0 to 2 years.
Cardboard - Sterile Water

Equitability (J)

Months

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Figure 4-13. Biodegradation Phase 2 - Wood treated with cellulose gel and soil.

Heterotrophic Plate Counts from Day 0 to 8 months.
Figure 4-14. Biodegradation Phase 2 - Wood treated with cellulose gel and soil, incubated at ambient RH. Heterotrophic Plate Counts from Day 0 to 8 months.
Figure 4-15. Biodegradation Phase 2 - Wood incubated in backfill soil unamended.

Heterotrophic Plate Counts from Day 0 to 8 months.
Wood - Soil only

Heterotrophic Plate Counts per Coupon vs. Months

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Figure 4-16. Biodegradation Phase 2 - Wood treated with cellulose gel only.

Heterotrophic Plate Counts from Day 0 to 8 months.
Figure 4-17. Biodegradation Phase 2 - Wood treated with R2B medium, cellulose gel, and soil. Heterotrophic Plate Counts from Day 0 to 8 months.
Figure 4-18. Biodegradation Phase 2 - Wood treated with *Scopulariopsis koningii* in R2B medium, cellulose gel, and soil. Heterotrophic Plate Counts from Day 0 to 8 months.
Wood - *Scopulariopsis koningii* in R2B medium, Cellulose & soil

- 1e+9
- 1e+8
- 1e+7
- 1e+6
- 1e+5
- 1e+4
- 1e+3

Heterotrophic Plate Counts vs. Months
Figure 4-19. Biodegradation Phase 2 - Wood treated with *Acremonium kiliense* in R2B medium, cellulose gel, and soil. Heterotrophic Plate Counts from Day 0 to 8 months.
Wood - *Scopulariopsis koningii* in R2B medium, Cellulose & soil

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Figure 4-20. Biodegradation Phase 2 - Wood treated with R2B medium, cellulose gel, and soil. Equitability values from Day 0 to 8 months.
Figure 4-21. Biodegradation Phase 2 - Wood treated with *Scopulariopsis koningii* in R2B medium, cellulose gel, and soil. Equitability values from Day 0 to 8 months.
Wood - *Scopulariopsis koningii* in R2B medium, Cellulose & soil

**Equitability (J)**

**Months**

0 2 4 6 8 10

Equitability (J)
Figure 4-22. Biodegradation Phase 2 - Wood treated with *Acremonium kiliense* in R2B medium, cellulose gel, and soil. Equitability values from Day 0 to 8 months.
Figure 4-23.  Biodegradation Phase 2 - Wood incubated in backfill soil unamended.

Equitability values from Day 0 to 8 months.
Wood - Soil only

![Graph showing equitability (J) over months]

- Equitability (J) decreases from month 0 to month 2, then stabilizes.
- The graph shows data points with error bars indicating variability.

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Figure 4-24. Biodegradation Phase 2 - Wood treated with cellulose gel and soil.

Equitability values from Day 0 to 8 months.
Figure 4-25. Biodegradation Phase 2 - Wood treated with cellulose gel and soil at ambient RH. Equitability values from Day 0 to 8 months.
Figure 4-26. Biodegradation Phase 2 - Wood treated with cellulose gel only.

Equitability values from Day 0 to 8 months.
Wood - Cellulose only

Equitability (J)

Months

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Figure 4-27. Biodegradation Phase 2 - Wood at Day 0 treated with *Scopulariopsis koningii* in R2B medium, cellulose gel, and soil. Scanning Electron Microscopy, 500x magnification.
Figure 4-28. Biodegradation Phase 2 - Wood after 2 months treated with *Scopulariopsis koningii* in R2B medium, cellulose gel, and soil.

Scanning Electron Microscopy, 500x magnification.
Figure 4-29. Biodegradation Phase 2 - Wood after 8 months treated with *Scopulariopsis koningii* in R2B medium, cellulose gel, and soil. Scanning Electron Microscopy, 650x magnification.
Figure 4-30. Biodegradation Phase 2 - Cardboard at Day 0 treated with *Scopulariopsis koningii* in R2B medium, cellulose gel, and soil. Scanning Electron Microscopy, 500x magnification.
Figure 4-31. Biodegradation Phase 2 - Cardboard after 2 months treated with

*Scopulariopsis koningii* in R2B medium, cellulose gel, and soil.

Scanning Electron Microscopy, 250x magnification.
Figure 4-32. Biodegradation Phase 2 - Cardboard after 2 months treated with *Scopulariopsis koningii* in R2B medium, cellulose gel, and soil. Scanning Electron Microscopy, 2000x magnification.
Figure 4-33. Biodegradation Phase 2 - Cardboard after 8 months treated with

*Scopulariopsis koningii* in R2B medium, cellulose gel, and

soil. Scanning Electron Microscopy, 430x magnification.
Figure 4-34. Biodegradation Phase 2 - Metal at Day 0 treated with SRBs, cellulose gel, and soil. Scanning Electron Microscopy, 5000x magnification.
Figure 4-35. Biodegradation Phase 2 - Metal after 2 months treated with SRBs, cellulose gel, and soil. Scanning Electron Microscopy, 1000x magnification.
Figure 4-36. Biodegradation Phase 2 - Metal after 8 months treated with SRBs, cellulose gel, and soil. Scanning Electron Microscopy, 1100x magnification.
Figure 4-37. Biodegradation Phase 2 - Metal after 8 months treated with cellulose gel only. Scanning Electron Microscopy, 1000x magnification.
Figure 4-38. Biodegradation Phase 2 - Metal after 8 months treated with cellulose gel and soil. Scanning Electron Microscopy, 2000x magnification.
Figure 4-39. Biodegradation Phase 2 - Metal after 2 months treated with Iron Oxidizers, cellulose gel, and soil. Scanning Electron Microscopy, 1000x magnification.
Figure 4-40. Biodegradation Phase 2 - Metal after 2 months treated with SRBs, cellulose gel, and soil. Scanning Electron Microscopy, 5000x magnification.
Figure 4-41. Biodegradation Phase 2 - Metal after 8 months treated with SRBs and cellulose gel. Scanning Electron Microscopy, 1500x magnification.
Figure 4-42. Biodegradation Phase 2 - Wood after 8 months treated with *Acremonium kiliense* in R2B medium and cellulose gel. Scanning Electron Microscopy, 2000x magnification.
CHAPTER 5

BIOFILM FORMATION AND MICROBIAL EFFECTS ON POLYETHYLENE ENCAPSULATING MATERIAL

This chapter has been prepared for submission to *Applied and Environmental Microbiology* and is presented in the style of that journal. The complete citation is:

Abstract

An investigation of long-term stability of encapsulation materials of low-level radioactive waste at the Nevada Test Site (NTS) was initiated to see if biofilm development could compromise the structural integrity of polyethylene (ASTM Designation G22-76, 1990, Shaulis, 1996). At the NTS, low-level radioactive waste is stored in shallow trenches which, after filling with waste containers, are backfilled and capped with excavated soil to provide a stable barrier against migration of radioactive waste material away from the storage site. Polyethylene may be used to encapsulate radioactively-contaminated waste contained in large waste canisters to provide an added barrier against migratory or degradative processes.

Overall, heterotrophic plate counts increased with time on polyethylene surfaces for up to two years, after which the culturable counts showed no increase, to slight decreases, from two to three years. Confocal laser microscopy generally showed increased biofilm depth and colonization of the coupon surfaces during the course of the treatment. Coupon weight change was negligible and there was no apparent degradation observed on the coupon surfaces, either visibly or with the use of the confocal laser and scanning electron microscopy. Scanning electron microscopy revealed bacilli and coccobacilli, arranged singly and in chains, and actinomycetes on the polyethylene surfaces. Aggregates of what appeared to be biofilm exopolymer and minerals attached to the coupon surfaces were also seen. Diversity and equitability indices indicated shifts in the number and proportion of microbial species during biofilm development in all treatment groups. Such parameters will be key indicators in monitoring microbial activity and possible polyethylene degradation for national repositories.
Environmental bacteria often exist in biofilms, a matrix of bacterially produced extracellular polymeric substances (EPS) and bacterial cells (Auerbach et al., 2000). Bacterial adhesion and cellular binary fission in concert with EPS production leads to the formation of biofilms on a number of different surfaces (Tolker-Nielsen et al., 2000). With certain bacteria, e.g., *Shewanella putrefaciens*, *Vibrio cholerae*, and *Staphylococcus epidermidis*, EPS is required for initial attachment to surfaces. However, in the case of *Escherichia coli*, a component of EPS, colanic acid, is required for establishing the complex 3-dimensional structure of *Escherichia coli*'s biofilm (Danese et al., 2000). Especially in aquatic systems, biofilm morphology is well characterized with stalked EPS-encapsulated cells growing from the substrate's surface and channels for fluid flow around and through the EPS-cellular stalks.

Biofilms, however, appear patchy or consisting of microcolonies in environments that lack a constant fluid flow. These biofilms exist in environments that are only transiently wet. Whereas in aquatic systems, channels in the biofilms act as conduits for exchange of oxygen and nutrients from the bulk fluid, unsaturated biofilms depend on the air-biofilm interphase for diffusion of gasses and nutrients (Auerbach et al., 2000).

Instead of channels, unsaturated biofilms are often seen with valleys between individual bacteria. EPS exhibits a variety of structures ranging from spherical arrays to worm-like configurations (Auerbach et al., 2000). In addition, atomic force microscopy (AFM) revealed bacteria at the base of unsaturated biofilms emerging out of the biofilm-substrate interface, indicating crowding and variation in cell size that could contribute to biofilm roughness.

Structure of biofilms can also be affected by the surfaces to which microorganisms attach. A marine bacterium, SW5, formed tightly packed biofilms on hydrophobic surfaces but only sparse colonization of chaining cells on hydrophilic
surfaces (Dalton, et al., 1994). The bacterium, *Vibrio cholerae*, used different pathways for adhesion depending on whether the surface was living or not (Ben-Ari, 1999).

Generalizations about physical properties such as solubility, viscosity, or gel formation can be made about factors that influence adhesion (Christensen, 1989). For example, enhanced solubility inhibits adhesion. Ionic groups, flexible linkages, or irregular chain structures influences solubility. These, along with environmental factors such as varying composition of the solvent and changing temperatures, could affect the 3-dimensional conformation of polysaccharide segments.

Certain biofilm polysaccharides have hydrophobic characteristics because of inclusion complexes such as amylose-iodine (Christensen, 1989). The iodine molecule is positioned within the amylose helix with the glucose residues oriented so that the hydrophobic portions are facing in one direction giving rise to a hydrophobic side of the polysaccharide chain. This can help to explain why apparent hydrophilic polysaccharides can adsorb to non-polar surfaces, such as polyethylene, and demonstrates the complexity of adhesion.

Biofilm formation can be considered a universal mode of survival and nutrient optimization for bacteria (Costerton et al., 1987). In general, conditioning films adsorb to substrates which concentrate scarce nutrients (Beveridge et al., 1997). However, this does not imply that eutrophic environments contain exclusively planktonic microorganisms (Lappin-Scott and Costerton, 1995) or that bacterial attachment and biofilm formation are based solely on nutrient scavenging. Other possibilities include resistance to washout, protection from predators, protection from dessication (Decho, 1994), heterotrophic relationships, syntrophic dependencies, and co-metabolism of recalcitrant compounds.

In nutrient binding and storage, large molecules associate with the EPS matrix through a combination of ionic, covalent, and Van der Waal interactions (Decho, 1994, Costerton and Boivin, 1991). Colloidal particles can also be trapped in the EPS where
they are stored and later hydrolyzed by extracellular and cell-surface enzymes. The EPS matrix slows the diffusion of these enzymes reducing the diffusion before hydrolysis is completed.

The biofilm matrix can slow not only the diffusion of extracellular enzymes but gaseous molecules such as oxygen (Geesey, 1991). Diffusion of oxygen through EPS is slowed, thus allowing for activities such as oxygen-sensitive nitrogen-fixation (Decho, 1994). The EPS matrix can also protect cells by binding and slowing the infiltration of toxic metals such as Cu, Cd, Pb, and Co (Ford and Mitchell, 1990). The charged binding sites on the matrix polymers can trap antibiotic molecules and afford protection to the innermost cells in the biofilm (Costerton et al., 1987, Davey and O'Toole, 2000). The susceptibility of biofilm bacteria to antibiotics could be diminished by the thick, hydrated anionic matrix which is a different solute phase from the bulk fluids of the system. Although, the EPS matrix is a barrier against antibiotic diffusion, the biofilm cells themselves are also phenotypically different from their planktonic counterparts which may affect antibiotic susceptibility as well (Costerton, 1995).

The sessile mode also triggers altered cell physiology such as higher rates of glucose assimilation and respiration (Fletcher, 1986). One facet of planktonic life is that consumed nutrients are primarily used for reproduction; however in mature biofilms, cell division may be an infrequent occurrence. The energy produced from consumed nutrients in biofilm-associated cells could be used to make exopolysaccharide which the cells can digest and use during periods of low nutrient availability (Watnick and Kolter, 2000).

EPS has also been reported to provide protection against dessication, UV radiation, osmotic shock, and shifts in pH (Davey and O'Toole, 2000). The EPS matrix acts like a highly, hydrated water storage reservoir (Decho, 1994) and has been shown to contain more than 95% water when fully hydrated (Neu, 1994). Work by Ophir and Gutnick, 1994, showed that EPS plays a role in dessication resistance as demonstrated...
by increased survival rates of EPS-producing mucoid strains of *Escherichia coli*, *Acinetobacter calcoaceticus*, and *Erwinia stewartii* compared to nonmucoid variants after exposure to vacuum dessication.

Immobilization of bacteria in biofilms enhances the microorganisms’ ability to survive at a higher rate than their planktonic counterparts. Through the use of artificial biofilms, *Escherichia coli*, demonstrated a higher rate of survival (viability and culturable counts) and displayed a lower susceptibility to aminoglycoside and beta-lactam antibiotics (Perrot et al, 1998). The distribution of bacteria within the agar disc (or artificial biofilm) was heterogenous with a higher cell concentration in the peripheral areas of the agar disc and less concentrated in the middle. This is consistent with the architecture of natural biofilms.

UV irradiation resistance in association with biofilms was demonstrated by fusing the recA gene (induced by DNA damaging agents) of *Pseudomonas aeruginosa* with the lux operon of *Vibrio fischeri* creating a bioluminescent biosensor for DNA damage (Davey and O'Toole, 2000). *Pseudomonas aeruginosa* containing the biosensor was immobilized in alginate, and after exposure to UV irradiation, no DNA damage response was elicited as indicated. It must be noted that the ability to resist dessication has also been associated with the ability to resist ionizing radiation (Mattimore and Battista, 1996). *Deinococcus radiodurans* is radiation and dessication resistant and response to both assaults requires the ability to repair extensive DNA damage.

Adherence behavior is also used by microorganisms to position themselves where the supply of organic nutrients is optimal, the pH allows utilization of specific growth factors, oxygen requirements are met, and the temperature range is compatible (Lappin-Scott and Costerton, 1995). *Thermothrix thiopara* is a classic example of positioning by adherence behavior. *Thermothrix thiopara* is a sulfur-oxidizing bacterium that attaches and positions itself at the sulfide-oxygen interface of thermal,
sulfur springs. Sulfide, located in the anaerobic zone of the sulfur spring, is oxidized by the sulfur oxidizing bacteria, an oxygen-requiring reaction.

Adherent positioning behavior can also lead to coaggregation of two more species of microorganisms resulting in the formation of, for example, sewage sludge granules (Macleod et al., 1990). One advantage to coaggregation is that it allows for syntrophic cross-feeding as in the syntrophic relationships observed between hydrogen-producing bacteria and methanogens in sludge flocs (Conrad et al., 1985). Syntrophic relationships are also seen when heterotrophic bacteria attach to cyanobacterial filaments. Oxygenic photosynthesis provides the needed oxygen for heterotrophs; and in turn, carbon dioxide, needed in photosynthesis, is provided by the heterotrophs to the cyanobacteria.

In this study, biofilm formation and its potential degradative effect on polyethylene were investigated. The effect of microorganisms native to NTS was studied by constructing microcosms with native soil and polyethylene coupons. Quantitative characteristics of biofilm formation were measured using heterotrophic plate counts (HPC), equitability indices, and soil and biofilm pH. Qualitative descriptions of biofilm formation and polyethylene integrity were recorded using scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM).

Materials and Methods

Soil Collection

Two different types of soil samples were collected from Nevada Test Site Area 5, waste burial pit 5 (Fig. 5-37). From the side of the pit, soil, for microbial analysis, was removed using a flame-sterilized auger. Backfill soil, for use in assembling microcosms, was collected from the base of the pit using a flame-sterilized shovel. Both soil sample types were placed on ice and transported to the laboratory within 6 hours of collection and subsequently stored at -20°C (Kieft, et al., 1997).
Microcosm Assembly

Approximately 200 microcosms were assembled to simulate burial conditions in the pit. Autoclaved glass jars, 250 mL volume (VWR Scientific, West Chester, PA), with Teflon-lined closures were used to house polyethylene coupons (5.08 cm x 5.08 cm x 0.3 cm) and native backfill soil. Assembly of the microcosms was completed in a sterile, laminar flow hood, followed by incubation at 30°C.

Water Activity and pH Measurements

At each timepoint, pH measurements were taken at the surface of the coupons (biofilm) and in the soil using a Beckman (Fullerton, CA) 110 ISFET pH meter. The water activity of each microcosm was measured using an AquaLab CX2 water activity meter, Decagon Devices, Inc. (Pullman, WA) (Gee et al., 1992).

Heterotrophic Counts

Biofilms were collected from the surface of the polyethylene coupons by scraping with sterile tissue culture scrapers (Costar, Corning Inc., Midland, MI) and vortexing the biofilm in R2B broth (APPENDIX I). Harvested biofilms were plated onto R2A (Difco/BD Diagnostics Systems, Sparks, MD) agar plates, and incubated for 2 wk at room temperature. Data were collected on culturable counts and Shannon-Weaver diversity indices (Atlas and Bartha, 1998) were calculated (See APPENDIX I).

Confocal Laser Microscopy

Progression of biofilm formation and coverage of the polyethylene surface was monitored using confocal laser scanning microscopy (CLSM) (See APPENDIX I). CLSM was conducted at the Center for Biological Imaging, Department of Biological Sciences, University of Nevada, Las Vegas.

Treatment Groups

Two control groups of coupons, sterilized dry backfill soil and dry backfill soil, were assembled. Dry backfill soil was used to compare the progress of biofilm

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formation on coupons that resulted from seven different amendments of native soil. Sterilized dry backfill soil was used to determine abiotic effects on the structural integrity of polyethylene in the absence of microorganisms, and to monitor possible contamination of the microcosms during the course of the investigation.

Two of the treatment groups were as follows: backfill soil amended with sterile, distilled water, and backfill soil amended with 10% R2B broth only (See APPENDIX I). The remaining five treatment groups consisted of backfill soil amended with 10% R2B broth and one of the following combinations of microorganisms isolated from the native soil: Paracoccus sp. (EPS producer), an acid producing isolate, Paracoccus sp. plus the acid-producing isolate, an actinomycete isolated from backfill soil, and a combination of all three bacterial types.

The timepoints for sacrificing microcosms were day 0, 2 mo, 6 mo, 1 yr, 2 yr, and 3 yr (for selected treatment groups). Duration of the experiment was determined based on how long it would take for biofilm to form on polyethylene under the various treatment groups described above.

Results

Heterotrophic plate counts increased for all treatments from $5 \times 10^3$ to $1 \times 10^6$ CFU at day 0 to $1 \times 10^6$ to $1 \times 10^8$ CFU between two and three years of incubation (Figs. 1 - 8). All treatments, except for the nutrient-treated microcosms (no microbial addition) and backfill soil controls, showed culturable counts which peaked after 2 mo, $1 \times 10^7$ to $1 \times 10^8$ CFU, and remained constant through the duration of the experiment. Nutrient-treated microcosms (no microbial addition) culturable counts peaked at $1 \times 10^7$ CFU after 6 mo and remained constant through the 3 yr timepoint (Fig. 3). Backfill soil controls showed culturable counts that peaked one log lower, $1 \times 10^5$ CFU after 1 yr of incubation and remained constant through the 2 yr timepoint (Fig. 1). Backfill soil controls,
without nutrient addition, also showed a sharp increase in heterotrophic plate counts from approximately $5 \times 10^3$ CFU at day 0 to $1 \times 10^6$ CFU after 2 mo of incubation (Fig. 1).

Equitability for most treatments cycled during the 2-3 yr incubation period (Figs. 9-16). For the first 2-6 months of incubation, equitability values increased from 0.4-0.6 at day 0 to 0.8-0.9 at 6 months. Equitability values decreased slightly to 0.6-0.7 after 1 yr of incubation and remained constant or increased slightly to 0.7-0.8 at the 2-3 yr timepoints.

Equitability values within dry backfill soil, water-treated, and nutrient-treated (no microbial addition) show that water-treated and nutrient-treated groups provided equitability values that increased from approximately 0.6 at day 0 to 0.8 after 2 years (Figs. 10, 11). Equitability values for the dry backfill soil decreased sharply from 0.8 at day 0 to 0.5 at 6 mo and remained constant for 2 years (Fig. 9).

Water activity values for unamended backfill soil remained constant at approximately 0.98 from day 0 to 6 mo. From 6 mo to 2 yr, water activity values decreased to approximately 0.2 (Fig. 17).

CLSM images of polyethylene surfaces buried in dry native soil showed gradual colonization of microorganisms that increased from 2 mo to 2 yr (Figs. 20-26). Microorganisms that colonized the polyethylene surfaces included chaining cocci and filamentous bacteria (Figs 23-26). CLSM images of polyethylene surfaces treated with water, nutrient, and bacterial inocula showed increasing biofilm formation over the polyethylene surface during the course of the study (Figs. 27-31). Depth code analyses showed increase in thickness of biofilm ranging from 2-4 um at day 0 to 8-16 um at 3 yr (Figs. 32-36).

After an initial small decrease from pH range 8.0-8.5, biofilm pH remained relatively stable for all treatment groups until 1 yr (Fig. 18). Beginning 1 and 2 yr, the water-treated and actinomycetes-treated microcosms produced biofilm pH values which remained at the 1 yr level of approximately 7.50-8.00 (Fig. 18). The microcosms
inoculated with the acid-producing bacterial isolate demonstrated a slight drop in biofilm pH at the 2 yr timepoint to slightly below pH 7.50. The remaining treatment groups demonstrated significant decreases in biofilm pH values, 5.50-6.50, after 2 years (Fig. 18). Of the latter, the Paracoccus sp. (EPS-producer) inoculated microcosms and the nutrient-treated (no microbial addition) microcosms produced biofilm pH values which increased to between 7.00 and 7.50 (Fig. 18). The microcosms inoculated with all three bacterial types remained stable from 2 to 3 years at slightly above pH 6.00 (Fig. 18).

After an initial small decrease as seen with biofilm pH, soil pH remained relatively stable for all treatment groups until year 2 timepoint (Fig. 19). At the 2 yr timepoint, the water-treated, nutrient-treated (no microbial addition), and actinomycete inoculated microcosms showed soil pH values that remained stable in a range between 8.00 and 8.50 (Fig. 19). Also at the 2 yr timepoint, the microcosms inoculated with all three bacterial types showed a sharp decrease in soil pH to slightly below 5.50 (Fig. 19). The three remaining treatment groups showed only slight decreases in soil pH values, 7.00-7.50, at the 2 yr timepoint (Fig. 19). The changes in soil pH were similar to those seen with biofilm pH changes, but more moderate. One exception was the treatment group involving all three bacterial types where the soil pH was lower, between 5.00 and 5.50, than the matching biofilm pH which was slightly greater than 5.50 (Fig. 19). Once again, the values returned to near initial readings by the 3 yr timepoint except in the microcosms containing all three bacterial types.

Discussion

Results obtained from the culturable counts during the course of this study show an increase for the first 2-6 months followed by relatively constant values through the 2-3 year incubation period (Figs. 1-8). All treatments, including the backfill soil control, gave similar results.
A number of possibilities appear plausible to explain changes observed in culturable counts within the biofilms. Two of these involve the hydrophobic nature and space constraints within biofilms. Polyethylene provides hydrophobic surfaces which can be colonized by microorganisms that generally have a net negative charge on their cell surfaces (Fletcher and Loeb, 1979). As a monolayer of attached bacteria is formed, the hydrophobic surface gradually becomes a negatively charged surface which is not favorable for bacterial attachment. Therefore, a decrease in rate of bacterial attachment occurs (Fletcher and Loeb, 1979). This phenomenon gives one explanation for the data resulting from this study.

Spatial constraints exist between bacteria within a biofilm. Using AMF, Auerbach et al. (2000), revealed multilayer biofilms with bacterial cells emerging from underneath other bacteria, giving the impression of limited space. Depending on the bacteria, such constraints could impede cell division. It is possible that in a mature biofilm, cell division is rare (Watnick and Kolter, 2000) and instead, cellular activities are directed towards the production of EPS which can be used as a food source during times of limited nutrient availability.

Culturable counts for the backfill soil control were lower at day 0, $5 \times 10^3$ CFU, as compared with the other treatment groups, $1 \times 10^6$ - $5 \times 10^6$ CFU (Figs. 1-8). The culturable counts did not peak until 1 yr for the backfill soil control, compared with the other treatment groups which peaked after 2-6 months of incubation. Except for the backfill soil control, all treatment groups were inoculated with either sterile water, R2B medium, or R2B medium containing microorganisms. The added water and nutrients made possible the dissolution and subsequent adsorption of organic substrates onto the surface. The diffusion of these organic molecules can be rapid, resulting in significant deposition, 0.8-1.5 mg organic matter/ m$^2$ surface within 15 minutes (Lappin-Scott and Costerton, 1995). These molecules include proteins, polysaccharides, nucleic acids, humic acids, fatty acids, and lipids (Geesey, 1994). An abundance of reactive binding
sites is available for further reaction with additional solute molecules or with chemical
groups expressed on bacterial surfaces. Concentration of nutrients onto the
polyethylene surface could become a chemoattractant for microbes, allowing them to
attach and grow (Marshall, 1988). The microcosms amended with water or nutrient
maintained culturable counts 10-100 fold greater than the dry, backfill soil controls
during the length of the experiment (Figs. 1-8).

Establishment of a biofilm in the backfill soil control corroborated earlier
studies of adhesion of bacteria to solid surfaces. It has been shown that bacteria were
rapidly attracted to solid surfaces, but irreversible attachment was only achieved after
bacteria were exposed to solid surfaces for a long period of time (Marshall, 1988).
During this phase of attachment, bacteria synthesized extracellular adhesive materials
(Marshall, 1988, Christensen, 1989). In addition, bacteria in natural environments were
confronted with low nutrient levels and, therefore, exhibited small size, reduced
metabolic activity, and increased adhesiveness. Increased adhesiveness provided
bacteria with an opportunity for growth under conditions of low-nutrient availability
(Geesey, 1994). The water activity in the backfill soil decreased below the optimum
level of $a_w = 0.96$ for most bacteria (Atlas and Bartha, 1998) after 6 months of
incubation in this study. The water activity level decreased to less than 0.8 at 1 yr and to
approximately 0.3 at 2 yr (Fig. 17). The decrease in water availability, and possibly
nutrient availability, most likely slowed metabolic activities of the microbes which
colonized and grew on the surface as they adapted to starvation conditions in order to
survive (Marshall, 1988).

The decrease in metabolic activities of microbes due to decreased nutrient and
water availability may be illustrated in the static level of culturable bacteria which
occurred after 2-6 months of incubation (Figs. 1-8). The water activity of backfill soil
decreased at six months from $a_w = 1.0$ to approximately 0.3 after 2 yr (Fig. 17).
As illustrated in the CLSM images (Figs. 27-31), biofilm formation on the polyethylene surface began with patchy colonization of bacterial cells. These patches developed into larger irregular-shaped colonies with peaks, valleys, and crater-like structures. Work by Tolker-Neilsen et al., (2000) showed similar biofilm developments using pure cultures of either *Pseudomonas* sp. strain B13 or *Pseudomonas putida* OUS82. Progression of the *Pseudomonas* sp. biofilms also began with small irregular patches of biofilm; however, depending on the bacterial species, the biofilm structures took on shapes ranging from dense ball-shaped microcolonies to loose irregularly shaped protruding structures.

Biofilms in environments that are only transiently wet, as in this study, may appear as patchy or dense microcolonies (Auerbach et al., 2000). Atomic force microscopic studies showed that these unsaturated biofilms, as opposed to biofilms in aquatic systems, had ridges and valleys (Auerbach et al., 2000) similar to the biofilm morphologies observed on the polyethylene coupons. It appears that biofilms show a range of morphologies depending on whether the environment is continuously wet or almost completely dry.

pH changes in the soil and polyethylene biofilm surfaces reflect the metabolic activities of the microorganisms. As microorganisms colonized the polyethylene surfaces and, assuming nutrient availability was adequate, the pH decreased due to the metabolic activity of the microbes. The breakdown of nutrients can result in end-products such as CO$_2$ which can dissolve in water to form carbonic acid. Anaerobic metabolism can result in the accumulation of alcohols and acids (McCabe 1990, Vroom, et al., 1999). Measured biofilm pH using two-photon excitation microscopy showed pH values decreased at the surface of the biofilm from 7.0 to 5.9 after sucrose was added. Breakdown products can also include ammonia which, along with the consumption of sulfate and nitrate, increase pH.
Our study demonstrated that most treatment groups shifted biofilm pH from initial values of 8.00-8.50 to pH values ranging from 5.50-6.50 after 2 years (Fig. 18). Soil pH for most treatment groups decreased from initial values of 8.00-8.50 to pH values ranging from 7.00-7.50 after 2 years (Fig. 19). The microcosms inoculated with all three bacterial groups showed a sharp decrease in soil pH, less than 5.50, after 2 years (Fig. 19) reflecting their mode of metabolism which may be fermentation. Measurements of pH in the soil and polyethylene biofilms of the actinomycetes-treated and water-treated groups showed only slightly decreased pH over 2 years with values ranging from 7.5-8.0 (Figs. 18, 19).

Biofilms are a major survival strategy for microorganisms and, although advantageous to the microbes, can be a major problem for maintenance and stability of manufactured devices or structures. This could be a problem for the encapsulation of low-level nuclear waste using polyethylene. The biofouling has been controlled to a certain extent through the use of paints and other coatings. However, such coatings can be detrimental to nontargeted organisms as well as toxic to humans.

One strategy is the exploitation of “smart” polymers which exhibit fouling release properties (Ista et al., 1999). These polymers can exhibit changes in solubility based on such environmental stimuli as pH, temperature, and light. For one particular type of “smart” polymer (PNIPAAm) at temperatures below a lower critical solubility temperature (LCST), the polymer is soluble and can be washed off along with any attached biofilm by rinsing with cold water (Ista et al., 1999).

Polyethylene is used as an encapsulating material for low-level radioactive waste stored at the NTS. Biodegradation and microbially-influenced corrosion are associated with the presence of biofilms. Therefore, the effect of biofilms on polyethylene encapsulating material was studied as one means of determining the stability of the low-level radioactive waste trenches at NTS. Our study demonstrated that microorganisms indigenous to NTS were capable of adhering and forming biofilms.
on polyethylene surfaces, however, CLSM and SEM images showed no signs of pitting. There were no weight changes in the sacrificed polyethylene coupons to indicate loss of material (data not shown). Stress testing showed no compromise in the structural integrity of treated polyethylene coupons compared to untreated polyethylene (data not shown). Under the conditions of this study, polyethylene remained stable against any potential biodegradative effects incurred by biofilms.

References


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Figure 5-1. Polyethylene incubated in unamended backfill soil. Heterotrophic Plate Counts from Day 0 to 2 years.
Backfill Soil

Heterotrophic Plate Counts

Months

1e+3
1e+4
1e+5
1e+6
1e+7
1e+8

0 10 20 30 40
Figure 5-2. Polyethylene incubated in backfill soil amended with water.

Heterotrophic Plate Counts from Day 0 to 2 years.
Backfill Soil & Water

Heterotrophic Plate Counts

Months

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Figure 5-3. Polyethylene incubated in backfill soil amended with nutrient (R2B broth). Heterotrophic Plate Counts from Day 0 to 3 years.
Figure 5-4. Polyethylene treated with Actinomycetes and nutrient (R2B broth).

Heterotrophic Plate Counts from Day 0 to 3 years.
Figure 5-5. Polyethylene treated with acid-producer (NTS isolate) and nutrient (R2B broth). Heterotrophic Plate Counts from Day 0 to 3 years.
Acid-Producer & Nutrient

Heterotrophic Plate Counts

Months

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Figure 5-6. Polyethylene treated with *Paracoccus* sp. (EPS-producer) and nutrient (R2B broth). Heterotrophic Plate Counts from Day 0 to 3 years.
Paracoccus sp. & Nutrient

Heterotrophic Plate Counts

Months

1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
0  10  20  30  40
Figure 5-7. Polyethylene treated with *Paracoccus* sp. (EPS-producer), acid-producer (NTS isolate), and nutrient (R2B broth). Heterotrophic Plate Counts from Day 0 to 3 years.
Paracoccus sp., Acid-Producer, & Nutrient

![Graph showing heterotrophic plate counts over months.](image-url)
Figure 5-8. Polyethylene treated with *Paracoccus* sp. (EPS-producer), acid-producer (NTS isolate), Actinomycetes, and nutrient (R2B broth). Heterotrophic Plate Counts from Day 0 to 3 years.
Paracoccus sp., Acid-Producer, Actinomycetes, & Nutrient

Heterotrophic Plate Counts vs. Months

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Figure 5-9. Polyethylene incubated in unamended backfill soil. Equitability indices from Day 0 to 2 years.
Figure 5-10. Polyethylene incubated in backfill soil amended with water. Equitability indices from Day 0 to 2 years.
Backfill Soil & Water

Equitability (J)

Months

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Figure 5-11. Polyethylene incubated in backfill soil amended with nutrient (R2B broth). Equitability indices from Day 0 to 3 years.
Backfill Soil & Nutrient

Equitability (J)

Months

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Figure 5-12. Polyethylene treated with Actinomycetes and nutrient (R2B broth).

Equitability indices from Day 0 to 3 years.
Figure 5-13. Polyethylene treated with acid-producer (NTS isolate) and nutrient (R2B broth). Equitability indices from Day 0 to 3 years.
Acid-producer & Nutrient

Equitability (J)

0.0 0.2 0.4 0.6 0.8 1.0

0 10 20 30 40

Months

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Figure 5-14. Polyethylene treated with *Paracoccus* sp. (EPS-producer) and nutrient (R2B broth). Equitability indices from Day 0 to 3 years.
Paracoccus sp. & Nutrient

Equitability (J)

0.0
0.2
0.4
0.6
0.8
1.0

0 10 20 30 40

Months

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Figure 5-15. Polyethylene treated with *Paracoccus* sp. (EPS-producer), acid-producer (NTS isolate), and nutrient (R2B broth). Equitability indices from Day 0 to 3 years.
Paracoccus sp., Acid-producer, & Nutrient

Equitability (J)

Months

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Figure 5-16. Polyethylene treated with *Paracoccus* sp. (EPS-producer), acid-producer (NTS isolate), Actinomycetes, and nutrient (R2B broth). Equitability indices from Day 0 to 3 years.
Paracoccus sp., Acid-producer, Actinomycetes, & Nutrient

Equitability (J)

0.0 0.2 0.4 0.6 0.8 1.0

0 10 20 30 40

Months

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Figure 5-17. Water activity values, from Day 0 to 2 years, for unamended backfill soil.
Figure 5-18. Biofilm pH values from Day 0 to 3 years.
Figure 5-19. Soil pH values from Day 0 to 3 years.
Soil pH vs Time

- EPS-producer
- Acid-producer
- EPS-producer & Acid-producer
- Actinomycetes
- EPS-producer, Acid-producer, Actinomycetes
- Water
- Nutrient

Time (Months)

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Figure 5-20. Polyethylene at Day 0 incubated in unamended backfill soil. Bright
Green and orange areas indicate microbial attachment. Confocal Laser
Scanning Microscopy, 400x magnification.
Figure 5-21. Polyethylene after 2 months incubation in unamended backfill soil.

Bright green and orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 5-22. Polyethylene after 2 months incubation in unamended backfill soil.

Bright green and orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 5-23. Polyethylene after 1 year incubation in unamended backfill soil. Bright green and orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 5-24. Polyethylene after 1 year incubation in unamended backfill soil. Bright green and orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.

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Figure 5-25. Polyethylene after 2 years incubation in unamended backfill soil. Bright green and orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 5-26. Polyethylene after 2 years incubation in unamended backfill soil. Bright green and orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 5-27. Polyethylene at Day 0 treated with *Paracoccus* sp. (EPS-producer), acid-producer (NTS isolate), and Actinomycetes. Bright green and orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 5-28. Polyethylene after 2 months treated with *Paracoccus* sp. (EPS-producer), acid-producer (NTS isolate), and Actinomycetes. Bright green and orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 5-29. Polyethylene after 1 year treated with *Paracoccus* sp. (EPS-producer), acid-producer (NTS isolate), and Actinomycetes. Bright green and orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 5-30. Polyethylene after 2 years treated with *Paracoccus* sp. (EPS-producer), acid-producer (NTS isolate), and Actinomycetes. Bright green and orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 5-31. Polyethylene after 3 years treated with *Paracoccus* sp. (EPS-producer), acid-producer (NTS isolate), and Actinomycetes. Bright green and orange areas indicate microbial attachment. Confocal Laser Scanning Microscopy, 400x magnification.
Figure 5-32. Depth code analysis for figure 5-27.
Figure 5-33. Depth code analysis for figure 5-28.
Figure 5-34. Depth code analysis for figure 5-29.
Figure 5-35.  Depth code analysis for figure 5-30.
Figure 5-36. Depth code analysis for figure 5-31.
Figure 5-37. Low-level radioactive waste, shallow trench disposal facility, Nevada Test Site.
CHAPTER 6

DISCUSSION

This investigation was based on subsurface microbes and their potential effect on manmade structures. Materials used for any structure must withstand variations in abiotic factors such as water availability, salinity, temperature, light, and radiation. Biotic factors (e.g., microorganisms) can have an influence on the structural integrity of substrates by their metabolic activities as they colonize surfaces such as metal, wood, stone, and concrete.

There are many examples where abiotic reactions in concert with microbial activities are involved in the weathering of manmade structures. For example, air pollutants were found to promote microbial colonization of natural stone leading to the deterioration of stone buildings (Mansch and Bock, 1998). Sulfuric acid produced by the metabolic activities of *Thiobacillus* sp. has caused severe damage to concrete sewer pipes, wastewater collection systems, and treatment plants (Vincke, et. al., 1999). Colonization by phototrophic bacteria and subsequent biofilm formation have caused significant damage to building structures of historical and cultural importance (Ortega-Morales, et. al., 2000).

The key to understanding microbial effects on the integrity of manmade structures is biofilms. Biofilms are exopolysaccharide matrices that protect bacteria
from adverse environmental conditions such as dessication, extreme temperatures, poor
nutrient availability, and predation. Bacteria involved in biodegradation and
microbially-influenced corrosion (MIC) are known to exist in biofilms and can form an
extensive, corrosive layer over any surface. Therefore, to assist the Department of
Energy - Yucca Mountain Project in ensuring the long-term stability of a nuclear waste
repository and the Nevada Test Site/ Nevada Operations in minimizing the risks of low-
level radioactive waste disposal, it was imperative to study biofilms in concert with
microbial degradation and microbially-influenced corrosion. It was necessary to
determine what conditions are required for biofilms to form as well as what conditions
will impede microbial attachment and biofilm formation. The study of biofilms is
important to issues concerning nuclear waste storage, the structural integrity of waste
packaging and structural support, and how these structures are affected by MIC and
biodegradation.

After initial placement of spent fuel rods in the repository at Yucca Mountain,
models predict that high temperature and low moisture conditions will prevail for an
extended period of time. Conditions which define the boundary for biofilm formation
were a central part of this research project. The finding of thermophilic bacteria in
calcite deposits in Yucca Mountain (Chap. 3) suggested that thermophiles might also
exist in the tuff and muckpile rock of Yucca Mountain which would further suggest that
canisters containing the spent fuel rods could be colonized. Therefore, temperatures of
30, 60, and 70°C were investigated (100% relative humidity (RH)) to determine if
thermophiles might be present and capable of forming biofilms on metal coupons under
these conditions. Results showed that at 60 and 70°C, heterotrophic plate counts were
negligible even after 18 months of incubation (Chap. 2). The apparent lack of thermophilic bacteria in the welded tuff of Yucca Mountain was further confirmed by the calcite deposition study (Chap. 3), i.e., thermophiles were only present in calcite deposits and not the native tuff material.

The most critical requirement for microbial metabolism and growth is water. Lack of water and nutrients puts cells into a state of starvation-survival (Costerton, et. al., 1987). Bacteria in this starvation mode decrease in size (Amy, et. al., 1993) and alter their cell surfaces (Kjelleberg and Hermansson, 1984) and patterns of peptide synthesis (Groat, et. al., 1986). In addition, they conserve their scarce metabolic resources in the exopolysaccharide (EPS) matrix until nutrient resources become favorable again (Costerton, et. al., 1987).

The water activity level for most bacteria needs to be 0.96 or greater for active metabolism (Atlas and Bartha, 1998). Results of the boundary for biofilm formation study (Chap. 2) showed that at 100% relative RH and 30°C, heterotrophic plate counts (HPC) from day 0 to 5 months on all three metal coupons increased from less than $1 \times 10^3$ CFU to $4-7 \times 10^4$ CFU per coupon. At relative humidities ranging from 86% to 32% at 30°C, culturable counts were negligible for the three metals tested.

The decrease in metabolic activities of microbes due to decreased nutrient and water availability may also be illustrated in the polyethylene biofilm study (Chap. 5) where static culturable counts for 2 - 3 years occurred after 2-6 months of growth on the coupons. The water activity of the backfill soil in the polyethylene biofilm study (Chap. 5) decreased below the optimum level of $a_w = 0.96$ for most bacteria (Atlas and Bartha, 1998) after 6 months of incubation. The water activity level decreased to less than 0.8 at
The decrease in heterotrophic plate counts (HPCs) seen in our study on boundary conditions for biofilm formation (Chap. 2) may be indicative of stress caused by nutrient deprivation. At 100% RH and 30°C, HPCs decreased after 5 months of incubation. It appears that the bacteria detached from the metal surface, accounting for the decrease in HPCs. Work done by Delaquis, et. al., 1989, demonstrated that the depletion of either glucose or nitrogen led to the active detachment of cells from a *Pseudomonas fluorescens* biofilm. Detachment resulting from nutrient depletion was also observed by Sawyer and Hermanowicz, (2000), working with *Aeromonas hydrophila* and *Pseudomonas aeruginosa* on glass surfaces.

Another possible reason for decreased HPCs is that microorganisms may undergo a shift from the culturable state to a viable, but nonculturable state. In this case, the nonculturable state could be a result of starvation. The process whereby bacteria adapt to starvation in order to survive is termed starvation-survival (Morita, 1982, Marshall, 1988). During starvation, the rapid and extensive degradation of ribosomes prevents a rapid response to new nutrient sources which decreases the culturability of cells (Becker, 1999).

The biodegradation study (Chap. 4) focused on ensuring adherence of microorganisms to the coupon surfaces by providing a water and nutrient reservoir. It was necessary to provide a water-based, nutrient-rich matrix mixed with microbial
inocula in order to achieve maximum attachment onto coupon surfaces and maintenance of microbial viability for colonization and biofilm formation. A microcrystalline, cellulose gel (Opta Food Ingredients, Inc) which absorbs liquids and becomes a paste-like substance was chosen (Potera, 2000).

Initial adherence of microorganisms to the coupons used in the biodegradation study (Chap. 4) increased with use of the cellulose gel compared to the adherence of microorganisms on substrate surfaces without the use of cellulose gel (See Chap. 4 results). The cellulose gel appeared to provide the necessary nutrients and water for adherence and colonization. Heterotrophic plate counts (HPCs) were higher at day 0 (compared to HPCs obtained without the use of the cellulose gel), and they increased over an 8 month period. SEM images showed extensive colonization of fungi with fungal hyphae embedded in the wood and cardboard. Extensive colonization of bacteria was seen on metal surfaces as well.

It was important to accelerate biodegradation to minimize the threat of subsidence, which could compromise the structural integrity of the low-level radioactive waste shallow trench disposal facility at the Nevada Test Site (NTS). Other industries have also attempted to accelerate biodegradation. Concerns for effectively degrading recalcitrant materials such as cellulose and lignin are present in agriculture. The poultry industry also has waste disposal concerns due to the rapid accumulation of recalcitrant materials such as beta keratin, a primary component of feathers (Ichida, et al., 1999).

Methods to monitor the degradation process in the UNLV Environmental Microbiology Lab’s study of accelerated degradation of waste packaging material were
similar to methods utilized in a study of biodegradation of poultry waste (Ichida, et. al., 1999). Culturable counts and SEM analyses were used to document the progression of biofilm formation and evidence of substrate degradation was recorded using SEM (Ichida, et. al., 1999, See Chapter 4). Comparing images from both studies, there was a striking similarity in the appearance of microorganisms and their biodegradative effects on feathers, cardboard and wood.

In the boundary conditions for biofilm formation study (Chap. 2), the crushed muckpile rock used to bury metal coupons was exposed to human and mechanical perturbation which likely increased the number and types of microorganisms present (Haldeman, et al., 1995). Because of this, the metal surfaces tested in the biofilm formation study (Chap. 2) were colonized by a variety of microbial species which formed microcolonies. Microcolonies are usually distributed in an uneven manner creating areas on the metal surface that are more heavily colonized than other areas (Geesey, 1991). Areas of a metal surface which are not colonized are aerated and cathodic compared to colonized sections which are anaerobic and anodic at the biofilm/metal interface. Essentially, a battery is created which allows for the flow of electrons from the anodic to the cathodic regions of the metal where metal dissolution occurs (Geesey, 1991). MIC resulting in metal dissolution is likely given appropriate water, nutrient, and temperature conditions as discussed earlier.

Another form of MIC that may be applicable to the metal coupons; elemental hydrogen absorbed by metal causing hydrogen embrittlement. Metal-sulfides (e.g., iron-sulfide) are produced by anaerobic SRBs (sulfate-reducing bacteria) and these may form the basis for corrosive capabilities by SRBs (Jones and Amy, 2000). Sulfide
promotes the ionization of many metals and, therefore, accelerates the anodic process (Geesey, 1991). Iron sulfides can accumulate on the surface of the metal thus stimulating the corrosion process by creating a galvanic cell with iron sulfide as the cathode and iron as the anode, causing dissolution of the iron (Cord-Ruwisch, 2000). SRBs would not be metabolically active if biofilms were not present to allow for the development of oxygen gradients creating anaerobic environments (a result of oxygen consuming activities of neighboring aerobes and facultative anaerobes). The presence of SRBs is a concern for the Yucca Mountain repository. SRBs have been isolated from the native tuff (Pitonzo, 1996, Castro, 1997). This along with the perturbation of the native tuff due to tunnel mining and other human presence could increase the risk of MIC.

Biofilms in environments that are only transiently wet, as created in the polyethylene study (Chap. 5), may appear as patchy or dense microcolonies (Auerbach et al., 2000). Atomic force microscopic studies showed that these unsaturated biofilms, as opposed to biofilms in aquatic systems, had ridges and valleys (Auerbach et al., 2000) similar to the biofilm morphologies observed on the polyethylene coupons (See Chapter 5). Even in an aqueous environment, the biofilms imaged by confocal laser scanning microscopy (CLSM) began as patches of colonies and later resulted in thick, dense biofilm structure (Tolker-Nielsen, et. al., 2000).

As in biofilms formed in aqueous environments, the physical structure of unsaturated biofilms has a purpose. Biofilms formed in aqueous environments have channels which act as conduits for nutrient supply and waste removal (Stoodley, et. al., 1994). In unsaturated systems, such as in our study of biofilm formation on
polyethylene (Chap. 5), water availability is limited and there is a lack of such channels for transport of nutrients and removal of wastes. The primary mechanism for mass delivery is the air-biofilm interphase and diffusion within the biofilm matrix (Auerback, et. al., 2000). Unsaturated biofilm morphology appears to remain stable with changing environmental conditions which occur especially in soils exposed to periods of wet and dry.

A separate investigation was conducted relating to the proposed, high-level nuclear waste repository at Yucca Mountain. This investigation was based on the presence of calcite deposits within fractures. There was a possibility that the calcite deposits resulted from an upwelling of subsurface, geothermally-heated water containing dissolved calcium carbonate. As the water cooled, the dissolved minerals precipitated forming calcite deposits. Thermophilic bacteria might have been enriched in thermal water. The presence of thermophilic, calcium-precipitating bacteria could lend support to the subsurface, geothermal water origin of Yucca Mountain’s calcite deposits.

The identification of thermophiles isolated from calcite deposits in Yucca Mountain suggests a historical warm water influence in Yucca Mountain. *Bacillus thermoleovorans*, isolated at 70° C, was originally described from soil near hot water effluent in Pennsylvania and in hot spring mud in Arkansas (American Type Culture Collection, Manassas, VA). *Bacillus flavothermus*, isolated at 60° C, is a thermophilic bacterium originally isolated from hot springs in New Zealand (Pikuta, et.al. 2000). These two thermophiles, are typically associated with hot springs, suggestive of presence in geothermal waters.
All of the thermophilic bacteria growing at 60 and 70°C were isolated from calcite or welded tuff mixed with calcite. No thermophilic bacteria capable of growing at 60 and 70°C were obtained from welded tuff lacking calcite deposits (Chap. 3). If thermal waters infiltrated Yucca Mountain, the calcite deposits could have formed from the precipitation of calcium carbonate due to its low solubility, $K_{sp} = 10^{-8.32}$ (Ehrlich, 1996) and as a result of high temperature which favors calcium carbonate precipitation (Morita, 1980, Ferrer, et. al., 1988). However, there are many examples of bacterially-precipitated calcium carbonate in the environment without hot water.

Microbes can precipitate calcium carbonate through their metabolic activities. Aerobic and anaerobic oxidation of carbon compounds generates carbon dioxide which (when combined with calcium) will precipitate as calcium carbonate (Ehrlich, 1996). There is also the aerobic and anaerobic oxidation of nitrogen compounds with the release of ammonia. The result of ammonia release is an increase in pH that precipitates calcium carbonate (Ehrlich, 1996). The hydrolysis of urea leading to ammonium carbonate and then calcium carbonate precipitation is responsible to a lesser degree.

What tends to occur in bacterially-induced calcium carbonate precipitation is that bacteria bind cations, including calcium, to their cell surface. There is evidence that points to the possible initiation of calcium carbonate crystal formation on the surface of bacterial cells (Morita, 1980). Once the initial calcium carbonate crystal is formed, it acts as a nucleation site for the deposition of more calcium carbonate. Examples of this can be found in calcite deposits in caves (Contos, et. al., 2001), thermal springs (Casanov, et. al., 1999, Jones and Renault, 1996), desert soils (Monger,
et. al., 1991), and marine and freshwater environments (Morita, 1980, Robbins and Blackwelder, 1992, Thompson, et. al., 1997). Bacterially-precipitated calcium carbonate has also been demonstrated in the laboratory (Stocks-Fischer, et. al., 1999, Appanna, et. al., 1997).

Whether bacteria acted as nucleating sites for calcium carbonate precipitation in Yucca Mountain has yet to be determined. Direct examination of calcite crystals obtained from desert soil, caves, and thermal springs has revealed bacteria and other microbes, such as fungi, within the calcite crystals (Monger, et. al., 1991, Contos, et. al, 2001, Casanova, et. al., 1999). Preliminary evidence indicates that to find bacteria within the Yucca Mountain calcite deposits may be difficult due to the small numbers of thermophiles which have been isolated so far. Whether this is due to small initial numbers of bacteria or survival of only a small percentage is not known. The presence of thermophilic bacteria exclusively in calcite deposits implies their deposition with warm to hot water. The thermophilic bacterial ability to precipitate CaCO₃ (Chap. 3) provides evidence that they could have contributed to the calcite deposition if conditions were favorable. Mineral precipitation without microbial influence could also result in calcite deposition. The deposits in Yucca Mountain may well be the result of both abiotic and biotic processes.

Recent work by Cline and Wilson (2000?) demonstrated that calcite deposits in Yucca Mountain were the result of warm water influence that has not occurred in the last two million years. Therefore, thermophilic bacteria isolated from the calcite deposits could conceivably be greater than two million years old if they were enriched by this ancient upwelling process.
There are examples of bacterial survival that span geologic time. Two *Staphylococcus*-like strains, AMG-D1 and AMG-D2, were isolated from plant and soil inclusions in 25-35 million year old, Dominican amber (Lambert, et al., 1998). What is of interest is how microbes remain viable for such long periods of time. Morita (2000) proposed the use of H\textsubscript{2} as an energy source for autotrophic and mixotrophic bacteria when food resources are scarce or nonexistent. H\textsubscript{2} is able to penetrate the microbial cell, has a low energy of activation, and is used in many biochemical reactions. Over geologic time, microbes have had sufficient time to develop this survival process.

The presence of H\textsubscript{2} gas is prevalent in igneous rocks and volcanic gases (Morita, 2000). Repeated volcanic ash deposition gave rise to the tuff that is present at the Nevada Test Site (Amy, 1997). Because hydrogen gas is found in volcanic gases and is permeable, it is probable that H\textsubscript{2} is present in the volcanic tuff in Yucca Mountain. Yucca Mountain is composed of thick layers of welded tuff separated by thinner layers of nonwelded, bedded tuff. The presence of microorganisms in the rock might have been due to transport in a water phase under unsaturated conditions or by advection (Kieft, et al., 1997). As previously mentioned, microorganisms could also have been deposited in fractures and cavities within the volcanic tuff by subsurface thermal water as indicated by geologic evidence (Wilson and Cline, in press) and the presence of thermophilic bacteria in the calcite deposits (Chap. 3). This adds support to the possibility of transport of gases such as H\textsubscript{2} as well.

Hydrogen gas is ubiquitous in the earth's upper mantle due to its permeability. The fact that many Archaea are capable of H\textsubscript{2} metabolism may be indicative of a physiologic remnant of ancient processes (Madigan, 2000). This would indicate that
hydrogen oxidation is metabolically successful, making it a good source for energy of survival.

The concept of gaseous feeding would be of interest in not only determining the means of survival of thermophilic bacteria in calcite deposits of Yucca Mountain, but the survivability of other microbes within Yucca Mountain as well. There are hydrogen-oxidizing bacteria such as *Ralstonia, Pseudomonas, Paracoccus,* and *Alcaligenes* which grow best microaerobically. There are carboxydrotrophic bacteria which can grow on carbon monoxide producing carbon dioxide and H₂ (Atlas and Bartha, 1998). Some methanogens can metabolize H₂ and carbon monoxide to form methane and water. Then there are the methanotrophs, methane-oxidizing bacteria, which also play an important role in the carbon cycle by converting methane back to cell material and carbon dioxide (Madigan, 2000). The concept of gaseous feeding could have implications for microbial effects on any manmade structure contemplated for the nuclear waste repository at Yucca Mountain.

The metabolic activities of microbes within biofilms can have the adverse effects of utilizing these substrates as nutrient sources, and/or producing metabolic end products capable of corroding or degrading the repository canister materials under consideration at Yucca Mountain. At the Nevada Test Site, the reverse is under consideration; to accelerate the biodegradation of waste packaging materials in order to reduce the risk of subsidence in the shallow trench disposal cells. In both situations, the safety of nuclear and radioactive waste storage can only be accomplished by ensuring the structural integrity of the radioactive waste disposal/storage facilities. This includes determining the suitability of Yucca Mountain as a high-level nuclear waste repository.
based on the origin of calcite deposits within the mountain and the survivability of indigenous microbes at Yucca Mountain and the Nevada Test Site.

References


APPENDIX I

METHODS FOR ANALYZING BIOFILM STRUCTURE AND THERMOPHILIC, CALCITE-PRECIPITATING BACTERIA
Enrichment Medium Formulation for Iron-Oxidizing Bacteria

Medium for iron-oxidizing bacteria, e.g., *Thiobacillus ferrooxidans*, contains two separate stock solutions which must be autoclaved separately prior to recombining for the final solution. Stock solution #1 contains: 3.0 g (NH₄)₂SO₄, 0.50 g K₂HPO₄, 0.50 g MgSO₄·7H₂O, 0.10 g KCl, 0.01 g Ca(NO₃)₂, and 1.0 mL 10N H₂SO₄ (in a final volume of 700 mL of distilled water, final pH = 3.0 - 3.6 at 25°C). Stock solution #2 contains: 44.22 g FeSO₄·7H₂O in 300 mL distilled water. The two stock solutions were autoclaved for 20 minutes at 121°C. The FeSO₄·7H₂O solution was then aseptically added to the first stock solution and mixed (Atlas, 1993; Manning, 1975).

Enrichment Medium Formulation for Sulfate-Reducing Bacteria

Medium for sulfate-reducing bacteria is prepared by combining 3 separate stock solutions which must be autoclaved separately prior to recombining for the final solution. Stock solution #1 contains: 3.14 mL 60% stock solution sodium lactate, 2.0 g MgSO₄·7H₂O, 1.0 g NH₄Cl, 1.0 g Na₂SO₄, 1.0 g yeast extract, 0.5 g K₂HPO₄, and 0.1 g CaCl₂·2H₂O (in a final volume of 980 mL of distilled water, final pH = 7.4 at 25°C). Stock solution #2 contains: 0.5 g of FeSO₄·7H₂O in 10 mL of distilled water. Stock solution #3 contains: 0.1 g ascorbic acid and 0.1 g sodium thioglycollate in 10 mL distilled water. All 3 stock solutions were autoclaved for 20 minutes at 121°C. Stock solutions 2 and 3 were aseptically added to stock solution 1 and mixed.

Enrichment Medium Formulation for Calcite-Precipitating Bacteria

Medium for calcite-precipitating bacteria contains: 10.0 g glucose, 4.0 g yeast extract, 2.5 g calcium acetate, and 15.0 g Bacto agar in one liter of distilled water, final pH = 7.0 (Monger et al., 1991). After mixing the ingredients, the medium was autoclaved for 20 minutes, 121°C. The agar medium was allowed to cool to approximately 45 - 50°C prior to pouring into 15 x 100 mm Petri plates (VWR, West
Chester, PA). The plates were filled to capacity to compensate for the high temperatures (45, 60, 70°C) that the calcite isolates grew in.

**Minimal Nutrient Medium for Environmental Bacteria**

Minimal nutrient medium (R2B) contains: 0.5 g Bacto yeast extract, 0.5 g Bacto proteose peptone No. 3, 0.5 g Bacto casamino acids, 0.5 g Bacto dextrose, 0.5 g soluble starch, 0.3 g sodium pyruvate, 0.3 g potassium phosphate dibasic, and 0.05 g magnesium sulfate in one liter distilled water, final pH = 7.21 (Difco/ BD Diagnostics Systems, Sparks, MD). After mixing the ingredients, the medium was autoclaved for 20 min, 121°C. The broth medium was allowed to cool and transferred to sterile one liter bottles before use.

**Total Bacterial Counts using Epifluorescent Microscopy**

Acridine orange (Sigma, St. Louis, MO) and DAPI (4', 6-diamidino-2-phenylindole dihydrochloride, Sigma) are two fluorescent dyes which bind to DNA and were used to detect the presence of viable and dead (total) bacterial cells in environmental specimens (Coleman, 1980, Hobbie et al. 1977). A 0.1% (w/v) stock solution of acridine orange and a 100 mg/mL stock solution of DAPI were used to stain suspensions of bacteria and biofilm harvested from substrate surfaces.

Bacterial/biofilm suspensions stained with acridine orange were filtered through 0.2 mm polycarbonate filters (Osmonics, Minnetonka, MN). A volume of 100 mL sample was mixed with 400 mL of 0.1% acridine orange. The sample was allowed to incubate for three minutes with the acridine orange before filtering. Using DAPI, samples were fixed with equal volumes of methanol and shaken for 30 minutes at room temperature. Following the fixation procedure, DAPI was added to a final concentration of 5 mg/mL and the sample was incubated for an additional 30 minutes,
room temperature. The entire stained sample was then filtered through a 0.2 mm polycarbonate filter.

A filtered specimen was placed on top of a single drop of immersion oil applied to a microscope slide’s surface. A second drop of immersion oil was placed on top of the polycarbonate filter prior to applying the coverslip. Using a Nikon Optiphot microscope, the samples were viewed with 100X Fluor objective lens and the bacteria counted using the following formula: on filters: \( \text{cells/mL} = \frac{\text{average number of cells per grid}}{5.16 \times 10^4} \times \text{dilution} \). The area of filter inside the apparatus is 3.14 cm\(^2\).

Species Richness and Equitability: Shannon-Weaver Index

There are mathematical indices that describe species richness, variety, or i.e., evenness, equitability, in a community. Species richness (diversity) can be expressed by ratios between total species and total numbers; it measures the number of species in a community, but does not indicate the number of individuals in a species. Equitability measures the proportion of individuals (colonies in this case) among the species and can detect if there are any dominant populations. Overall, diversity indices can reflect the complexity of a community structure.

A commonly used measure of diversity is the Shannon-Weaver Index (Atlas and Bartha, 1998). This diversity index is sensitive to species richness and relative species abundance. The one problem with using the Shannon-Weaver Index is that it is sensitive to sample size, in particular, small samples. The Shannon-Weaver Index of diversity (\( H \)) equals: \[ H = C/N (N \log N - \sum n_i \log n_i) \] where \( C = 2.3 \), \( N \) = number of individuals, and \( n_i \) = number of individuals in the \( i^{th} \) species. Equitability (\( J \)) equals: \[ J = \frac{H}{H_{\text{max}}} \] where \( H \) is the Shannon-Weaver diversity index and \( H_{\text{max}} \) is the theoretical maximal Shannon-Weaver diversity index for the population examined and assumes each species has only one member.
Heterotrophic plate counts were done two weeks after plating harvested biofilms. Dilutions were prepared so that 30 - 200 culturable counts were obtained on each plate. Species were identified by colony morphology which was described and counted separately for each species of bacteria.

Confocal Laser Microscopy

Scanning confocal laser microscopic analysis was performed at the Center for Biological Imaging, Department of Biological Sciences, University of Nevada, Las Vegas using a Zeiss LSM 5. Sacrificed coupons were fixed in 4% glutaraldehyde (Sigma, St. Louis, MO) for a period of 18 hours (overnight). The coupons were removed from the glutaraldehyde and allowed to air dry for an additional 24 hrs. due to the fact that the wood and cardboard coupons absorb a significant amount of liquid. Each coupon is affixed to a glass slide using 2-sided tape and stained by flooding the exposed side of the coupon with 0.1% acridine orange. After three min., the excess acridine orange is aspirated off and the coupon surface is allowed to dry before viewing.

The confocal laser microscope can optically section a specimen to form a Z-stack. The Z-stack is obtained by moving the objective lens vertically along the z-axis until the bottom and top layers of the biofilm are observed and marked. The computer calculates the thickness of each layer and number of layers needed for the Z-stack of the biofilm. Each layer is recorded and compiled by the computer resulting in a 3-dimensional image which can be viewed as a projected image, stereo image, or depth plot.

The best imaging was obtained using the Zeiss LSM 5 argon laser and an excitation wavelength of 488 nm. The collection filter for Channel 1 was 650 nm and for channel 2 was 505-550 nm. The field was chosen by viewing the coupon surface using reflected epifluorescence prior to using the lasers. There was difficulty in working with the cardboard and wood coupons due to the porosity and irregularity of
their surfaces. The 40x and 63x objectives required several applications of immersion oil and coverslips were not used. Once the selected field was focused, it was necessary to work quickly to obtain the Z-stack for 3-dimensional imaging because the cardboard and wood surfaces continued to absorb immersion oil.

Scanning Electron Microscopy

SEM was performed by the Electron Microscope Facility, Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona and by the EPMA/SEM Lab, Department of Geosciences, University of Nevada, Las Vegas.

Dehydration procedures followed by Northern Arizona University were as follows: (1) the cardboard coupons were washed in water (3 x 30 min. changes), 50% ethanol (1 x 20 min.), 70% ethanol (1 x 20 min.), 95% ethanol (1 x 20 min.), 100% ethanol (3 x 20 min.), (2) the wood coupons were washed in water (8 hours to overnight, 3x), 50% ethanol (1 x 24 hrs.), 70% ethanol (1 x 24 hrs.), 95% ethanol (1 x 24 hrs.), 100% ethanol (3 x 12 hrs.), (3) the metal coupons were dehydrated using the same protocol as the cardboard coupons (Sellers, 2001). These dehydration procedures were followed due to the thickness of the coupons which harbored a significant amount of moisture. After dehydration, all coupons underwent critical point drying.

Scanning electron microscopy performed at the EPMA/SEM Lab, University of Nevada, Las Vegas used a model JSM-5600 Jeol (Peabody, MA) scanning electron microscope. The coupons prepared at this facility were much thinner and smaller in dimension than those prepared at Northern Arizona University. Due to less moisture content, sacrificed coupons were fixed in 4% glutaraldehyde (Sigma, St. Louis, MO) for a period of 18 hours (overnight). The coupons were removed from the glutaraldehyde and allowed to dry for a period of one week in a dessicating chamber (VWR, West Chester, PA). Coupons were mounted and coated using a Pelco Model 3 sputter coater before viewing.
Polymerase Chain Reaction Reagents

Proteinase K was prepared by dissolving lyophilized Proteinase K in sterile, distilled water to a concentration of 1.0 mg/mL. Proteinase K was kept on ice while in use and stored at -20°C when not in use.

K-Buffer contains: 4.0 mL Tris HCl (40 mM), 0.2 mL Igepal CA630 at same volume, 1.0 mL Tween 20 (Polyoxyethylenesorbitan), and 20.0 mL EDTA at 1M for a final concentration of 0.2 mM (in a final volume of 100 mL of distilled water, pH 8.0 at 25°C).

TE buffer working stock was prepared by taking 10.0 mL of 100x TE buffer and diluting with 990 mL dH2O, pH 8.0.

The dNTP Master Mix contains: 500 mL dH2O (0.2 um filtered, autoclaved), and 125 mL of each dNTP (10 mM stock solutions of adenine, thymidine, guanine, and cytosine). The dNTP Master Mix was stored at a final volume of 1 mL at -20°C.

The 16s-27f and 16s-1492r primers were supplied by Integrated Technologies, Inc. (Coralville, IA) and reconstituted to a final volume of 1.0 mL each using TE buffer (0.2 mm filtered Tris EDTA, pH 8.0). The initial concentration of 16s-27f (Primer “A”) was 62.61 nM/mL and that of 16s-1492r (Primer “B”) was 110.21 nM/mL. The primers were then stored at -20°C.

Working stocks of the primers were prepared at a final concentration of 2.5 nM. Primer “A” working stock contains 7.9 mL of the original stock solution diluted with 192 mL dH2O (0.2 mm filtered, autoclaved). Primer “B” working stock contains 4.54 mL of the original stock solution diluted with 195 mL dH2O (0.2 mm filtered, autoclaved). Primer “A” and Primer “B” working stocks were stored at -20°C.

The DNA Ladder standard used was the AmpliSize (Bio-Rad, Hercules, CA) 50-2000 bp Ladder. Five mL of the DNA ladder was mixed with 1.0 mL of tracking dye before loading onto the agarose gel.
Agarose gels for electrophoretically separating the PCR products contains: 80 mL 1x TE buffer pH - 8.0, 0.53 g agarose, and 4.0 mL ethidium bromide. The buffer, agarose, and ethidium bromide were mixed and then microwaved on high setting for 1 - 2 minutes until boiling. Combs were set in the gel molds and the agarose gel poured. The agarose gel was allowed to cool and set for approximately 1 - 2 hours prior to use. Electrophoretic equipment used were Owl Scientific, Inc. Easy-Cast Electrophoresis System Model #B1 and Bethesda Research Laboratories Power Supply Model #100.

The ingredients for the PCR Master Mix contains: 16.0 mL dNTP Master Mix, 10.0 mL 10x buffer from Taq DNA polymerase kit (Qiagen, Valencia, CA), 20.0 mL Q Solution from Taq DNA polymerase kit, 2.0 mL MgCl2 from Taq DNA polymerase kit, 5.0 mL Primer “A”, 5.0 mL Primer “B”, and 2.0 mL Taq Polymerase (in a final volume of 100 mL of 0.2 mm filtered, autoclaved distilled water). All of the reagents for the PCR Master Mix were kept on ice during the preparation. The PCR Master Mix was spun in the microfuge (Tomy High Speed Microcentrifuge Model #MC-150) for 1 minute at the lowest setting and gently rocked, in order to mix the reagents properly without denaturing them.

Polymerase Chain Reaction Procedures

Standardized bacterial cell suspensions were prepared by dispensing into pre-weighed 1.8 mL eppendorf tubes 1.0 mL of dH2O (0.2 um filtered, autoclaved). Approximately, 30 mg of cells from each pure culture were scraped from culture plates and added to the eppendorf tubes. The bacterial suspensions were vortexed, centrifuged, and the supernatent poured off and discarded. The eppendorf tubes were reweighed and the cell weight recalculated. Distilled water was added to the bacterial pellets to make a final concentration of 30.0 mg bacterial cells per 1 mL volume.

To obtain bacterial cell lysates for DNA, 40 mL standardized cell suspension,
10 mL Proteinase K, and 50 mL K buffer were combined and centrifuged on low to
mix. The cell suspensions were heated at 60° C for exactly 20 minutes, immediately
transferred to 100° C for 5 minutes, and quickly placed on ice until cool. The lysates
were centrifuged at 10,000 to 15,000 rpm for 10 minutes. The supernatents were
removed and stored at -20° C.

For the PCR reactions, 8 mL of PCR Master Mix and 2 mL of DNA template
(sterile dH2O for the negative control) were combined in sterile PCR tubes. The PCR
tubes were centrifuged at the lowest setting for 1 minute to mix the reagents. The
GeneAmp 2400 PCR System thermocycler (Perkin Elmer Biosystems, Wellesley, MA)
program for the PCR reaction was 94° C - 5 minutes pre-ramp, followed by 40 cycles of
94° C - 30 seconds, 55° C - 45 seconds, and 72° C - 55 seconds. At the end of these 40
cycles, there was a post-ramp of 72° C - 7 minutes followed by a hold temperature of
10° C until the PCR tubes can be removed and stored at 4° C until needed.

DNA Sequencing Reagents

The 2.5x sequencing buffer (Perkin-Elmer Applied Biosystems, Wellesley, MA)
contains 5mM MgCl₂ and 200mM Tris pH 9.0 which were mixed by vortexing. A
modified 2.5x sequencing buffer using 25 mM MgCl₂ (Qiagen PCR Reagent Kit,
Valencia, CA) contains 4 mL 25 mM MgCl₂ and 16 mL 250 mM Tris pH 9.0 which
were vortexed before use. The 20 mL total volume of modified sequencing buffer was
sufficient for 5 sequencing reactions.

The sequencing reaction master mix contains: 10.7 mL dH₂O (0.2 mm filtered,
autoclaved), 0.3 mL primer (either the forward or reverse primer, only 1 primer per
reaction), and 4.0 mL 2.5x modified sequencing buffer. The sequencing master mix
was spun in the microcentrifuge at the lowest setting for 1 minute to mix and then
placed on ice. For weak sequencing signals, the volume of dH₂O used was decreased
by the same volume increase in DNA template.
DNA Sequencing Reaction Procedures

The following reagents were used to complete the sequencing reaction: 15.0 mL sequencing reaction master mix, 4.0 mL Terminator Ready Reaction Mix (Perkin Elmer Applied Biosystems, Wellesley, MA), and 1.0 mL DNA (cleaned PCR product). All reagents were kept on ice while setting up the sequencing reaction PCR tubes. The PCR tubes were then placed in the GeneAmp 2400 PCR System thermocycler (Perkin Elmer Biosystems, Wellesley, MA) using the following program: 96° C - 10 seconds, 50° C - 5 seconds, 60° C - 4 minutes (25 cycles), followed by a hold temperature of 10° C for an indefinite period until PCR tubes can be removed and placed at 4° C.

The DNA was precipitated and washed with a series of ethanol dilutions. To a labeled 1.5 mL eppendorf tube, 20.0 mL sequencing reaction product was added along with 180 mL of 60% ethanol (room temperature). The ethanol and DNA were mixed by finger tapping and allowed to sit for 15 minutes prior to centrifuging at 12,000 rpm for 20 minutes. After centrifugation, the supernatent was poured off and the edge of the eppendorf tube tapped gently onto a Kimwipe to remove excess supernatent. This washing procedure was repeated a second time using 180 mL of 60% ethanol, followed by a third washing using 75% ethanol. The eppendorf tubes were allowed to air dry for 60 minutes before taken to be sequenced or stored at -20° C. The sequencer used was the Perkin Elmer Biosystems (Wellesley, MA) ABI Prism 3700 Analyzer.

Sequencing results were submitted online to “BLAST Search” at www.ncbi.nlm.nih.gov. The “Basic Search” option was used and the sequence was copied and pasted where the prompt indicates. Results of sequences were e-mailed to the submitter’s e-mail address as requested by “Blast Search”.

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References


APPENDIX II

BIODEGRADATION PHASE I: HETEROTROPHIC PLATE COUNTS
AND EQUITABILITY INDICES
Metal - Backfill Soil

Heterotrophic Plate Counts per Coupon vs Months
Wood - Sterile Water

Heterotrophic Plate Counts per Coupon

Months

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Metal - Sterile Water

Heterotrophic Plate Counts per Coupon vs. Months

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Wood - *Streptomyces* sp.

![Graph showing heterotrophic plate counts per coupon over time for Wood - *Streptomyces* sp.](image-url)

**Axes:**
- **Y-axis:** Heterotrophic Plate Counts per Coupon
- **X-axis:** Months

**Data Points:**
- 0 months: $1 \times 10^8$
- 5 months: $1 \times 10^7$
- 10 months: $1 \times 10^6$
- 15 months: $1 \times 10^5$
- 20 months: $1 \times 10^4$
- 25 months: $1 \times 10^3$
- 30 months: $1 \times 10^2$
Cardboard - *Streptomyces* sp.

![Graph showing heterotrophic plate counts per coupon over months.](image)
Cardboard - *Paracoccus* sp.

Heterotrophic Plate Counts per Coupon

Months

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Metal - *Paracoccus* sp.

Heterotrophic Plate Counts per Coupon

Months

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Wood - Sterile Water

Equitability (J)

Months

0 5 10 15 20 25 30

0.0 0.2 0.4 0.6 0.8 1.0

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Cardboard - Sterile Water

Equitability (J)

Months

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Wood - *Streptomyces* sp.

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Wood - *Paracoccus* sp.

Equitability (J)

Months

0  5  10  15  20  25  30

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Metal - *Paracoccus* sp.
APPENDIX III

BIODEGRADATION PHASE II: HETEROTROPHIC PLATE COUNTS

AND EQUITABILITY INDICES
Metal - Cellulose only

Heterotrophic Plate Counts

Months

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Metal - Cellulose & soil

Heterotrophic Plate Counts

Months

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Metal - Cellulose & soil, ambient RH

Heterotrophic Plate Counts

0 2 4 6 8 10
Months

1e+3 1e+4 1e+5 1e+6 1e+7 1e+8 1e+9

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Wood - R2B medium, Cellulose only

Heterotrophic Plate Counts

Months

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Cardboard - R2B medium, Cellulose only

Heterotrophic Plate Counts

Months

10
8
6
4
2
0
10
8
6
4
2
0
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Wood - *Scopulariopsis koningii* in R2B medium & Cellulose

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Cardboard - *Scopulariopsis koningii* in R2B medium & Cellulose
Cardboard - *Scopulariopsis koningii* in R2B medium, Cellulose & soil

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Wood - *Acremonium kiliense* in R2B medium & Cellulose
Cardboard - *Acremonium kiliense* in R2B medium & Cellulose
Cardboard - *Acremonium kiliense* in R2B medium, Cellulose & soil

Heterotrophic Plate Counts

Months

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Metal - SRB medium, Cellulose only

Heterotrophic Plate Counts

Months

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Metal - SRBs, Cellulose & soil

Heterotrophic Plate Counts

0 2 4 6 8 10
Months

10^3 10^4 10^5 10^6 10^7 10^8 10^9
Metal - FeOx medium, Cellulose only

Heterotrophic Plate Counts vs. Months

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Metal - FeOx microbes, cellulose only

Heterotrophic Plate Counts

Months

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Metal - FeOx microbes, Cellulose & soil

Heterotrophic Plate Counts

Months

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Cardboard - Soil only

Equitability (J)

Months

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Metal - Soil only

Equitability (J)

Months

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Metal - Cellulose only

Equitability (J)

Months

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Cardboard - Cellulose & soil

Equitability (J)

0.0  0.2  0.4  0.6  0.8  1.0

0  2  4  6  8  10

Months

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Cardboard - Cellulose & soil, ambient RH

Equitability (J)

Months

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Metal - Cellulose & soil

Equitability (J)

Months

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Cardboard - R2B medium, Cellulose only

Equitability (J)

0.0  0.2  0.4  0.6  0.8  1.0

0  2  4  6  8  10

Months
Wood - *Scopulariopsis koningii* in R2B medium & Cellulose

- Equitability (J)
- Months

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Cardboard - *Scopulariopsis koningii* in R2B medium & Cellulose

Equitability (J)

Months

0.0 0.2 0.4 0.6 0.8 1.0

0 2 4 6 8 10

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Cardboard - *Scopulariopsis koningii* in R2B medium, Cellulose & soil

Equitability (J)

Months

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Wood - *Acremonium kiliense* in R2B medium & Cellulose

![Graph showing the relationship between Equitability (J) and Months.](Image)

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Cardboard - Acremonium kiliense in R2B medium & Cellulose

Equitability (J) vs. Months
Cardboard - *Acremonium kiliense* in R2B medium, Cellulose & soil

Equitability (J)

Months

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Metal - SRB medium, Cellulose only

Equitability (J)

0.0 0.2 0.4 0.6 0.8 1.0

0 2 4 6 8 10

Months

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Metal - SRB medium, Cellulose & soil

Equitability (J)

0.0
0.2
0.4
0.6
0.8
1.0

0
2
4
6
8
10

Months
Metal - SRBs, Cellulose only

Equitability (J)

Months

0  2  4  6  8  10

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Metal - FeOx medium, Cellulose only

Equitability (J)

Months

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Metal - FeOx medium, Cellulose & soil

Equitability (J)

Months

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Metal - FeOx microbes, Cellulose only

Equitability (J)

Months

0.0 0.2 0.4 0.6 0.8 1.0

0 2 4 6 8 10

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