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Review of Microbial Responses to Abiotic Environmental Factors in the Context of the Proposed Yucca Mountain Repository

Examen des réponses microbiennes aux facteurs abiotiques dans le contexte du dépôt proposé du mont Yucca

A. Meike, S. Stroes-Gascoyne

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REVIEW OF MICROBIAL RESPONSES TO ABIOTIC ENVIRONMENTAL FACTORS IN THE CONTEXT OF THE PROPOSED YUCCA MOUNTAIN REPOSITORY

by

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REPORT PREPARED FOR THE U.S. DEPARTMENT OF ENERGY, YUCCA MOUNTAIN SITE, CHARACTERISTION OFFICE UNDER AECL TECHNOLOGIES INC. CONTRACT # DE-AC08-95-NV11784

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EXAMEN DES RÉPONSES MICROBIENNES AUX FACTEURS ABIOTIQUES DANS LE CONTEXTE DU DÉPÔT PROPOSÉ DU MONT YUCCA

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RAPPORT RÉDIGÉ POUR L'U.S. DEPARTMENT OF ENERGY, SITE DU MONT YUCCA, BUREAU DE CARACTÉRISATION EN VERTU DU CONTRAT D'AECL TECHNOLOGIES INC. N° DE-AC08-95-NV11784

RÉSUMÉ

Un atelier sur les activités microbiennes au mont Yucca (mai 1995, Lafayette, CA) a eu lieu dans le but de compiler des renseignements sur tous les aspects pertinents de l'activité microbienne qui s'appliquent à un dépôt éventuel au mont Yucca. Les résultats de cet atelier ont suscité un certain nombre de mesures visant à incorporer finalement les conséquences du comportement microbien dans les modèles d'évaluation des performances. Une de ces mesures était d'élargir une méthode de modélisation existante pour englober les caractéristiques distinctives d'un dépôt au mont Yucca (p. ex., conditions non saturées et charge thermique importante). En même temps, on a entrepris un certain nombre d'études expérimentales ainsi que la compilation de documentation pertinente pour étudier plus en profondeur les paramètres physiques, chimiques et biologiques qui auraient un effet sur l'activité microbienne dans des conditions semblables à celles du mont Yucca. Cette recherche de documentation (terminée en 1996) est l'objet du présent document.

La documentation rassemblée peut être divisée en quatre catégories, 1) facteurs abiotiques, 2) dynamique de la communauté et aspects *in situ*, 3) nutriments et 4) transport des radionucléides. La bibliographie complète (donnée à l'annexe A) représente une ressource considérable, mais est trop importante pour pouvoir être traitée dans un seul document. Par conséquent, le rapport actuel est axé sur la première catégorie, les facteurs abiotiques, et sur un examen de ces facteurs afin de faciliter l'élaboration d'un modèle pour le mont Yucca.

La première partie du rapport (chapitres 1 à 3) est un examen des états microbiens généraux, des phases et des critères de prolifération, des conditions relatives à la «prolifération normale» et d'autres types de prolifération, stratégies de survie et mort de cellule. Elle contient principalement des idées bien établies en microbiologie. Les capacités microbiennes de survie et d'adaptation aux modifications de l'environnement sont examinées parce qu'un dépôt placé au mont Yucca aurait deux effets. En premier lieu, l'environnement naturel serait perturbé par l'excavation et la construction du dépôt et en second lieu, cet environnement modifié serait alors perturbé par les déchets radioactifs eux-mêmes au cours de la durée de vie du dépôt (chaleur, redistribution d'humidité et éventuellement radioactivité).

Dans la deuxième partie (chapitres 4, 5 et 6), on traite des capacités microbiennes établies dans la première partie, dans un cadre propre au site qui contribuera à définir les limites de l'activité microbienne prévue. L'évolution prévue d'un dépôt potentiel au mont Yucca ainsi que les facteurs relevés qui peuvent influer sur l'activité microbienne (c.-à-d. hautes températures, changements du pH, dessiccation, changements de salinité et rayonnement) sont examinés. On y traite des prévisions d'activité microbienne, dans la mesure du possible dans le contexte où elle contribue concrètement aux décisions de conception pour le dépôt du mont Yucca envisagé.

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ABSTRACT

A workshop on Microbial Activities at Yucca Mountain (May 1995, Lafayette, CA) was held with the intention to compile information on all pertinent aspects of microbial activity for application to a potential repository at Yucca Mountain. The findings of this workshop set off a number of efforts intended to eventually incorporate the impacts of microbial behaviour into performance assessment models. One effort was to expand an existing modelling approach to include the distinctive characteristics of a repository at Yucca Mountain (e.g., unsaturated conditions and a significant thermal load). At the same time, a number of experimental studies were initiated as well as a compilation of relevant literature to more thoroughly study the physical, chemical and biological parameters that would affect microbial activity under Yucca Mountain-like conditions. This literature search (completed in 1996) is the subject of the present document.

The collected literature can be divided into four categories, 1) abiotic factors, 2) community dynamics and in-situ considerations, 3) nutrient considerations and 4) transport of radionuclides. The complete bibliography (included in Appendix A) represents a considerable resource, but is too large to be discussed in one document. Therefore, the present report focuses on the first category, abiotic factors, and a discussion of these factors in order to facilitate the development of a model for Yucca Mountain.

The first part of the report (Chapters 1-3) is a review of general microbial states, phases and requirements for growth, conditions for 'normal growth' and other types of growth, survival strategies and cell death. It contains primarily well-established ideas in microbiology. Microbial capabilities for survival and adaptation to environmental changes are examined because a repository placed at Yucca Mountain would have two effects. First, the natural environment would be perturbed by the excavation and construction of the repository and second, that modified environment would then be perturbed by the radioactive waste itself during the lifetime of the repository (heat, redistribution of moisture and possibly radioactivity.

Part Two (Chapters 4, 5 and 6) discusses the microbial capabilities established in part one, in a site specific framework that will help define the limits of expected microbial activity. The expected evolution of a potential repository at Yucca Mountain is reviewed as well as those factors identified to be potential issues to microbial activity (i.e., high temperatures, changes in pH, desiccation, salinity changes and radiation). Expectations for microbial activity, where possible in the context of explicit input to design decisions for the proposed Yucca Mountain Repository, are discussed.

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FOREWORD

The lack of sufficient data and understanding to build a model that could assess the significance, or predict the influence, of microbial activity on physical and chemical properties of a nuclear waste repository on par with existing abiotic models came into focus through a workshop of microbiologists, geologists and Yucca Mountain Project engineers. This Workshop on Microbial Activities at Yucca Mountain (May 1995, Lafayette, CA) was held with the intention to compile information for application to a potential repository at Yucca Mountain (Horn and Meike 1995).

An assessment of the perturbations of the natural environment as a result of the presence of a repository, the capabilities of the microbes with respect to affecting a repository's performance, and the duration of time over which it would be necessary to determine the performance of a repository, made it clear that a purely abiotic model would be incomplete. The findings of this workshop set off a number of efforts intended to eventually incorporate the impacts of microbial behaviour into performance assessment models for a proposed Yucca Mountain repository.

One effort was to expand an existing modelling approach (McKinley and Hagenlocher 1993) to include the distinctive characteristics of a repository at Yucca Mountain, e.g., unsaturated conditions and a significant thermal load. This resulted in the so-called TSPA Near Field Model (TSPA 1998). At the same time, a number of experimental studies were undertaken (Meike et al.1999, Chen et al. 1999), and a compilation of relevant literature (Appendix A of this report) to more thoroughly study the physical, chemical and biological parameters that would affect microbial activity under Yucca Mountain-like conditions. Both efforts were undertaken to provide data that could further develop and strengthen the model.

The literature search, (Appendix A, by D. Haldeman, University of Nevada, Las Vegas 1996) addressed a large number of significant factors elicited from the Workshop on Microbial Activity at Yucca Mountain (Horn and Meike 1995):

1. Microbial growth and responses to environmental parameters - abiotic factors (growth properties, pH, temperature, osmoregulation, desiccation, radiation, mutation).

2. Survival (general survival, dormancy, spores, resuscitation, viable versus non-culturable organisms, cryptic growth, injury, stress proteins, bacterial size).

3. In-situ considerations on attachments (biofilms, polymers, polysaccharides) and interactions (microbe-microbe, consortia, competition, colonization, succession).

4. Diversity of microbiota (general diversity, sulphate-reducing bacteria, anaerobes, acetogens, microaerophiles, iron-reducing bacteria, autotrophs, hydrogen-oxidizing bacteria, etc.) and the genetic methods for detecting and identifying microbes.

5. Nutrients and cycling.

continued...

6. Degradation of introduced materials (hydrocarbons (diesel exhaust), concrete, PVC, rubber, bitumen, styrene, polysaccharaides, blasting residues, etc.).

7. Sorption/adhesion and migration: transport of metals and radionuclides.

A complete bibliography of all literature collected in 1996 from this search is given in Appendix A. The bibliography represents a considerable resource; too large to present in one document when it is combined with the necessary discussion. Therefore, the entire bibliography is planned to be presented in four annotated units: 1) abiotic factors, 2) community dynamics and in-situ considerations, 3) nutrient considerations and 4) transport of radionuclides. The present document focuses on the first category, abiotic factors, and a discussion of these factors in order to facilitate the development of a model for Yucca Mountain.

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1. INTRODUCTION

A large number of abiotic physical and chemical factors influence the survival, metabolic activity and growth of microorganisms. These factors, described in texts such as Atlas and Bartha (1987), Brock and Madigan (1991) and Madigan et al. (2000), include nutrients (organic and inorganic compounds), temperature, pH, water activity, salinity and osmotic pressure, radiation, pressure and redox potential. The relative importance of these factors varies from organism to organism. The dominant microbes in an environment will be those less sensitive to the environmental extremes of that environment or those that can take advantage of the local conditions. A general assumption is that the potential for survival of any microorganisms exists anywhere but that the environment selects. Given the major environmental perturbations that would occur during the construction and operation of a high level nuclear waste repository at Yucca Mountain, it is clear that there may be a shift in the favored microbial populations. From this perspective, then, it is clear that more than an assessment of native microbial populations and processes would be necessary to understand their potential impact on that repository environment. An assessment of microbial responses to expected environmental changes, and the kinds of organisms that will be favored is, therefore, also in order.

The potential importance and effects of microbial activity on the performance of a nuclear waste repository have been discussed in a number of recent publications (e.g., Meike and Horn 1995, Meike 1996, Meike 1998a,b, Stroes-Gascoyne and West 1996, 1997, Pedersen and Karlsson 1995, Pedersen 2000, West et al. 2000). In brief, microbes can affect water chemistry (pH, Eh), corrosion of waste containers and waste forms and radionuclide migration (sorption, complexation, colloid formation). Horn and Meike (1995) established that microbes are expected to be present in the proposed Yucca Mountain repository environment. Indigenous species exist in the rock and new organisms will be introduced. Some aspects of microbial activity that are potentially significant to the ability to predict processes at Yucca Mountain during the lifetime of the proposed repository were presented. Meike (1996, 1998a,b) has pointed out that the repository environment will be different from the natural geological environment due to the importation of water and potential nutrient sources (large volumes of construction and packaging materials). Furthermore, it was made clear that little data exist that apply directly to the expected environment of the proposed Yucca Mountain site (i.e., an unsaturated environment). It was argued that, although microbes might be expected to be inactive during excessively dry or hot periods, the repository drifts would not become "sterile", due to the wide-ranging capabilities and adaptive strategies that microbes possess. This argument is supported by a recent field study in which microbes installed in heater holes of unsaturated rock have been demonstrated to migrate but they also reappeared in holes after more than a year of heating above 100°C (Chen et al. 1999). Meike et al. (1999) have used microbial communities derived from Yucca Mountain Topopah Spring Tuff to demonstrate that microbial activity can significantly impact abiotic chemistry and the character of that impact can be determined by species selected through nutrient supply and limitation. The impact of these effects depends on the actual activity of the microbes in the repository environment, which is determined by and must also be weighed against, abiotic factors. Consideration of these microbial facts and the interrelated character of the abiotic and biotic factors suggests that abiotic models intended to

describe processes in such a perturbed environment over geological periods of time are incomplete. It is desirable to ultimately develop models that integrate biotic factors.

This report assesses, in some depth, the effects of the physical changes that a Yucca Mountain repository would impose on the indigenous and introduced (during repository construction and operation) microbial population at Yucca Mountain. The ultimate goal is to define those parameters (based on the current state of knowledge in microbiology) that control microbial activity in this particular environment such that those parameters can be included in performance assessment models for the site. The present work represents part of an effort intended to better bound the possible consequences of microbial activity over geologically significant periods of time in the specific environment conditions expected in a potential repository at Yucca Mountain. Four major categories have been identified: 1) abiotic factors, 2) community dynamics and in-situ considerations, 3) nutrient considerations and 4) transport of radionuclides. This report deals primarily with the first category: microbial survival strategies and the influence of abiotic environmental parameters on their success. Identification of these processes and potential consequences will contribute to a more accurate prediction of the local aqueous chemistry and radionuclide migration at Yucca Mountain.

A very simple model, Microbial Impacts to the Near-Field Geochemistry (MING), for assessing microbial influence has been constructed for the Yucca Mountain environment (Sassani et al. 1997, 1998, TSPA 1998), using many of the basic assumptions of McKinley and Grogan (1991) and McKinley et al. (1997), but differing in the requirements and special conditions of the unsaturated Yucca Mountain environment. Although this model (MING) has been key in the demonstration of the many ways that microbial processes integrate into factors that are presently acknowledged as significant to the repository, the modelling capabilities in this area fall short of the level of the models (hydrological, chemical and radionuclide transport) with which they must interface. Due to the lack of information the model simplifies microbial activity in ways that could provide unrealistic results.

Fundamentals of microbial processes are presented here to indicate directions for more detailed models that can reduce uncertainty in performance assessment models, as well as some recommendations for design, such that microbial factors do not negatively influence the intended attributes of the design. For example, some of the stress factors such as radiation and nutrient availability are strongly influenced by repository and waste package design decisions.

Although it is unlikely that a first principles model of microbial processes will be developed in a time frame that is useful to the Yucca Mountain Project, it is possible to incorporate microbial processes into models at a number of levels, related to the level of knowledge in a particular area. Some processes, such as changes in biomass, may ultimately be described by a constitutive equation, while others may be described in less detail or by an envelope delimiting the range of general microbial activity. Such limits can be referred to as "on/off switches". In addition, those environmental factors that may determine shifts in microbial population, and thereby modify associated microbial processes, are highlighted.

Much of the information collected in the bibliography is based on laboratory experiments which form probably a gross simplification of the natural environment. For example, some of the

experiments represent nutrient-enhanced and accelerated processes, others are conducted in water saturated environments. Neither of these are true of the Yucca Mountain environment over the long term. However, it provides a framework and perspective for the incorporation of microbial processes into models of repository behavior and make design decisions. A level of detail is described in this report that will probably not be necessary in the models but that will expose the significant complex relationships that should be reflected in predictive models. Consideration of this complexity helps to define the tightest bounds possible for various aspects of microbial activity without resorting too often to oversimplification that can lead to unrealistic models.

This report consists of two parts, and although it is largely based on the 1996 literature search (Appendix A), more recent publications are included to illustrate the continuous development in this field. The first part (Chapters 2 and 3) defines microbial states and general microbial capabilities and adaptation strategies. The second part (Chapters 4, 5 and 6) focuses on Yucca Mountain specific factors, examines the anticipated environmental evolution, and discusses expected trends in microbial activity in that context in order to provide bounds in time and type of activity. Chapter 2 is a review of general microbial states, phases and requirements for growth (e.g. nutrients), conditions for 'normal growth' and other types of growth, survival strategies (i.e., starvation, dormancy, sporulation, resuscitation) and cell death (i.e., what is sterile?). It contains primarily well-established ideas in microbiology. Microbial capabilities for survival and adaptation to environmental changes (Chapter 3) are examined because a repository placed at Yucca Mountain would have two effects. First, the natural environment will be perturbed by the excavation and construction of the repository and second, that chemically and physically modified environment will be perturbed by the radioactive waste itself during the lifetime of the repository (heat, redistribution of moisture and possibly radioactivity). Mutations and mutants, which are a natural part of microbial growth and an important adaptive strategy, and that can vary in occurrence rate by a range of stresses, are also discussed in Chapter 3.

Part two (Chapters 4, 5 and 6) discusses the microbial capabilities established in general form in Chapters 2 and 3 in a site specific framework that will define the limits of expected microbial activity. First the expected evolution of a potential repository at Yucca Mountain is reviewed (Chapter 4). Those factors identified to be potential issues to microbial activity (i.e., high temperatures, changes in pH (concrete induced and otherwise), desiccation, changes in gas chemistry, salinity changes (as a result of high temperatures and resulting drying and evaporation) and radiation (should the design include non-self-shielding containers)) are discussed in Chapter 5. In Chapter 6, the expectations for microbial activity, where possible in the context of explicit input to design decisions for the proposed Yucca Mountain Repository, are discussed.

2. MICROBIAL STATES

As outlined in the Introduction, the information in this chapter represents some of the fundamentals of microbial behaviour, which has been summarized in many texts, and is presented here to provide the context for the site specific discussion in Chapters 4 and 5. Unless

otherwise noted, Chapter 2 summarizes aspects of Chapters 4,5,6,7, and 9 of Lim (1989) and Chapter 8 of Atlas and Bartha (1987).

2.1 "NORMAL GROWTH"

A vegetative cell is capable of growing actively. This state can be contrasted with a number of inactive states which include endospores, resting stages, and cell death. In single celled microorganisms cell death is defined as the point at which re-initiation of cell division is no longer possible. It is important to note that the determination of cell death is dependent on the manner in which growth re-initiation is attempted. In particular, some microbes are revived by methods that would result in permanent lack of growth in other growth environments (e.g., chemical, heat or cold shock). The vegetative cells of many bacteria can undergo some degree of differentiation into structurally and physiologically distinct forms, classified variously as endospores, exospores, myxospores, cysts, akinetes and resting stages. However, microbial growth does not refer to an advancement along a series of structural stages, or an increase in size which defines growth in multi-cellular organisms. Rather, microbial growth refers to the increase in the number of cells, which occurs by cell division.

Standard bacterial growth curves track cell growth using some measure of cell number. The most often used models of microbial growth apply to unicellular organisms which divide by division and age only in response to external factors such as exhaustion of a substrate, effect of an inhibitor, or changes in the physicochemical environment (these models cannot apply without caution to other types of microbial growth, i.e., mycelial (fungi) growth or budding (yeasts) (Aragno 1981)). The fundamental definitions of procaryotic cell division are bacterial generation time, which is also known as its doubling time, or the duration of one binary fission from having just divided to the point of having just completed the next division. Generation times vary with organism and environment. A fast growing bacterium can have a generation time on the order of 20 minutes under ideal conditions. Slowly growing bacteria or those in less ideal conditions can have generation times of hours or days.

2.1.1 Reaction Kinetics to Describe Microbial Substrate Transformation

In zero-order reactions the rate of transformation of a substrate is unaffected by changes in the substrate concentration, because the reaction rate is determined by some factor other than the substrate (e.g., the amount of catalyst) (Paul and Clark 1989):

$$DA/dt = -k, \text{ or } A_t = A_0 - kt$$

Where:

A_t	=	amount of substrate remaining at any time
A_0	=	initial concentration of substrate
k	=	rate constant (concentration . $time^{-1}$)
t	=	time since initiation of reaction
$t_{1/2}$	=	$A_0/2k$ (50% of initial substrate gone)
t_{mrt}	=	A_0/k (mean residence time)

(At high substrate concentrations at which substrates are not limiting, enzymatic reactions are usually zero-order).

In first-order reactions, the rate of transformation of a substrate is proportional to the substrate concentration (Paul and Clark 1989):

$$DA/dt = -kA \text{ or } A_t = A_0e^{-kt} \text{ or } \ln (A_t/A_0) = -kt$$

Where:

A_t	=	amount of substrate remaining at any time
A_0	=	initial concentration of substrate
k	=	rate constant ($time^{-1}$)
t	=	time since initiation of reaction
$t_{1/2}$	=	0.693/k
t_{mrt}	=	1/k

Many reactions found in nature have rate constants in which the graph of product versus time yields a curve approaching some maximum value. This is best described by a hyperbolic equation, which is known as the Michaelis-Menten equation in enzyme chemistry but as the Monod equation when used to describe microbial growth. In physical chemistry the use of a curve approaching an asymptote to describe adsorption phenomena is called a Langmuir equation. All three use identical principles (Paul and Clark 1989):

$$V = V_{max} A/(k_m + A)$$

Where:

V	=	rate of reaction (concentration . $time^{-1}$)
A	=	substrate concentration
V_{max}	=	maximum reaction rate (concentration . $time^{-1}$)
K_m	=	(half-saturation) constant or affinity

When

V	=	$1/2V_{max}$, $k_m = A$

If $A >> k_m$, $V = V_{max}$ (zero order reaction)
If $A << k_m$, $V = kA$ (first order reaction), where $k = V_{max}/k_m$

The hyperbolic equation can be linearized to solve, or computer fitting can be used. The great applicability of such hyperbolic equations stems from the fact that the whole asymptotic curve can be described by two values, V_{max} and k_m (Paul and Clark 1989).

2.1.2 Growth Phases

The standard bacterial growth curve reflects the stages of growth a pure culture of bacteria will go through, beginning with the addition of cells to sterile medium and ending with the death of all of the cells present. When added to fresh medium in the laboratory, bacteria usually progress

through four phases of growth: "lag phase", "log (logarithmic or exponential) phase", "stationary phase", and "decline phase" (e.g., Madigan et al. 2000).

The *lag phase* is a slowing of cell division which occurs, for instance, when differences exist in the chemistry or concentration of the old and new medium before responding to the new environment with renewed cell division. In some cases the lag phase involves an increase in cell size in the absence of division. It is associated with a physiological adaptation to the new environment. Individual cells continually undergo changes in mass. They increase in mass as a precursor to division, then divide, and as a result the number of cells increases as the mass of each cell is diminished. Note however that size is not always a determinant of division. Bakken and Olsen (1987a,b) studied the potential relationship between cell size and growth. They observe that whereas the small diameter bacteria (<0.4 μm) increased to greater than 0.4 μm before growth, very few (0.2%) were observed to be viable in the sense of showing growth. Due to this different behavior, Bakken and Olsen (1987a) commented that the smaller bacteria may not be smaller forms of the normal sized bacteria. The length of the lag phase can vary from an hour or two to several days. It depends on the bacterial species and the characteristics of the new and the old media. It has been observed that organisms that are adapted to limited nutrients and large accumulated wastes of old cultures take longer to adjust to a new medium than those from a relatively fresh, nutrient-rich medium .

Logarithmic or *exponential* growth is a physiological state marked by back-to-back division cycles such that the population doubles in cell number every generation time. Exponential growth is not accompanied by a change in average cell mass. Logarithmic growth can be easily achieved in a culture (sometimes after a lag period) and can be maintained until nutrient exhaustion or self-inhibitory effects occur. Provided that the cells are fully adapted to the growth medium, and that all nutrients are present in excess such that the nutrient transport systems of the cells are saturated, and that no change occurs in the medium that affects the whole cell activity, the doubling time of the biomass in a culture will be constant at any time. Thus, cell numbers increase exponentially with time. Logarithmic growth represents balanced growth if biomass and its components (e.g., number of cells, proteins, nucleic acids enzyme activities) increase at the same rate.

A *stationary phase* is achieved when the number of cells reaches a constant value. The simplest way to achieve this is that no cells die or divide. However, it is more commonly a steady-state equilibrium where the rate of cell growth (division) is exactly balanced by the rate of cell death. These two possibilities are distinguished by comparing viable counts with total counts. If neither changes then the former possibility is true, if total count increases and viable counts remain constant, then the latter is true. The stationary phase usually occurs when cell concentrations are such that some aspect of the environment is no longer able to serve the requirements of exponential growth. The stationary phase is also a time of significant physiological change and adaptation of cells to survival. Cell death or least lack of cell division is brought about by the absence of important nutrients, which may have been incorporated into cells during log-phase growth, or the introduction of toxins, which may be products of microbial activity released during log-phase growth.

The *decline or death phase* occurs when cell death exceeds division such that a reduction in the number of cells is observed. Specifically, the viable count declines. Typically the decline in the number of cells occurs exponentially. Vegetative cells die when exposed to harsh conditions for too long a period of time. During the decline phase, cells commonly undergo involution, in which they assume a variety of unusual shapes.

Although the language and observations are often transferred to describe bacteria in natural settings, the "batch culture environment" is different from many natural environments that experience continuous or pulsed influxes of nutrients. This review examines mostly phases of activity and inactivity that occur when cells are stressed but do not die. If, in contrast to the above described batch culture, fresh medium is supplied, then a population may experience only some of these phases or may be captured at a single growth phase for an extended period of time. This is the fundamental principle of the "chemostat".

2.2 REQUIREMENTS FOR GROWTH

All living organisms require nutrients for growth and reproduction. These nutrients are used inside the cells, after transporting them from the environment through the cell walls, either in catabolic processes to generate energy for cellular processes, or in anabolic processes to create chemical building blocks for cell growth. Based on the nature of their energy source, microorganisms are divided into two categories, phototrophs and chemotrophs. Phototrophs capture the radiant energy of sunlight and transform it into chemical energy that is stored in the bonds of carbohydrates and other molecules. Phototrophic microorganisms (algae, cyanobacteria and photosynthetic bacteria (Rhodospirillaceae, Chromataceae, Chlorobiaceae and Chloroflexaceae)) are not important in subsurface environments such as that of Yucca Mountain and will, therefore, not be further considered here. Most microorganisms are chemotrophs and must rely on the oxidation of reduced chemical compounds as a source of energy.

2.2.1 Energy

Chemotrophy can be subdivided into two groups, the chemo-organotrophs and the chemolithotrophs. The former obtain their energy from the oxidation of reduced organic compounds such as carbohydrates, organic acids and proteins. The latter obtain their energy from the oxidation of reduced inorganic compounds such as H_2S, H_2, NO_2^-, NH_3 and Fe^{2+}. Although chemolithotrophy is restricted to only a few types of bacteria (and is not found in higher life forms), chemolithotrophic bacteria are widely distributed in soil and water.

All primary energy-yielding reactions utilized by living organisms are oxidation-reduction reactions. Table 2.1 shows the reaction couples known to be used by living organisms to gain energy for growth (Kristjansson and Hreggvidsson 1995).

In microbes (and other organisms), energy is released in catabolic metabolic pathways and stored in chemical form in adenosine triphosphate (ATP) which subsequently can be used in anabolic pathways to build cell material.

TABLE 2.1

THE REDOX REACTION COUPLES KNOWN TO BE USED BY LIVING ORGANISMS TO GAIN ENERGY FOR GROWTH

Reductant	Oxidant (electron acceptor)						
	(CH_2O)	O_2	S	SO_4^{2-}	NO^{3-}	CO_2	Light
(CH_2O)	+	+	+	+	+	+	+
CH_4	-	+	-	-	-	-	-
CO	-	+	-	-	-	-	-
H_2	+	+	+	+	+	+	+
H_2S	-	+	-	-	+	-	+
S	-	+	-	-	+	-	+
NH_3	-	+	-	-	-	-	-
NO^{2-}	-	+	-	-	-	-	-
Fe^{2+}	-	+	-	-	-	-	-
H_2O	-	-	-	-	-	-	+

2.2.2 Water

Water is essential for all active microbial cell processes. The availability of water in an environment varies, and depends not only on the actual amount of water, but also on the concentration of solutes in the water. Many of the original survival studies were conducted on microorganisms that exist in a marine environment. The marine environment is mostly a low nutrient environment where water availability is not a limiting factor. Fundamental differences have been noted between marine and soil bacteria. For example, Bakken and Olsen (1987a,b) suggest that dwarf cells (cells with diameters <0.4 μm) can be found in most natural environments. In soils they are the most numerous but represent only 10-20% of the bacterial biovolume. Unlike marine bacteria, which seem to shrink during starvation episodes, the soil dwarf bacteria seem to be a distinct organism, with very different apparent nutrient needs. Only 0.2-0.8% can grow on nutrient agar. Given this basic difference, and the known microbial strategies for surviving episodes of desiccation, it is possible that survival strategies observed in a water saturated environment may not be directly applicable to a non-saturated environment.

The influence of water activity (a_w) and water potential on microbial survival, activity and growth and the relationship to the environment at the Yucca Mountain is discussed in detail in Chapter 4. Water is also fundamental to the migration of bacteria through soil or fractures. Bacteria can sorb and, therefore, transport radionuclides. Spores can be airborne, but vegetative cells require water for movement. The relationship between water and bacterial movement is discussed in Chapter 5.

2.2.3 Carbon

Carbon is required by living organisms as the basic building block of cellular structures and metabolic compounds. Heterotrophs use organic carbon as their source, autotrophs use inorganic carbon (CO_2) as carbon source for organic material (CO_2 fixation). Autotrophic bacteria include photoautotrophs (energy from light) and chemoautotrophs (energy from oxidation of reduced chemical compounds). Most microorganisms are unable to use CO_2 as principal carbon source and require instead preformed organic compounds for carbon, in so-called heterotrophic processes. Chemoheterotrophs oxidize chemical compounds for energy and require organic forms of carbon for cell material.

The types and substrates a microbe uses for its energy and/or carbon sources depends on many factors, e.g., availability of the substances, environmental conditions, and the presence of functional biochemical pathways in the organism for the metabolism of the compounds. Metabolic pathways can be catabolic (generating energy for cell processes), anabolic (providing building blocks for the synthesis of cell material) or amphibolic. The latter is a metabolic pathway that functions in the dual roles of catabolism and anabolism. Many important metabolic pathways in bacterial cells are in fact amphibolic, including the Embden-Meyerhof pathway and the tricarboxylic acid (TCA) or Krebs cycle. As an example, glyceraldehyde-3-phosphate in the Embden-Meyerhof pathway can be catabolized to form pyruvate. This same compound can also be used as a precursor for amino acids such as serine and glycine. In the TCA cycle, the intermediates α-ketoglutarate and oxaloacetate are also used as precursors for the biosynthesis of amino acids. Coenzymes nicotineamide-adenine-dinucleotide (NAD) and NAD-phosphate (NADP), involved in oxidation-reduction reactions, are involved in "steering" metabolic intermediates into catabolic or anabolic reactions, respectively. This type of control is one way in which the cell regulates rates of catabolism and anabolism.

2.2.4 Nitrogen

In living organisms, N is a component of proteins, nucleic acids, coenzymes, cell walls and other cellular constituents and, as such, indispensable. Bacteria obtain N from either inorganic sources such as NO_3^-, NH_3, and N_2, or from organic sources such as amino acids, purines and pyrimidines. The assimilation of N from NO_3^- into proteins and other cellular molecules is called assimilatory nitrate reduction, a multi-step process involving a series of reactions which add electrons to nitrate, eventually producing ammonia with the help of a number of specific enzymes. The electrons are donated by reduced nicotinamide-adenine dinucleotide ($NADH + H^+$). Assimilatory nitrate reduction is widespread and the major process by which N is incorporated into cellular material. Ammonia is assimilated by bacteria into a number of different organic compounds. A principal pathway is reversible incorporation into the amino acid glutamic acid which requires $NADPH + H^+$ and a specific enzyme (the low-efficiency but no energy requiring pathway) or ATP and another specific enzyme (the high efficiency energy expensive pathway). Nitrogen fixation, a process unique to certain bacteria, is an energy-requiring highly reductive process in which N_2 is reduced to two molecules of NH_3 (by the enzyme nitrogenase) which can then be converted to other fixed chemical forms of nitrogen for metabolic use. It occurs either in non-symbiotic free-living bacteria or in symbiotic associations between bacteria and certain plants such as legumes.

2.2.5 Phosphorus

Phosphorus is an element found in nucleic acids, membrane phospholipids, coenzymes and many intermediate compounds associated with metabolism and energy storage (e.g., ATP). It is obtained from both inorganic and organic sources. Inorganic phosphate is utilized directly by bacteria, but organic phosphate compounds need to be hydrolyzed to release the phosphate before it can be incorporated into other substances.

2.2.6 Sulphur

Sulphur is required for certain amino acids (cysteine and methionine), transfer ribonucleic acid (tRNA) and some coenzymes. Most microbes are able to use inorganic sulphate as S source. Sulphate is reduced to H_2S by assimilatory sulphate reduction, and the H_2S formed is incorporated into the amino acid cysteine, from where it can be transferred to other compounds in the cell. Sulphur can also be supplied directly to the cell in organic form by addition of the amino acids cysteine and methionine to growth media.

2.2.7 Other Required Elements

Other elements are required in smaller to much smaller amounts than C, N, P and S. These include K, Mg, Ca, Fe and for some organisms, Si and Na. K is a principal inorganic cation and a cofactor for some enzymes. Mg influences the stability of ribosomes, membranes and nucleic acids, is a component of chlorophyll, and a cofactor for many enzymes. Ca is associated with heat resistance of endospores, and a cofactor for some enzymes. Fe is a constituent of cytochromes (electron carriers), and a cofactor for some enzymes.

2.2.8 Organic Growth Factors

Growth factors such as amino acids, purines, pyrimidines and vitamins are organic nutrients required for growth but not necessarily synthesized by the microorganism that needs it. Amino acids (constituents of proteins and sometimes precursors of intermediates in metabolic pathways) must either be synthesized by the organisms or provided as exogenous nutrients in the form of free amino acids or small peptides that can be degraded by bacterial proteases. Bacteria unable to synthesize certain amino acids generally lack one or more enzymes in the specific biosynthetic pathways for those amino acids. Purines and pyrimidines are needed in the synthesis of nucleic acids, and of many coenzymes and certain antibiotics. If they are not formed by the bacterial cell they must be provided exogenously. Vitamins and similar compounds are needed as constituents of enzymes and coenzymes. Some bacteria have extensive requirements for vitamins whereas others are self-sufficient.

2.2.9 Gaseous Requirements

Bacteria are divided into four groups on the basis of their oxygen sensitivity and requirement. Aerobic bacteria grow in the presence of oxygen. Microaerophilic bacteria grow best at oxygen concentrations lower than those in air. Facultative anaerobic bacteria grow in the presence (respiration) or absence (fermentation) of oxygen. Anaerobic bacteria require the absence of oxygen for growth. Within each of these categories, there are ranges of sensitivity to, and of requirement for oxygen, e.g., anaerobic microorganisms vary from obligate anaerobic to aerotolerant.

Bacteria possess enzymes that convert oxygen to different chemical compounds including the toxic substances superoxide free radical (O_2^-), peroxide (O_2^{2-}) which usually appears in the form of hydrogen peroxide (H_2O_2) and hydroxyl free radical (OH^-). These compounds cause oxidation of the cell and are therefore lethal. Most aerobic, microaerophilic and facultative anaerobic bacteria possess enzymes that convert these harmful compounds to harmless ones. For instance the enzymes superoxide dismutase and catalase are involved in converting O_2^- and H_2O_2 to O_2 and H_2O. Aerotolerant anaerobes possess superoxide dismutase but lack enzymes to degrade H_2O_2.

2.3 OTHER STATES

2.3.1 Longevity, Cell Death and the Definitions of 'Sterile' and 'Viable'

Microorganisms are known to survive for long periods of time. Sneath (1962) pursued various methods of determining the longevity of microorganisms. He obtained samples from collections that had been either sealed or preserved in environments that were not conducive to the growth of bacteria. The samples ranged from soils attached to the roots of botanical specimens to fecal samples. He was unable to culture organisms more than a hundred years old, but suggested that the older samples (to 500 years old) were not preserved in a conducive manner. He postulated that, theoretically, under the right conditions, spore survival would be limited by the decay of ubiquitous potassium-40, which he estimated at 10^9 years. Viable bacteria were indeed recovered from a Roman archeological site (Seward et al. 1976).

More modern approaches are not limited to culturing of microbes, and culturing techniques also have improved to include a broad range of conditions. Presently there is ample evidence that microorganisms can exist in the sub-surface for very long periods of time. This survival is known to continue under extremely adverse conditions. For example, Nedwell et al. (1994) isolated microorganisms from organic remains at Shackelton's (1907) and Scotts (1910-1911) camps in the Antarctic. Others were found at elevated temperature and pressure near hydrothermal vents on the ocean floor. However, some of these survival states are not part of the growth cycle described in Section 2.1. In the discussions in this report, which encompass long periods of time, distinction must be made between inactive bacteria that have the capacity to be resuscitated, and cell death. Also, experimental definition of terms and tests may succeed for the short term but do not necessarily over the long term. For example "sterilization" does not necessarily imply that all of the bacteria are dead. Medical sterilization is defined as 2 positive results in 100. Over the relatively short period of time that is important for medical purposes, it is clear that this definition is roughly equivalent to the total destruction of bacteria. However, it is also clear that

the same definition does not lead to a sterile environment over long periods of time in a repository environment where small populations can multiply over time because of their adaptive capabilities.

Moreira et al. (1994) emphasized the distinction between mortality and culturability in their studies of survival of bacteria inoculated in sterile tap and mineral waters. The mortality was low but culturability decreased markedly. In fact, Morita (1988) illustrated that very slow growth rates and no growth rates are common, if not the standard, in natural conditions, and that "no growth" should be considered a biological state, rather than as cell death. However, death is often determined by the failure to multiply in a laboratory medium that has been chosen as representing a favorable environment. Therefore, many bacteria which do not grow on normal laboratory media would be considered dead by this definition, but are more appropriately termed "nonviable". Even assessment of viability is fraught with difficulty. Button et al. (1993) discussed techniques used in assessing the viability of organisms, pointing out that some approaches favor the most nutrient tolerant rather than the most abundant organisms. They favoured enumerations of multiple cultures in a range of media dilutions in order to achieve a more representative assessment of the viable population in a sample.

Postgate and Hunter (1962) used the culturability of cells as a determinant of cell death and viable population. They recorded the progression and speed of events on a molecular level during cell death and noted that intracellular RNA broke down rapidly during death, and that phosphate and base fragments were released back into the medium. Most of the ribose was metabolized. After a lag, protein degraded, but intracellular polysaccharide DNA showed little evidence of degradation. Reeve et al. (1984) pointed out that without the ability for protein degradation, the capability of carbon starved *Salmonella and Escherichia* sp. to survive is reduced. Kurath and Morita (1983) suggested that when live cell counts are needed as a basis denominator for interpretation of observed activities it is rather the number of actively respiring cells that should be used. It is possible to detect the active respiration of bacteria using a fluorescent redox probe (Rodriguez et al. 1992). Another colorimetric indicator (tertrazolium salt) has been used by Roslev and King (1993).

2.3.2 Starvation, Metabolic Arrest, Dormancy and Resuscitation

Environmental conditions are fundamental in determining the survival of bacteria under starvation conditions. According to Morita (1993), long-term starvation-survival is a mechanism by which a species creates a situation analogous to the bacterial spore (Section 2.3.3), i.e., all metabolic systems cease to function. This state is called metabolic arrest (Morita 1993) or shutdown.

The majority of starvation and resuscitation studies have been conducted with marine organisms. Nystrom et al. (1990) studied multiple nutrient starvation (glucose, amino acids, ammonium and phosphate) in marine *Vibrio* sp. and recognized three phases of starvation in the rate of RNA synthesis, which took place over a matter of hours. They noted that the earlier a protein was formed in the starvation sequence, the more important it was for survival.

Nystrom et al. (1992) demonstrated that carbon and nutrient starvation conditions promoted long-term starvation resistance in marine *Vibrio* sp. Such starvation conditions also increased the bacteria's resistance to heat, ultra-violet (UV) radiation and $CdCl_2$ stress. However, nitrogen or phosphorus deficiencies alone did not induce such long-term survival capability or resistance to those stresses.

Moyer and Morita (1989a) demonstrated a link between starvation patterns and cell sizes when they varied growth rates using marine psychrophillic vibrio bacteria that were previously grown in dilute and rich media. They considered cell size reduction to be an adaptive strategy in that it reduced predation and increased surface area per unit volume for maximum nutrient uptake. Three phases of starvation were identified based on trends in nucleic acid and protein concentration.

Moyer and Morita (1989b) examined the impact of growth rate on DNA, RNA and protein concentrations during starvation using bacteria that were previously grown in rich media. The three phases of starvation observed in these studies differed somewhat depending on the growth rate. They attributed the fluctuations that are characteristic of the first phase (about 14 days) to redistribution of cellular constituents in preparation for long term starvation. During the second stage (about 75 days), the impact of drastic environmental changes was buffered by the lower metabolic activity that had been induced. During this stage, the authors also noted that the viable count was reduced to 0.3% of the total cell numbers and remained that low for the duration of the experiment because metabolism had slowed to a point that it was difficult to catabolize large molecules and usable carbon had been exhausted. In the final stage (day 90 and beyond), the cells underwent final changes into a state of metabolic arrest. In such a state, microbes can maintain essential parts of their system for extremely long periods of time.

Amy and Morita (1983a) as well as Stevenson (1978), suggested that microbes can remain in the dormant state for many years. Amy and Morita (1983a) demonstrated in marine *Vibrio* sp. that recovery time increases with starvation time. The demonstrated recovery times, however, are in the range of days for several weeks of starvation. Cells starved for supposedly very long periods of (geologic) time have been cultured in the laboratory (Amy et al. 1993).

Amy and Morita (1983b) have shown that changes occur in the patterns of protein occurrence during the starvation of marine psychrophilic *Vibrio* sp. The pattern is complex since proteins not only disappear but new proteins also appear. Amy and Morita (1983b) discriminated between a short-term starvation state and a long-term starvation state based on the protein pattern. Both patterns are different from the growth phase pattern. Both studies by Amy and Morita (1983a,b) suggested that during starvation microorganisms go through a succession of stages on the way to total dormancy in which the lag time before revival is correlated to the commitment to dormancy.

Amy et al. (1983) noted progressive changes in the levels of DNA, RNA, protein, ATP glutathione and radioactivity associated with ^{35}S methionine-labeled cellular protein during the starvation of psychrophilic heterotrophic *Vibrio* sp. The process occurred over a 6-week period and ultimately resulted in a stable long-term pattern of dormancy.

Even during metabolic arrest certain changes can occur that can increase the cell's chances of ultimate resuscitation when the environment becomes conducive to growth. Caldwell et al. (1989) investigated the impact of starvation survival on plasmid expression and maintenance in *P. aeruginosa, P. putida, E. coli* and *P. cepacia*, during 250 days of immersion in sterile well water. (Some resistances to antibiotics and heavy metals are plasmid expressions and the loss of expression, therefore, indicates the degradation of the plasmid.) They observed all possible patterns of plasmid usage during starvation: no loss of expression; loss on initial recovery with subsequent expression; and complete loss of plasmid expression.

Carbon starvation has been studied primarily in marine and experimental chemostat settings. Not all carbon is bioavailable and in fact, not even all organic carbon is bioavailable. Morita (1990) pointed out that most organic matter is recalcitrant and that, therefore, most microorganisms do not have sufficient energy to carry on their metabolism for growth and reproduction. Roslev and King (1994) demonstrated that under anoxic conditions, carbon starved methanotrophs could maintain a condition that allowed the rapid recovery when beneficial conditions returned. Carbon starvation under oxic conditions, however, caused significant changes in morphology and loss (28-35%) of cell protein.

Schultz and Matin (1991) identified more than 30 proteins associated with the onset of carbon (glucose or succinate) starvation. Their work suggested that the production of these proteins prepares the cell to survive a period of starvation. Tunner et al. (1992) manipulated the protein production in *E. coli* by using glucose starvation. Davis and Robb (1985) studied uptake of mannitol in strains of *Vibrio* and *Pseudomonas* during starvation. They showed that *Vibrio* species maintained its original rapid uptake system for a long period (5 weeks) of starvation, after which a system with a higher affinity for mannitol was observed. The *Pseudomonas* species, on the other hand, suppressed the mannitol uptake system shortly (30hours) after the onset of starvation. The suppressed system, however, could be induced by supplying mannitol, even after 6 weeks of starvation.

Blum et al. (1990) described the regulation of some genes induced by carbon starvation in *E. coli*. Kim et al. (1995) have studied genes that are related to carbon starvation sensitivity and have suggested that it is the s^{54} promoter sequence part of the gene that regulates that sensitivity.

2.3.3 Sporulation

The endospore is the intracellular product of sporulation or sporogenesis, occurring in certain bacteria. An endospore is a tough, dormant form of a bacterial cell that can exist for long periods of time. For example regenerative spores have been found in Egyptian mummies. At least part of the toughness associated with a spore is found in its very tough outer layers, called a coat. Although many organisms other than bacteria form spores, the bacterial endospore is unique in its degree of resistance to adverse conditions. The structure of bacterial spores is much more complex than that of the vegetative cell in that it has many layers. Bacterial spores resist desiccation, heat, and a variety of chemical and radiation treatments that would be lethal to the vegetative bacteria (Brock and Madigan 1991).

Sporulation is a normal part of the growth cycle of some bacteria. Because spores are more resistant to unfavourable environmental conditions, spores are a major coping mechanism for the organisms that possess that capability, i.e., some bacteria and most fungi. Endospores are capable of germinating despite harsh treatment, and thus can potentially produce actively replicating cells where none were previously present. However, it is difficult to assess the metabolic contributions of sporeformers to habitats because of the problem of determining whether they are present as dormant spores or active, vegetative cells. In many cases, spores are constitutively dormant and require a 'trigger' (chemical, heat or cold shock) to initiate germination. Endospores of thermophilic bacteria can be recovered from sediments in cold lakes and ocean cores where they have accumulated and may persist for very long periods because they are protected from sunlight and other forms of environmental damage.

There are three signals a bacterial cell uses to trigger the initiation of sporulation: a nutritional signal, a population density signal and a cell cycle signal. Nutritional signals act in either direction. Starvation of any of the major nutrients (carbon, nitrogen, or phosphorus) can induce sporulation. On the other hand good carbon sources repress sporulation. Population density is seen as a trigger for sporulation because it could not be induced efficiently in cells maintained at low population density. This was not the case when cells were suspended at low density in medium previously conditioned by growth of cells at high density. This observation suggested that the effect was not due to depletion of an essential growth factor, but rather, the production of a substance such as an oligopeptide, that would trigger, or be essential for, sporulation.

There are many biochemical changes that accompany the morphological changes during sporulation but these are not considered here. Initiation of sporulation can occur only at a specific point in the cell division cycle and the process of cellular differentiation leading to spore formation is described in three phases. During the first, or differentiation, stage there is a single cell type, which eventually contains two completed chromosomes. The phase is completed with the division of this cell into two cells that are similar in most respects other than size. In the second phase the differentiation becomes fixed; the two cells have their own genomes, and by the end of this phase the two cell types differ. The mature spore, however, and the development of its properties only takes place in the third stage of development.

Resistant structures are further discussed in Chapter 3 (Section 3.6).

2.3.4 Cryptic Growth

Cryptic growth describes the re-utilization of lysis and leakage products, by intact cells of the same population, as carbon energy substrates and nutrient sources (Mason and Hamer 1987). Cryptic growth can account for the survival pattern in cultures under starvation (non-growth) conditions. In survival studies in which cryptic growth is possible, it should be expected to be the rule rather than the exception (Mason and Hamer 1987).

Banks and Bryers (1990) also showed that cryptic growth and turnover of cellular biomass can be significant under situations of low substrate flux or starvation conditions. The following scenario was postulated for the net accumulation of cells. Primary soluble carbon energy substrate is metabolized by microbial cells forming more cells, soluble metabolic byproducts and

respired CO_2. Processes such as maintenance energy requirements detract from cell and byproduct formation by intercellular carbon recycling. Energy dissipation can also occur by an external cycle involving the lysis of intact cells followed by either scavenging of the released soluble organic carbon by intact cells (i.e., cryptic growth) or solubilization of particular cellular debris followed by growth of intact cells on the solubilized material (Banks and Bryers 1990). Both processes are considered cryptic growth.

Cryptic growth probably occurs in rock-inhabiting (lithobiontic) microorganisms, which are widespread in nature (Friedman and Ocampo-Friedman 1984) and can be found in a wide variety of extreme climates and environments, ranging from hot deserts to Antarctica, from alpine to submerged marine and freshwater rocks and from caves to the surfaces of monuments and buildings. Cryptoendolithic communities are 'closed ecosystems' (on the biological timescale) where the flow of matter is significantly restricted. CO_2 exchange through the rock crust is very low, indicating that an internal CO_2 pool exists in the endolithic airspace. This may well serve as the control mechanism in regulating photosynthesis, a necessity in the physically limited space of the cryptoendolithic microenvironment. Generally, therefore, these communities occur at or near the surface (on a mm scale) and the depth of the occupied zone depends on the physical properties of the rock and the complexity of the microbial community. These cryptoendolithic ecosystems are very simple and insulated habitats which may form a last foothold in a gradually deteriorating environment. They can serve as models for certain exobiological scenarios. Changes in microbial colonization patterns in desert rocks can be used as paleoclimate indicators. Biogenous rock weathering, due to the activity of cryptoendoliths, is geomicrobiologically significant (Friedman and Ocampo-Friedman 1984).

2.4 INTERACTIONS BETWEEN MICRO-ORGANISMS

2.4.1 Diversity and Heterogeneity

Heterogeneity of microbes in spatial distribution is related to their diversity (Rutter and Nedwell 1994). Heterogeneity is to be expected in natural habitats due to competition between microbes as well as the presence of physical and chemical gradients of many types. Spatial heterogeneity and its impacts are often lost in laboratory cultures, which are conducted on small scales or deliberately agitated to achieve homogeneity.

Wolfaardt et al. (1993) pointed out that laboratory tests which use a single concentration of a test compound favour the proliferation of a small group of closely related organisms. Natural systems, however, have spatial heterogeneity. They also pointed out that under conditions of a gradient, certain steady-state conditions are developed within a microbial community because diffusional pathways are developed along those gradients such that complex consortia can develop that include slow growers, which are capable of complete mineralization of a compound.

2.4.2 Consortia and Cooperative Activity

Studies of consortia for bioremediation are also useful in another general way for the purpose of the present work. That is, the experimental consortia are often pre-selected from the natural environment by subjecting them to the desired conditions for significant periods of time (years).

Thus these consortia represent adaptive capabilities of microbial populations to a changing environment, and the development of strategies, not on the part of single species, but whole communities to take advantage of a potential nutrient source or changing environmental conditions.

For example, Bright et al. (1994) demonstrated that iron-, manganese- and sulfate-reducing microbial consortia, as well as broad spectrum anaerobic heterotrophic mixed cultures all produced methylarsenicals in an a subarctic aerobic environment. They contrasted this behavior with methylation of mercury, which is primarily associated with sulfate reducers. Bright et al. (1994) suggested that this general methylation behavior was a detoxifying response to the increased bioavailability of arsenic in anaerobic environments that may be necessary for the range of organisms that are found in that environment. Detoxification of the environment was also invoked by Frischmuth et al. (1993) who were able to associate the resistance of certain microbial strains to $HgCl_2$ to the transformation of Hg^{2+} to Hg^0 thus making it less bioavailable. Sorption of metals by bacteria is also sometimes a product of consortial activity. For instance, Guan et al. (1993a,b) demonstrated the sorption of Cr^{6+} by a consortium of denitrifying bacteria. Their results indicated that the removal of the chromate ion from solution may be associated with microbial metabolic processes.

2.4.3 Competition

In nature, exponential growth is not the rule. Stanier et al. (1976) calculated that a single bacterium with a doubling time of 20 minutes would produce progeny roughly 4,000 times the weight of the earth (2.2×10^{31} grams) in approximately 1 and a half days.

The ability to successfully capture the available nutrients is of major importance and competitive abilities are an important determinant of the survival of the species. Other important factors are the ability to survive during periods of starvation (by maintaining the ability to move chemicals across the cell membrane) and motility to move to nutrient sources.

Experiments intended to understand competition in a more controlled environment than a natural setting have shown the importance of the form in which the nutrient is provided in competition (e.g., the competitive success of a microbe can vary depending on the form of nitrogen available, NH4, NO_3 or N_2 gas) (Keith and Herbert 1985). Part of the success or competitive advantage of a species is its ability to survive the products of its own and other's activity. Physical factors such as temperature can affect success in a competition (Thomas and Wimpenny1993).

2.4.4 Succession

Clements (1916), quoted by McCormick et al. (1991) stressed the importance of individual population dynamics in succession. The progression is defined in a linear manner in which the status of the new population is dependent on and facilitated by the preceding population. This is contrasted to Gleason (1926), who put forward the idea that the community response and development consists of the sum of the responses of more than one population to changes that occur in time and in space. Gleason's (1926) idea is more accepted at present. He suggests the following stages of succession: pioneer colonization, early successional species, and increased

abundance of mid to late successional species. In experiments it was determined that loss of the mid to late successional species does not significantly affect the distribution of the other species. However, the early successional species and pioneer species did appear to be attenuated by the existence of mid and late successional species in the environment. This led to the conclusion that while species specific characteristics may dominate the process of succession in an isolated system, the relative importance of species interactions increases in a pre-populated or non-isolated system.

Microbes can determine succession not only within the microbial realm, but also in those of other species. Chapin et al. (1994) have traced the primary succession of higher organisms (tree species) to the characteristics of colonizing bacteria. They point out that the "black-crust algal/microbial community" enhanced the survivorship of some species of alder. In the studies by Zak et al. (1990) of three types of late successional forests, carbon and nitrogen cycles were found to be strongly correlated to the accrual of plant and microbial biomass.

Guiral et al. (1994) discuss natural succession of microbial species after a perturbation (emptying and liming) of a tropical pond. It was observed that opportunistic species were favored originally by the availability of nutrients. However, as the nutrient supply dwindled these species were eliminated. The presence of a large amount of organic compounds allowed bacterial communities to develop and finally phytoplankton. A general observation in this perturbation situation was that succession was based at first on proliferation and species collapse. In the second phase more complexity was developed, which reduced the propensity of the population in general to be susceptible to small perturbations. Once a mature population was established, diluted areas were observed to recolonize with bacteria in a matter of a few hours and evolution of the mature community was observed in 24 days.

2.4.5 Summary

This chapter reviews general microbial states, such as the normal phases of growth, the requirements of growth (nutrients), conditions for normal growth and other types of growth, growth kinetics, general survival forms (dormancy, sporulation), and, briefly, some interactions between organisms, such as heterogeneity, consortia, competition and succession.

The potential Yucca Mountain repository would be a quite unusual environment from a microbial perspective, with a number of environmentally extreme characteristics. The following chapter, therefore, discusses microbial capabilities and adaptive strategies in response to a number of physical factors such as heat, desiccation, pH extremes, and radiation, all expected to influence the environment of a repository at Yucca Mountain.

3. MICROBIAL CAPABILITIES AND ADAPTIVE STRATEGIES

This chapter discusses, as outlined in the introduction, general microbial capabilities and adaptive strategies, at a mechanistic level, to survive in a perturbed and changing environment. Case studies, specifically those relevant to the Yucca Mountain environment, are given in

Chapter 5 following a definition and discussion (in Chapter 4), from a microbial perspective, of the natural conditions and the evolution of the Yucca Mountain environment that is expected to result from the construction and operation of a repository at that site. The topics reviewed in this chapter are adaptations to changes in water content (including desiccation and osmoregulation responses), adaptations to pH shifts and extremes, adaptation to nutrient levels and adaptation (survival) following radiation damage.

3.1 WATER

3.1.1 Water Inside Microbial Cells

It is widely accepted that about 70% of a bacterial cell is water, distributed amongst several structurally distinct compartments: the cytoplasm, the periplasm and the capsule (if present). It is unknown whether the properties of water may cause discrete subpartitioning of physiological zones within the cell compartment. Living cells undergo continuous shifts in the net concentrations of intracellular water, salts, lipids, macromolecules, trace metals and cofactors. Cells can be considered membrane-bound bags of water that contain on average some 2000 different proteins (some of which may be present in up to 100 000 copies), one or more chromosomes of several mm in length when uncoiled, about 6000 different mRNAs and >10 000 ribosomes. Whether these components are distributed in the cell according to an inherent organization or chaotically has thus far not been resolved (Potts 1994).

When bacterial cells are exposed to a gas phase with a water activity (a_w) lower than in the cell compartment, the cells will lose water. Depending on the magnitude in a_w differences, this loss may lead to rapid shrinkage of the cytoplasm or , if a_w is sufficient to allow some growth, the cells may synthesize a compatible solute and achieve a water balance. This drying stress is termed 'matric water stress' and the removal of substantial amounts of the bulk water from cells through matric stress is called desiccation. The difference, therefore, between osmotic and matric stress is that the former occurs in a liquid environment and allows the cell access to water if it can form compatible solutes. Matric stress removes water from the system and the cell does not have access to this water until it is re-added to the system by rainfall, irrigation, tidal water etc. Matric stress, therefore, would play a large role in the activity of bacteria in the Yucca Mountain subsurface.

Because of the difficulty of measuring it, there are few data for the turgor pressures generated in bacterial cells. Measurements available were made with liquid culture cells, and values ranging from about 2 to 4.5 bars have been measured. The turgor pressure varied considerably from cell to cell but was independent of cell size. It is very difficult to measure intracellular water because bacterial cells may contain substantial amounts of extracellular water, in attached fibrils and polymers. Measurements have indicated that some cells hold in excess of 95% of their own weight in water, most of which can be removed upon air drying (Potts 1994).

3.1.2 Water Retention with Respect to Cell Membrane and Cell Wall Structure

3.1.2.1 Cell Membrane

The cell membrane is a thin structure that completely surrounds the cell. It is only about 8 nm thick and forms the critical barrier that separates the cytoplasm from the external environment. The cell membrane is a highly selective barrier which enables the cell to concentrate specific metabolites and excrete unwanted material (Brock and Madigan 1991). The cell membrane consists of a phospholipid bilayer. In aqueous solution the phospholipids arrange themselves such that their hydrophilic portions (i.e., the glycerol parts) face outward and remain exposed to the aqueous environment, while the hydrophobic portions (i.e., the fatty acid parts) point inward towards each other in a hydrophobic environment. The bilayer character of the cell membrane probably represents the most stable arrangement of lipid molecules in an aqueous environment (Brock and Madigan 1991). The lipids of eubacteria (and eucaryotes) have ester bonds linking the fatty acids to the glycerol molecules. Archaebacteria have ether linkages between glycerol and hydrophobic side chains. In addition, the hydrophobic side chains are not fatty acids but consist of isoprene in archaebacteria, which makes these membranes considerably different from membranes composed of glycerol and fatty acids. Interestingly, all extreme halophilic bacteria (Appendix B) appear to be archaebacteria (Larsen 1981).

The cell (plasma) membrane is permeable only to water molecules which are sufficiently small and uncharged to pass between the phospholipid molecules. Some other small non-polar and fat-soluble substances such as fatty acids, alcohols and benzene may penetrate cell membranes by dissolving in the lipid phase. But passive movement of polar molecules (such as organic acids, amino acids, and inorganic salts, which are hydrophilic) does not really occur. These molecules must be specifically transported by a number of transport proteins embedded in, and unique to, the cell membrane. There are three classes of transport proteins: *uniporters* transport a substance from one side to the other side of the membrane; co-transport of two substances occurs in the so-called *symporters* (both substances are transported in the same direction); and *antiporters* in which substances are transported in opposite directions (e.g., the Na^+/H^+ antiport system important in pH homeostasis (Section 3.3.2). This highly specific carrier-mediated transport allows a cell to accumulate or excrete specific substances against a concentration gradient (Brock and Madigan 1991).

The cell or plasma membranes are readily permeable to water but present a more effective barrier to most other solutes (Csonka and Hanson 1991). The intracellular environment of any organism must remain relatively constant with respect to cell volume, ionic composition, pH and metabolite levels for active metabolism to occur, and these limits are very similar amongst most species. In order to maintain this internal environment, cells actively adapt to a stress by changing the concentration of a few solutes that are not greatly inhibitory to cellular processes, either through de novo synthesis or through transport from outside the cell. These substances are called compatible solutes (or osmoprotectants) and include K+, amino acids (e.g., glutamate, proline), disaccharides (e.g., trehalose), N-methyl substituted amino acids (e.g., glycine betaine, proline betaine), peptides (e.g., glutathione) and a number of other substances. They do, as a rule, not cross cell membranes rapidly without the aid of transport systems. For the most part they do not carry a net electrical charge near pH 7, which aids in their non-reactive character. However,

K^+ ions and glutamate are noteworthy exceptions (Csonka 1989). Compatible solutes and osmoprotectants are further discussed in Section 3.1.3.

3.1.2.2 Cell Wall

Because of the concentration of dissolved solutes inside a bacterial cell, a considerable turgor pressure develops. To withstand these pressures, bacteria have cell walls, which also give shape and rigidity to the cell. The existence of a cell wall distinguishes procaryotes from eucaryotes. Bacteria can be divided in two major groups, Gram-positive and Gram-negative bacteria, on the basis of differences in cell wall structure and composition. The Gram-negative cell wall is a multilayered structure and quite complex, while the Gram-positive cell wall consists of primarily a single type of molecule and is often much thicker. There is also a significant textural difference between the two types. Although various archaebacteria stain Gram-positive or Gram-negative, the chemistry of archaebacterial cell walls differs in major ways from the cell walls of eubacteria (Brock and Madigan 1991).

In the cell walls of eubacteria, there is one rigid layer which is primarily responsible for the strength of the wall. In most bacteria, other layers are present outside this rigid layer. The rigid layer consists of peptidoglycan, cross-linked to different extents in different bacteria. The shape of a cell is determined by the lengths of the petidoglycan chains and by the manner and extent of cross-linking of the chains. Almost 100 different peptidoglycan types are known. In Gram-positive bacteria, as much as 90% of the wall consists of peptidoglycan, although a small amount of teichoic acid is also present. In Gram-negative bacteria, only 5 to 20% of the wall is peptidoglycan and the other 80% or more of the wall is an outer layer (outside the peptidoglycan layer) made of lipopolysaccharide. This layer (called the LPS layer) is effectively a second lipid bilayer but is not constructed solely of phospholipids as is the plasma membrane, but also contains polysaccharide and protein. This outer layer is frequently toxic to animals and hence the pathogenic character of some bacteria.

Unlike the plasma membrane, the outer membrane of Gram-negative eubacteria is relatively permeable to small molecules (even though it is basically a lipid bilayer) because of the presence of porins, which are proteins that serve as membrane channels for the entrance and exit of low molecular weight substances. However, although the outer layer is thus relatively permeable to small hydrophilic molecules, it is not permeable to enzymes or other large molecules and one of the main functions of the outer layer may be its ability to keep certain enzymes, which are present outside the cytoplasm, from diffusing away from the cell. This region between the outer layer and the plasma membrane is called the periplasmic space in Gram-negative bacteria and houses a number of hydrolases for macromolecular nutrients, binding proteins for metabolites, and receptors for chemotactic signals. This space occupies 20-40% of the total volume (*E. coli*) and is maintained as a separate compartment during steady-state growth at all conditions of osmolarity. Gram-positive bacteria do not have an LPS outer layer or periplasmic space, but they do have teichoic acids attached to their cell wall which are partially responsible for the negative charge of the cell surface as a whole (Brock and Madigan 1991).

3.1.3 Cell Wall Response to Physical Signals

The regulation of most biological responses depends on the recognition of signal molecules by specific receptors. However, for some responses the information from the environment is not a specific molecule, but a physicochemical parameter, that is, by physical rather than chemical signals (e.g., the regulation of expression of genes by pressure, viscosity and temperature, dessication and increase in salinity), and the exact mechanism of signal transduction is not known. Osmoregulation is inseparable from general ionic and metabolic regulation and cytoplasmic osmolarity is controlled by a diverse set of homeostatic feedback mechanisms that respond to various intracellular signals. However, the exact nature or functioning of these signals is not fully known. Possible signals include isotropic pressure changes, pressure differentials across the inner membrane peptidoglycan complex, changes in the internal or external solute levels or water activities and changes in the inner membrane area.

3.1.4 Compatible Solutes and Osmoprotectants

There are several ways in which a population of bacterial cells (with no sheaths or outer investments) may be subjected to a water deficit. The most usually considered deficit is an "osmotic stress" in which the cells are suspended in an aqueous solution containing a solute that cannot enter the cells. There is a net efflux of water until there is a balance between the water activities in solution and in the cells. The opposite happens when cells accumulate compatible solutes or osmoprotectants, either by transport from the surrounding environment (e.g., the culture medium) or through de novo synthesis or both. Compatible solutes include K^+ ions, glutamate, glutamine, proline, quaternary amines (glycine, betaine) and sugars such as trehalose, sucrose and glucosylglycerol. The role of the solute is most likely that of stabilizing proteins and membranes. Under conditions of moderate water deficit, many compatible solutes appear to be important components of mechanisms that contribute to the maintenance of viability. Under the most extreme water deficit, however, only the disaccharides trehalose and sucrose seem to afford protection. For any compound to be an acceptable compatible solute, it must not have any excessive inhibitory effects on any metabolic processes.

3.1.4.1 Potassium

Potassium ions are the most prevalent cations in the cytoplasm of bacteria and, consequently, serve as one of the major intracellular osmolytes that maintain turgor. The intracellular concentration of K^+ in a wide assortment of bacterial species has been found to be nearly proportional to the osmolarity of the growth medium and there is a positive correlation between the intracellular content of this cation and the ability of bacteria to tolerate conditions of high osmolarity. Increased concentrations of K^+ is elicited only by high concentrations of solutes that can not diffuse across the cell membrane (e.g., glucose, sucrose, NaCl) and K^+ concentrations are only dependent on the osmolarity of the medium, not on kind of solute. Glycerol diffuses freely across cell membrane and K^+ accumulation is not observed when the solute is glycerol. K^+ influx and efflux stimulation, in response to exposure to media of hyper- or hypo-osmolarity, respectively, occurs very rapidly and does not require an energy source. The mechanism for this is not known, but it is possibly a direct effect of turgor on the proteins that mediate K^+ entry and exit.

3.1.4.2 Glutamate and Glutamine

The cytoplasmic level of glutamate increases in most procaryotes after exposure to high osmolarity media. In Gram-negative bacteria, osmotic stress can elicit greater than 10-fold increases in the level of glutamate such that in some organisms grown in high osmotic strength media more than 90% of the free amino acid content is glutamate. Glutamine levels also increase but the overall concentration is much lower such that a glutamine increase likely does not play a role in maintaining cytoplasmic osmolarity. Basal glutamate levels are up to 10-fold higher in Gram-positive bacteria but the relative increase in concentration during growth in high osmotic strength media is much less and much slower. It is possible that the accumulation of K^+ under conditions of osmotic stress is the regulatory signal for the synthesis of glutamate. However, the uptake of K^+ must be balanced by uptake of anions, or expulsion of other cations. Glutamate is the most abundant anion, but still accounts only for 50% of K^+ in media of high osmolarity and therefore other anions are needed to maintain the proper membrane potential upon accumulation of K^+ under osmotic stress. Expulsion of protons and the accumulation of some uncharacterized anion has been proposed as well as excretion of the divalent cation putrescine. It appears that the accumulation of glutamate and the efflux of protons (or perhaps the accumulation of an unknown anion) are sufficient to provide the charge balance for the K^+ accumulation that occurs upon osmotic stress.

3.1.4.3 Trehalose and Sucrose

Trehalose has been found to be synthesized in a number of bacteria in response to osmotic stress, but the production of trehalose may also respond to other signals (e.g., water or desiccation stress). Synthesis of trehalose entails the condensation of glucose 6-phosphate and uridine diphosphate glucose, yielding trehalose 6-phosphate which subsequently dephosphorylates to trehalose. Mutations which result in an impairment of the accumulation of trehalose result in increased sensitivity to osmotic stress.

Many desiccation-tolerant cells accumulate large amounts (sometimes in excess of 20% of their dry weight) of either or both of the disaccharides trehalose and sucrose in response to water stress. Trehalose is the only non-reducing oligosaccharide of glucose, a second representative is sucrose (a glucose-fructose oligosaccharide). It is generally believed that trehalose is physiologically more relevant than sucrose in terms of its efficiency and the stoichiometric amounts required for protection. The exact mechanism by which these sugars protect the cells from desiccation effects are under discussion (Potts 1994). Trehalose and succrose may not act as compatible solutes, but rather may replace the shell of water around macro-molecules, circumventing damaging effects during drying. This is the "water replacement hypothesis" proposed by Clegg (1986).

The lethal effects of desiccation have been attributed to amino-carbonyl reactions between cell membrane proteins and reducing sugars. Removal of water enhances such reactions while addition of non-reducing sugars can protect membranes by competing for binding with reducing sugars. Disaccharides served as the best protecting agents (Hensel 1994) and this is probably explained by the presence of OH groups in the protecting molecules (Louis et al. 1994). There is

also evidence for a direct interaction between trehalose and lipids. The sugar is thought to replace water molecules around the polar head of the phospholipid in the dry state (Clegg 1986). The work by Clegg provided some possible mechanisms as to how dried cell components might remain viable. What this study and others cannot explain fully is how these components can be redirected instantaneously, and in perhaps an ordered and stringent fashion, to resume integrated metabolism upon rehydration of the dried cell (Potts 1994). For instance, it has been observed that trehalose in desiccated yeast cells turned over rapidly upon cell rehydration. Stressed *E. coli* cells appear to regulate the cytoplasmic level of trehalose by a futile cycle involving overproduction, excretion and degradation to glucose, which is reutilized.

There are also reports in the literature that show no protection against desiccation in the presence of trehalose, or that trehalose is not always the preferred solute accumulated and the amount present does no always correlate with the magnitude of the water stress (Potts 1994).

3.1.4.4 Betaine

The amino derivative betaine is an important osmoprotectant present in several groups of bacteria. For instance, Kets and de Bont (1994) found that cells of *Lactobacillus plantarum* cultivated under osmotic stress of 0.6 M NaCl and in the presence of betaine showed an increased survival after air drying and subsequent vacuum desiccation to an a_w of 0.12.

3.1.4.5 Proline, Glycine Betaine and Choline

Proline can alleviate growth inhibition imposed by osmotic stress but it has not been fully resolved whether it is increased synthesis or increased uptake of proline that makes it work. In some bacteria, it seems to be increased synthesis (many species of Gram-positive bacteria) and in others it seems to be increased transport. In general, Gram-negative bacteria achieve high intracellular concentrations of proline during osmotic stress only by enhanced transport and accumulated concentrations are proportional to the osmotic strength of the medium. Some bacteria (enterics) have three independent proline transport systems, but it has not been resolved yet whether some higher-order mechanism is coordinating the activities of these three systems.

Another important osmoprotectant compound accumulated by bacteria under conditions of hyperosmolarity is glycine betaine (N,N,N-trimethylglycine). Cyanobacteria and some other CO^2 fixing procaryotes are able to carry out de novo synthesis of glycine betaine, but most other bacteria are unable to do so and are dependent on transport for accumulation. Although the ability to respond to exogenous glycine betaine or proline as an osmoprotectant is wide spread among bacteria, not all species are able to do so. (Table 1 in Csonka (1989) gives many examples of the effectiveness of proline and glycine betaine as osmoprotectants in various bacteria.)

Proline and glycine betaine also accumulate in plants during osmotic stress. Two hypotheses have been proposed to explain what makes these compounds so special. According to the first hypothesis, proline and glycine betaine have special interactions with proteins which protect proteins from denaturation in the presence of high concentrations of electrolytes, by coating the proteins with a hydrophilic shell that would enhance solubility. The second hypothesis proposes

that these two compounds are merely inert compatible solutes that are used to maintain cell turgor in media of high osmolarity and that their special properties are derived from the fact that they do not interact with proteins, because solutes that are excluded from protein surfaces tend to favour the native configuration of the proteins. Much of the current evidence supports the second hypothesis. The observation that proline and glycine betaine suppress the osmotic accumulation of K^+ in enteric bacteria is consistent with the notion that these compounds are less toxic to cellular processes than K^+ and are accumulated preferentially over K^+ by the cells as a means of maintaining turgor (Csonka 1989). There are a number of structural analogs of proline and glycine betaine that also have osmoprotecting effects.

E. coli can convert choline to glycine betaine under conditions of osmotic stress, such that choline is also an osmoprotectant. The formation of glycine betaine from choline entails two oxidation steps for which an electron acceptor (such as O_2) is needed. Therefore, choline cannot be used as an osmoprotectant anaerobically.

3.1.4.6 α, β-type Small Acid-Soluble Proteins (SASP)

Many factors are involved in spore resistance to UV radiation, heat and hydrogen peroxide, but one common factor is the saturation of spore DNA with a group of α, β-type small acid soluble proteins (SASP). These proteins are made in the forespore late in sporulation in amounts sufficient to cover the spore chromosome completely but are degraded in the first minutes of spore germination. Fairhead et al. (1994) found that binding of these α, β-type SASP to spore DNA was also a significant factor in spore resistance to freeze drying. Clearly, the presence of high levels of these unique DNA-binding proteins in spores of *Bacillus subtilis* as well as Clostridium species may play a major role in the extreme resistance of such spores to a variety of harsh conditions and thus to their long-term survival both in the laboratory and in natural environments.

3.1.5 Summary

In summary, microorganisms can protect themselves against the effects of desiccation and/or osmotic stress by taking up or synthesizing a number of compatible solutes and osmoprotectants that maintain balanced water activities inside and outside the cell, or protect macromolecules (proteins, lipids, DNA etc.) inside the cell against these effects.

3.2 TEMPERATURE

One of the most important environmental factors for microbial growth is temperature. Each organism has a defined temperature range over which it is capable of growing and if a temperature too high or too low is used, satisfactory growth will not occur. The optimum temperature is the temperature at which the organism grows the fastest. Some microorganisms have optimum temperatures as low as 5°C, and others are known with optima as high as 105°C. However, no one organism spans more than a small part of this temperature range. As a general rule, it can be stated that the most appropriate temperature for culture of a microorganism is near the temperature of the habitat in which that microorganism is growing (Brock and Madigan

1991). It is important to distinguish between the temperature bounds to microbial activity in general and temperature bounds for the activity of certain types of microbes that result in a population shift or utilization of resources by new strategies in order to cope with the stress of both increasing and decreasing temperature.

3.2.1 Growth Rate and Temperature

Growth is an integration of most of the metabolic activities of a microorganism and is chosen most conveniently to test the effect of temperature on the organism as a whole. Growth rates vary with temperature, and cardinal temperatures, as a rule constant for a given strain, are: minimum temperature (under which no growth occurs); optimal temperature (at which the growth rate is maximal); and maximum temperature (above which no growth occurs).

The optimum growth temperature is a compromise between effects that activate and effects that inhibit, and is much closer to the maximum than to the minimum temperature (Aragno 1981). Cells grown at optimal temperature are, therefore, already partially inhibited through temperature. A physiological optimum would be the highest temperature where the Arrhenius plot is still linear, a few degrees below the growth optimum. But even at the optimum temperature, growth will eventually be limited by other factors such as toxicity or starvation (Chapter 2), as is represented, for example by Michaelis-Menton or Monod equations (Paul and Clark 1989).

In a defined interval, bacterial growth follows approximately the Arrhenius Law (Aragno 1981):

$$\ln v = -E/RT + C,$$

where

- v = the reaction velocity
- E = activation energy of the reaction
- R = gas constant
- T = absolute temperature (K)
- C = constant

In the interval of bacterial growth where a linear relationship with temperature exists, it is possible to define an apparent activation energy of bacterial growth. Near the upper and lower limits of growth the curve abruptly drops to zero.

3.2.2 Upper Temperature Limits of Growth

It is difficult to categorize microorganisms according to their temperature relationships because of the absence of very precisely defined boundaries. Also, different publications define slightly different temperature ranges or boundaries. According to Aragno (1981) it is understood that psychrobiotic or psychrophilic means growth below 20°C, mesobiotic or mesophilic between 20 and 45°C and thermobiotic or thermophilic over 45°C. Brock and Madigan (1991) give as temperature ranges <0-20°C for psychrophiles, with an optimum temperature around 15°C, 14-45°C for mesophiles with an optimum near 38°C, 42-68°C for thermophiles with an optimum

temperature near 62°C and 66-96°C for extreme thermophiles with an optimum around 85°C. According to Kristjannson and Hreggvidsson (1995), a "normal" microbial environment is between 4 and 40°C and the thermophile boundary is put at 55 to 60°C for several reasons. Temperatures below this boundary are common in nature and microorganisms that grow optimally at or below this temperature are also very common. They can be isolated from normal soils and even from permanently cold places such as Antarctica. Temperatures above this boundary are rare in nature and occur only under special conditions, such as in composts, self-heated hay or in geothermal areas. Organisms that grow optimally above 55 to 60°C are all prokaryotes, so, using this boundary only prokaryotes can be thermophiles. Two other boundaries defined by Kristjannson and Hreggvidsson (1995) are extreme thermophile or hyperthermophile with a growth temperature above 85°C and pyrophile with a growth temperature above 100°C.

The lack of growth in microbes above a determined temperature is evidently due to changes in at least one structure or function indispensable to the growth process. Heat-sensitive structures in the cell may be membranes (particularly the physical state of their lipids), enzymes and other proteins and the protein-synthesizing apparatus (nucleic acids and ribosomes). The melting temperature of membrane lipids might determine the upper temperature limit for growth. Alternatively, the fatty acid composition of membrane lipids may be altered by changes in growth temperature, thus enabling the occurrence of adaptation. For example, in *E. coli*, an increase in growth temperature increased the ratio of (higher melting) saturated fatty acids over unsaturated fatty acids, which allowed preservation of the physical state of the lipids in the membrane (Aragno 1981).

Life at higher temperature requires thermostability of all essential proteins in the cell and genetic adaptation to thermophily would seem to require simultaneous mutations to enhance thermostability of essential proteins, a highly unlikely scenario (Aragno 1981). However, in mesophiles, many enzymes are active at temperatures higher than the maximum growth rate temperature so it is likely that adaptation to higher growth temperatures requires adaptation of only a small amount of enzymes and/or other proteins. Also, enzymes isolated from thermophiles denatured in vitro at growth temperatures, implying that their stability in vivo is enhanced. Moreover, there appear to be no unique or large differences in structure between thermophilic and mesophilic enzymes which implies that enhanced thermostability depends on only a few key subtituents in the primary structure. Therefore, the number of differences implied in the alteration of temperature maximum might be much smaller than would be expected (Aragno 1981).

3.2.3 Lower Temperature Limits of Growth

According to Arrhenius Law, growth should continue (at reduced rates) at lower temperatures until the medium freezes (Aragno 1981). This is the case for phsychrophiles but in other bacteria growth will stop at temperatures considerably above the freezing point of the medium, possibly because the cells biosynthetic capabilities cannot make the required changes to the cell membrane (i.e., higher proportion of low-melting unsaturated fatty acids). For instance, isolation of mutants of mesophilic enteric bacteria with a decreased minimum temperature has not been successful, likely because a number of mutations are required to decrease minimum temperature.

Isolation of mutants of mesophilic pseudomonads with a decreased minimum temperature has had some success because most pseudomonads are facultative phsychrophiles such that the number of mutations necessary to gain phsychrophily might be expected to be small in mesophilic strains of pseudomonads. On the other hand, mutants with increased minimum temperature are easily isolated and occur with approximately the same frequency as isolates with decreased maximum temperatures. Such cold-sensitive conditional mutants show an increase in both the minimal temperature and the apparent activation energy in the mid-range of temperature while their optimum and maximum temperatures are unaffected (Aragno 1981).

3.2.4 Adaptation To Changing Temperature Conditions

Increasing the maximum growth temperature (or lowering the minimum temperature) by mutations is as a rule a highly improbable event, which would imply the simultaneous modifications of a number of genes. On the contrary, mutations lowering the maximum growth temperature or increasing the minimum temperature are much more likely to occur because they may require only one mutation (Aragno 1981). Another type of adaptation occurs when strains are cultivated at an intermediate temperature with subsequent culturing at the desired higher temperature. For instance, *Bacillus stearothermophilus* did not grow when transferred directly from 37 to 55°C but after cultivation at 46°C in a rich medium they could be adapted to 55°C (Aragno 1981).

In some cases the cardinal temperatures of a given organism may vary according to the nutrients provided (Aragno 1981). Distinction should be made between fundamental processes (DNA replication and transcription, RNA translation, ribosome formation and function, ATP synthesis etc.) and "facultative" processes, such as transport and catabolism of a given nutrient source, because microorganisms are versatile in their use of nutrient resources. It is possible that the temperature limits of facultative processes are narrower than those of the fundamental processes, e.g., *Kelbsiella pneumoniae* can grow at 37°C with ammonium salts or organic nitrogen, but growth is prevented with N_2 or NO^{3-} as N sources. The nitrogen fixation enzymes are not inhibited at 37°C but the expression of the 'nif' genes (i.e., genes encoding the components of the nitrogen fixation system) is affected.

Leroi et al. (1994) studied the evolutionary adaptation of *E. coli* to a temporally varying environment. Results from that study do not support the hypothesis that evolution in a temporally varying environment will favour increased acclimation ability or phenotypic flexibility. Instead, it appears that bacterial adaptation to the constant component temperatures was more important than, and may even have traded off with, adaptation to sudden transitions in temperatures. Nonetheless, the results indicate that phenotypic acclimation ability itself has an underlying genetic basis and so may respond evolutionary. However, the direction of evolution in acclimation ability may be more complex than simple models would suggest, so that predicting the evolution of acclimation ability may depend on detailed knowledge of genetic and environmental correlations.

Probably a more important factor in microbial adaptation is the diversity of responses to environmental conditions, rather than the adaptive capabilities of a given species. This is particularly true for temperature. A good example is the succession of populations during the

processes of aerobic composting of hay or manure accompanied by self-heating phenomena. Even though in vitro pure cultures often show a broad temperature range for growth, in natural sites the temperature limits may possibly be much narrower (Aragno 1981).

3.2.5 Thermophiles and Hyperthermophiles

Thermophiles have been known from sources such as hot springs for over a century but hyperthermophiles are a recent discovery in microbiology. Hyperthermophiles have optimal temperatures between 80 and 110°C and are unable to grow at temperatures below 60°C, while some are unable to grow at temperatures below 80°C. Hyperthermophiles belong to phylogenetically distant groups and may represent rather ancient adaptations to heat (Blochl et al. 1995, Stetter 1992, Woese et al. 1990).

Most of the currently known hyperthermophilic species are strictly anaerobic, reduce elemental sulfur (S_o) with H_2 to H_2S and are obligate heterotrophs, utilizing mostly peptides instead of carbohydrates. Growth in vitro is generally only obtained on complex proteinaceous substrates and media typically containing one or more of yeast-, bacterial- or meat extracts, peptone or tryptone. The actual growth substrates are somewhat unclear. Metabolic products (typically including acetate, isovalerate and isobutyrate) indicate a fermentative-type metabolism. Few hyperthermophiles are saccharolytic and they have a limited substrate range (Kelly and Adams 1994).

3.2.6 Mechanism of Heat Resistance in Thermophiles and Hyperthermophiles

The principles of heat stabilization of cell components such as DNA, RNA, proteins, ATP and NAD in hyperthermophiles are still unknown and a challenging topic for basic research. Peak et al. (1997) studied the extreme resistance to thermally induced DNA backbone breaks in the hyperthermophilic Archaeon *Pyrococcus furiosus*. They measured the effect of elevated temperatures (up to 110°C) on the molecular weight of DNA in intact cells of *Pyrococcus furiosus*, compared with the effect on DNA in the mesothermophilic bacterium *E. coli*. At 100°C, DNA in *Pyrococcus furiosus* cells was about 20 times more resistant to thermal breakage than that in *E. coli* cells and 6 times fewer breaks were measured in *Pyrococcus furiosus* DNA after exposure to 100°C for 30 minutes than in *E. coli* DNA at 95°C. The hypothesis for this remarkable stability of DNA in a hyperthermophile is that it posesses endogenous protective mechanisms such as highly effective DNA ligation and repair enzyme systems, as well as protective proteins that protect against hydrolytic damage.

Although resting forms such as spores have never been observed in hyperthermophiles, cultures of *Pyrodictium* sp. grown at 110°C exhibit an extraordinary heat resistance and survive even autoclaving for 1 hour at 121°C. Under these conditions, about 70% of the soluble protein of the cells consists of a heat shock protein (Stetter 1995). Some hyperthermophiles contain glycoproteins which may help make them temperature resistant. (Glycoproteins are also found in the blood of Antarctic fish and protects them from freezing, so they may play a role in protecting primitive organisms from the extremes of hot and cold) (Kelly and Adams 1994).

Narberhaus et al. (1994) examined the response to heat stress in the thermophile *Thermoanaerobacterium thermosulfurigenes*. Upon a temperature shift from 50 to 62°C, four heat shock proteins were synthesized at an elevated level. The heat shock response in this thermophile was transient, with a maximum synthesis of heat shock proteins between 10 and 15 minutes after the shock. In mesophiles, heat shock produces larger numbers of heat shock proteins than in thermophiles and in hyperthermophilic Archaea, only a few heat shock proteins are involved. The relative abundance of these proteins increases because of a significant decrease of all other proteins under heat stress conditions. When reagents that disrupt normal protein synthesis were present during heat shock, enhanced thermotolerance was prevented (Trent et al. 1994).

Reeve (1994) speculates that modern mesophilic enzymes might differ systematically from primitive, thermostable enzymes. However, if only one or two amino acid substitutions were sufficient to reduce the operating temperature of an enzyme, then most of the original 'thermostabilizing' residues might well remain but would not be recognized when comparing contemporary mesophilic and thermophilic enzymes. Many features of the very early hyperthermophile enzymes may be retained in today's enzymes in mesophiles. This could imply that in man-induced hot environments, these enzymes may mutate back and become heat-resistant again.

It is unknown what molecule(s) limit(s) the maximum growth temperature of hyperthermophiles. Reeve (1994) suggests that their Achilles' heel could be a small universal metabolite such as ATP, NADH, glyceraldehyde-3-phosphate, or carbamyl phosphate because these molecules have half lives at 100°C in vitro that are measured in seconds. Many hyperthermophiles contain an unusually thermostable form of ferredoxin and a real novelty in some of their metabolic pathways is the use of tungsten-containing enzymes. Tungsten is found in unusually high concentrations in the sulphides that precipitate from thermal vent fluids (Reeve 1994).

3.2.7 Summary

One of the most important environmental factors for microbial growth is temperature. Each organism has a defined temperature range over which it is capable of growing and if the temperature is too high or too low, satisfactory growth will not occur. The melting temperature of membrane lipids might determine the upper temperature limit for growth. Alternatively, the fatty acid composition of membrane lipids may be altered by changes in growth temperature, thus enabling an adaptation to occur. Temperature changes in the mesophile range often cause ecological shifts in the populations present. Life at higher temperature requires thermostability of all essential proteins in the cell. Hyperthermophiles appear to protect themselves by producing heat shock proteins and perhaps by a very efficient DNA repair system.

3.3 pH

Each microorganism has a pH range within which growth is possible, and each usually has a well-defined pH optimum. Most natural environments have pH values between 5 and 9 and organisms with pH optima in this range are most common and are called neutralophiles. Only a few microorganisms can grow at pH values below 2 or above 10 (Brock and Madigan 1991).

Organisms that thrive at low pH's (as low as pH 1) are called acidophiles. Fungi as a group tend to be more acid tolerant than bacteria and many fungi grow optimally at pH 5 or below. A few fungi grow quite well at pH values as low as 2. Organisms that thrive at high pH's (as high as pH 11) are called alkalophiles. For most acidophiles and alkalophiles, the key to surviving the extreme pH regime is the ability to maintain a near-neutral interior cell pH. Neutralophiles can grow over a wide range from pH 5 to 9 because of a physiologically triggered pH homeostasis mechanism that maintains a relatively constant pH inside the cell (pH_i) over the broad range of external pH values (pH_o). In addition, pH gradients are not uncommon in the natural environment and are sometimes utilized by bacteria.

3.3.1 pH Homeostasis

The basis for pH homeostasis is the apparent modulation of primary cellular H+ pumps as well as K^+/H^+ and Na^+/H^+ antiport systems (Foster and Hall 1991). This process appears subject primarily to allosteric control (such as the pH set point for pump activation) since pH homeostasis functions normally in the presence of protein synthesis inhibitors. As far as genetic control, the system can be referred to as constitutive (this means that it does not have to be induced first by the environment and is not dependent on the synthesis of certain proteins) (Foster and Hall 1991).

There are a variety of mechanisms that can protect a cell from extreme acid stress. These include an increase in the internal buffering capacity or in the proton extrusion rate, as well as a decreased membrane proton conductance. Also, cells can prevent or repair acid damage as a result of lower pH_i. Microorganisms have a wide range of molecular defense mechanisms to repair or prevent injuries as a result of external stresses. Many of these defense strategies have been studied at the biochemical, genetic and molecular level. However, the system that protects cells from external acidification is still not fully understood (Foster 1992).

In acidic environments, the pH homeostasis mechanism enables a cell to maintain a differential pH ($\Delta pH = pH_i - pH_o$) of about 2 pH units with the interior of the cell being more alkaline relative to the external environment. Controlling this system of proton pumps is of integral importance to growth and survival in moderately acid environments. However, with increasing acidity, the system of pH homeostasis eventually fails. The system works very successfully down to pH values where ΔpH is close to 2.0, but below those pH values, ΔpH collapses and the cells begin to die. Classical pH homeostasis has failed as a result of pH_i being lowered too much. Values of pH_i below 5.5 are lethal and will damage internal proteins. The loss of viability is not due to external damage of the cell.

In alkaline environments, pH homeostasis is based on Na^+/H^+ antiporter activity and fails in the absence of sufficient Na^+.

3.3.2 The Role of Na^+ in pH Homeostasis and Solute Uptake in Alkalophiles

Several properties are shared by all extreme alkalophiles (Krulwich and Guffanti 1989). These include a specific composition of cell membrane lipids and of the membrane lipid/protein ratio, very high levels of respiratory chain components in the cell membrane, a generally more acidic

amino acid composition of proteins that are exposed to, or excreted into, the external milieu, and a Na^+ cycle that facilitates solute uptake and pH homeostasis. Any or all of these properties could be prerequisites of alkalophily. Studies with some non-alkalophilic mutants have established that at least the Na^+/H^+ antiporter that is involved in pH homeostasis is a necessary function for life at high pH.

Extreme alkalophiles that grow optimally at pH 10.0 - 11.0 maintain a cytoplasmic pH typically at least two pH units below the external pH. The aerobic alkalophilic Bacilli all exhibit primary proton pumping out of the cell during respiration (ATP production) but the net accumulation of protons by the cells requires Na^+. Suspension of cells of extreme alkalophiles in the absence of added Na^+ results in immediate alkalinization of the cytoplasm pH_i to equal pH_o. A pH gradient (ΔpH) with the cell interior more acidic is maintained by an electrogenic Na^+/H^+ antiporter that exchanges intracellular Na^+ (or Li^+) for external H^+. The antiporter is probably an abundant membrane protein that catalyzes rapid cation exchange. The stoichiometry of the exchange is not precisely known, but is such that the number of protons translocated into the cell is greater than the number of sodium ions translocated out. The potential difference ($\Delta\Psi$) set up by proton pumping during respiration (ATP production) energizes the electrogenic Na^+/H^+ antiport. A pH gradient and an inwardly directed chemical gradient of Na^+ are formed and a trans-membrane electrical potential is maintained. The crucial involvement of the Na^+/H^+ antiporter is supported by the isolation of nonalkalophilic mutant strains that can no longer grow well above pH 9 and have lost Na^+/H^+ antiporter activity (Krulwich and Guffanti 1989).

Many of the properties of the alkalophile Na^+/H^+ antiporter have been elucidated. The general properties include the use of Na^+ or Li^+, linear dependence upon the $\Delta\Psi$, and inhibition by a high internal proton concentration. It is probable that in many organisms the antiporter is required for growth under moderately alkaline conditions, such as pH 8.5 - 9.0. Less clear is whether there are other ion fluxes (such as K^+) or other mechanisms that play a role in pH homeostasis in the alkaline range.

Extreme alkalophiles have a bioenergetic dilemma with respect to oxidative phosphorylation, because of the proton pumping by the respiratory chain on the one hand, necessary for ATP synthesis and the electrogenic Na^+/H^+ antiport trying to keep protons in the cell on the other hand. There are various models proposed to explain this apparent conundrum with regard to oxidative phosphorylation and antiporter activity for pH homeostasis. These models, and the state of evidence supporting them have recently been reviewed by Krulwich (1995). Extreme alkalophiles probably solve this dilemma by their very high concentration of respiratory-chain components in their cell membranes, that may be instrumental in a very rapid recycling of extruded protons and by maximizing productive, proton-transferring collisions between respiratory chain components and the ATPase. Membrane lipids may also play a variety of roles in these processes. Alkalophiles possess somewhat high membrane lipid/membrane protein ratios and their membranes have a fatty acid composition consistent with a very fluid membrane. Obligate alkalophiles fail to grow at neutral pH because their membranes become leaky.

If the extrusion of Na^+ is a prerequisite for pH homeostasis, then the operation of a multitude of Na^+-coupled solute porters (symporters) may provide the means for Na^+ reentry. In other words, symporters are part of the pH homeostasis mechanism and presumably act by recycling the Na^+.

Most solutes are actively transported by alkalophilic bacteria in symport with Na^+ and at least some of the solute porters cannot substitute Li^+ for Na^+ as can the Na^+/H^+ antiporter of the same organism. However, although some insight has been gained into the characteristics of coupled transport in alkalophiles, still relatively little is known about the cation and anion transport systems in alkalophiles, and, likewise, little is known about the few carbohydrate transport systems that are Na^+ independent and seem to depend on ATP (Krulwich and Guffanti 1989, Ikeda et al. 1994).

The motility of the extreme alkalophiles (as well as that of at least some moderately alkaline-tolerant marine bacteria) depends upon the presence of Na^+ and appears to be energized by an electrochemical gradient of Na^+. Thus far, the motility of the alkalophilic bacilli has appeared to be dependent exclusively upon Na^+ with neither substitution by Li^+ at high pH nor any energization by a ΔpH at near-neutral pH. Even facultative alkalophilic strains that grow over a broad pH range have shown this Na^+ specificity for both motility and Na^+-coupled solute symporters.

3.3.3 Acid Adaptation and Tolerance

Foster and Hall (1991) and Foster (1992) studied the so-called acid tolerance response (ATR) in enteric bacteria (such as Salmonellae) that face particularly severe pH stresses in the human intestinal tract. The ATR is an ability of acid-adapted cells to keep pH_i 0.5 to 0.9 units more alkaline than in unadapted cells. Although the exact mechanism of ATR has not been elucidated, evidence that the ATR system provides the mechanism(s) for augmenting pH_i homeostasis was found in that adaptive enhancement of pH_i requires synthesis of new proteins during adaptation to slightly stressed pH levels (the preshock ATR system). These new proteins then protect or minimize acid denaturation of internal proteins during severe pH values (at which the normal pH homeostasis mechanism would have collapsed), by maintaining the internal protective pH_i. Normal constitutive pH homeostasis does not depend on the synthesis of new proteins and functions even when protein synthesis is absent or blocked.

Therefore, a two-phase model was envisioned by Foster and Hall (1991), to explain how cells cope with low pH stress. The first phase of protection, preshock, occurs as the environmental pH approaches 5.8, when the cell will induce the ATR associated pH homeostasis system. The primary function of the preshock ATR system, then, is to maintain pH_i and, in so doing, to retain viability. During severe acid stress, this system will keep pH_i near 5.5 and minimize acid denaturation of internal proteins. The second phase of this protective model involves the acid shock proteins induced once the pH drops to between pH 5 and pH 3. Acid shock is a response distinct from ATR. A completely different set of proteins are induced during acid shock. This shift also induces several heat shock proteins, which are possibly important both in preventing acid denaturation and in refolding denatured proteins (Foster and Hall 1991).

Foster and Hall (1991) conclude that there are several lines of evidence that implicate the proton-translocating ATPase as an important component of acid tolerance. The proton-translocating ATPase is required in the cell for oxidative synthesis of ATP and for generating proton motive force under anaerobic conditions. The ATPase might serve as a proton pump to extrude protons during the ATR. They have also implicated the ferric uptake regulator in the regulation of the

ATR. Mutations in the ferric uptake regulator locus produce an acid-sensitive phenotype, deregulate the production of several ATR proteins, eliminate the expression of several acid-regulated genes and prevent the development of the inducible pH_i homeostasis mechanism.

By inhibiting protein synthesis after preshock adaptation but prior to acid shock, acid tolerance can effectively be abolished. However, once the post-acid-shock proteins have been synthesized, inhibiting protein synthesis has no effect on acid tolerance. Acid shock alone is not sufficient for acid tolerance. Both preshock and postshock systems must be induced for the cells to survive severe acid stress.

This stepwise process makes sense considering that in nature gradual transitions in pH are more likely than sharp severe changes. The first step keeps pH_i around 5.5 and in the second step proteins may minimize DNA damage and internal protein denaturation, both of which occur when the internal pH falls below pH 5.5 (Foster 1992). Although ATR specific homeostasis has been studied mostly in enteric bacteria, it is conceivable that other organisms, including those occurring naturally, possess this system.

3.3.3.1 Acid Tolerant Mutants

Some organisms are spontaneous acid tolerant mutants. Often, these organisms are auxotrophs, requiring one or more specific growth substances (e.g., various amino acids). Some mutants appear to increase their internal buffering capacity, in some cases probably by accumulating large quantities of intercellular citrate or isocitrate. Since the pK_i for these acids is 6.4, they could conceivably buffer the pH_i towards this value, preventing it from dropping below the critical pH 5.5 point vital for survival during external pH stress. The isolation of acid-tolerant mutants that possess an elevated internal buffering capacity also provides proof that acid-induced cell death is directly related to a lowered pH_i and not to external acid damage to cell surface components essential for cell viability.

Other mutants have a dramatically improved pH homeostasis capability below pH 4 where normal pH homeostasis breaks down. These mutants have, therefore, an enhanced ability to handle severe acid stress in the same range affected by the ATR (discussed above). The exact mechanism of this enhanced ability is not clear.

3.3.4 Summary

In summary, all bacteria use Na^+/H^+ antiporter systems to support pH homeostasis at moderately alkaline pH values. Neutralophilic organisms can substitute K^+ for Na^+, unlike the extreme alkalophiles who need Na^+ (or Li^+). Na^+ reentry occurs via solute uptake systems that are coupled to Na^+ uptake and via motility that is coupled to Na^+ and, in high sodium environments, some leaking may cause reentering of sodium into the cells.

Acid tolerance is more complex than initially perceived, with induction of acid tolerance being a two-stage process. The first stage (pre-acid shock), triggered at pH_o below 6.0, induces synthesis of the ATR specific pH homeostasis mechanism that augments pH_i when the internal pH falls below pH 4.0. The second stage (post-acid shock) triggered below pH 4.5, induces a different set

of proteins that by themselves will not afford protection against severe acid. However, the acid shock proteins are important for survival when coupled with the inducible homeostasis system. This stepwise process seems logical considering that in nature gradual transitions in pH are more likely than sharp and severe changes. The first step keeps pH_i around 5.5 and the second step proteins may minimize DNA damage and internal protein denaturation, both of which would occur if the internal pH falls below 5.5 (Foster 1992).

3.4 NUTRIENTS

Kjelleberg (1984) noted the diversity in the microbial responses during periods of nutrient limitation. Bacteria have many mechanisms that allow them to survive. Some species sporulate, while others form multicellular aggregates or fruiting bodies. Others undergo an evolution that results in a more starvation resistant and less metabolically active state. It is, however, essential that the cell sustain some level of endogenous metabolism in order to allow the cells to preserve some ATP (or other high energy compounds) as well as the proton motive force across the membrane (Siegele and Kolter 1992). As a result of low nutrient conditions, changes in the cell wall composition and its surface properties, in the shape and size of the cell as well as in the topology of the chromosome may occur, that, according to Siegele and Kolter (1992) give the cell some of the properties of spores.

3.4.1 Nutrient Transport Across Cell Walls

Many of the nutrients required by microbes cannot pass freely through the cell membrane because they move against a concentration gradient, are too large or too highly charged. Microbes have a number of transport systems to transport nutrients into their cells. This enables them to take up a wide variety of substrates for growth and metabolism. Different organisms may transport the same substrate by different mechanisms.

Passive diffusion involves the movement of small molecules across the cell membrane down a concentration gradient. Water and small molecules generally enter or leave the cell by passive diffusion but it is a slow process and the rate of diffusion of large molecules is too slow to be of use to the cell.

Facilitated diffusion involves membrane-bound carriers (permeases or transport proteins) that attach to substrates for which they have an affinity. These substrates are then carried down a concentration gradient to the other side of the membrane. The process does not require energy and is more rapid than passive diffusion, but limited by the number of available carriers, competition for sites by substances resembling substrates and subject to mutation effects in the carriers.

Active transport is the energy-requiring, carrier-mediated movement of molecules across the membrane against a concentration gradient. Sugars, amino acids and other organic molecules are transported by this mechanism. This process can move substances against large gradients, but is subject to availability of energy and carriers and is affected by mutation of the carriers.

Group translocation is the process during which a molecule undergoes chemical modification during its movement across the membrane to ensure that it cannot pass out of the cell. This process requires energy, but is more energy efficient than the active transport process. Group translocation is a common transport mechanism for many types of sugars as well as purines, pyrimidines and fatty acids. It is widespread amongst anaerobic and facultative anaerobic organisms in which energy yields are lower than in aerobic organisms (fermentation versus respiration).

3.4.2 Nutrient Limitation and Starvation Survival

The physiology of the starvation response was reviewed by Matin (1992), who examined the generation of energy for survival, the increased scavenging capabilities, the increased resistance and the regulation of starvation responses. Starvation-survival was described by Morita (1982, 1993) as the physiological state resulting from an insufficient amount of nutrients, especially energy, for increase in size and multiplication (growth) of microorganisms. Two reviews of the cellular aspects of starvation response such as the endogenous metabolism rate and storage of polymers in starvation survival were published by Kjelleberg et al. (1987) and Dawes (1976).

It has long been recognized that populations harvested from the stationary phase of growth are less fragile than those from the exponential phase (Postgate and Hunter 1962). Kolter (1992) showed that *E. coli*, which survived in the stationary phase for extended periods of time, demonstrated enhanced survival compared to those that had only been exposed to the onset of the stationary phase. A study by Kramer and Singleton (1992) indicated that starvation survival and recovery mechanisms in *Vibrio* sp. were linked to the physiological state at the onset of starvation. Bacteria subjected to a gradual downshift in nutrients recovered more rapidly than those that had reached a starvation condition suddenly.

A study by Stevenson (1978) indicated that bacteria may become dormant in aquatic systems, stressing that groundwater and seawater are far less nutritious than laboratory cultures. However, Novitsky and Morita (1977,1978) observed an initial 200-fold increase in cell number of a type of marine organism (psychrophilic marine vibrio (Ant-300)), when suspended at low cell density in natural or synthetic seawater. This phenomenon occurs in the presence or absence of organic compounds. The authors attribute the phenomenon to a survival strategy, because after long periods (70 weeks) more cells (15x) were still viable.

MacDonell and Hood (1982) investigated <0.2 μm bacteria recovered from estuarine waters. The bacteria, subjected to increasingly nutritious preparations, increased in size and growth rate. Their work suggested that smaller size and low growth rate represents a successful adjustment to poor nutrient conditions.

James et al. (1995) suggested that the observed changes in cell morphology during starvation of a surface colonizing *Acinetobacter* sp. were physiological adaptations that increased the likelihood of attachment and colonization during low nutrient conditions, and dispersion under high nutrient conditions.

Amy and Morita (1983a) investigated starvation-survival patterns of 16 open ocean bacteria. In addition to observing significant changes in shape and size of the bacteria, they also observed three different patterns of survival, all of which resulted in a rapid and linear response to glutamic acid after 8 months of starvation. All three patterns were similar in that a constant number of viable cells was achieved after an initial phase. The initial phase varied in how that final constant number was achieved, i.e., after an initial increase; after an initial decrease; or after an increase followed by a decrease.

For bacteria that do not undergo differentiation into different structures, there are several mechanisms available to survive starvation. New proteins can be synthesized which support the economical usage of a limited nutrient, or provide bacteria with a better ability to resist stress. Matin et al. (1989) reviewed starvation and survival in non-differentiating bacteria within the genetic framework. RNA and protein degradation are expected when cells are heat stressed or starved of a nutrient. In addition, nitrogen deficiency induces bacteria to increase the production of enzymes that capture nitrogen, the proteins that favour it and/or the ability to utilize more nitrogen-containing resources. Phosphorous-deprived bacteria create diversity in transport systems. If iron concentrations fall below 1 mM, bacteria synthesize high affinity iron chelators (siderophores). The primary response of carbon-starved organisms is to enhance the number of carbon-bearing substrates that can be utilized by increasing the concentrations of enzymes that perform that function and creating intermediary metabolic functions.

Nutrient deprivation studies by Hood et al. (1986) demonstrated that the total number of lipids and carbohydrates declined rapidly within the first seven days of starvation, as did poly-β-hydroxybutyrate. DNA and proteins declined at a more constant rate over thirty days. RNA declined little. Phospholipids declined more rapidly than neutral lipids, and five-carbon sugars decreased relative to six-carbon sugars, especially glucose. Although ribosomes showed no apparent structural change, the integrity of the cell wall and membrane diminished substantially. Guckert et al. (1986) noted statistically significant trends in the fatty acid profiles during starvation experiments. C*is*-monoenoic fatty acids declined in comparisons to the saturated fatty acids cyclopropyl derivatives of *cis*-monoenoic fatty acids and *trans*-monoenoic fatty acids, which increased. Hood et al. (1986) suggested that the molecules that were maintained (i.e., certain lipids and carbohydrates) likely provided the resources needed to prepare for dormancy to preserve the cell under nutrient starvation. Nelson and Parkinson (1978) observed similar results in a *Pseudomonas* sp., a *Bacillus* sp. and an *Arthrobacter* sp. The levels of endogenous substrates, such as carbohydrate and protein showed a rapid decrease within the initial 20 hours of starvation. This was followed by a more gradual decline over the remainder of the test period (10 days). A positive correlation was observed between the rate of endogenous metabolism and the survival rates during starvation (i.e., the lower the rate of metabolism, the greater the survival rate).

Exoprotease activity, which is associated with nutrient starvation was examined in two *Vibrio* species by Albertson et al. (1990). They found that starved cells showed more exoprotease activity than that of cells at the onset of starvation. Although the activity tapered off during the period of starvation, the level of activity was still considered significant at the end of the study (120 hours). The authors conclude that in order for exoproteases to be produced and/or released

extracellularly, protein synthesis was necessary during starvation. It was further determined that the exoprotease activity was conducted by different mechanisms in the two species studied.

Yet another survival technique was described by Wrangstadh et al. (1990). Exopolysaccharide (EPS) production, which is closely associated with the cell surface during growth of marine *Pseudomonas* sp. was induced, by starvation, to be more peripheral. Changes in viscosity of the EPS were also noted. Wrangstadh et al. (1990) proposed that during starvation, short-chain EPS is modified into longer-chain units by de novo protein synthesis.

Blom et al. (1992) subjected *E. coli* to low concentrations of nine chemicals considered to be "model micropollutants". Their results indicated that even at concentrations below that which inhibited microbial growth, stress proteins were induced. Some of the proteins had been previously identified in relation to heat shock or carbon starvation. However, others were specific to the particular micropollutant.

3.4.3 Summary

Amy (1997) summarizes starvation-survival characteristics to include nearly total metabolic arrest in some cell types, miniaturization of cells, changes in cellular densities, changes in macromolecular quantities, and a general increase in resistance to a number of stressors. Cell membrane transport mechanisms may be enhanced, and plasmid and chromosomal DNA may change in quantity and the ability to be expressed. Specific starvation-related proteins may be synthesized under conditions of nutrient deprivation, either as unregulated constitutive proteins or de novo synthetic products. It is, however, essential that the cells sustain some level of endogenous metabolism in order to allow the cells to preserve some ATP (or other high energy compounds) as well as the proton motive force across the membrane.

3.5 RADIATION

Ionizing radiation such as gamma rays cause water and other substances to ionize, and mutagenic effects are brought about indirectly through this ionization. The various forms of radiation that can do damage are discussed in Appendix B. The most potent chemical species formed by ionizing radiation are chemical free radicals, of which the most important is the hydroxyl radical $OH^{\cdot}$. Free radicals react with, damage and may inactivate DNA molecules microbial (and other) cells.

3.5.1 Radiation Protection and Repair Mechanisms

Microbes use a number of structures or strategies as protection against radiation damage. These structures and mechanisms include spores, the cell wall structure, pigments, heat shock proteins and protection by other molecules. If these strategies fail and radiation damage of DNA occurs, a complex cellular mechanism, called the SOS regulatory system, is activated which initiates a number of DNA repair processes in microbes. In the SOS system, DNA damage serves as a distress signal to the cell, resulting in the coordinate de-repression (= activation) of a number of cellular functions involved in DNA repair. The SOS system is normally repressed by a protein called the LexA protein, but *LexA* is inactivated by *RecA*, a protease that is activated as a result

of DNA damage. In the SOS system, DNA repair occurs in the absence of template instruction, and many kinds of mutations can arise as a result of faulty repair of damage induced in DNA. But once the DNA has been repaired, the SOS system is switched off and further mutagenesis ceases. Not all DNA repair occurs in the absence of template instruction. Cells generally have a DNA repair system which requires template instruction and leads to proper DNA repair. This system works apparently most of the time but is not sufficient to repair the large amounts of damage done at high radiation doses (Brock and Madigan 1991). Then the SOS repair system is activated.

The studies by Arrage et al. (1993a,b) showed that the majority of UV-resistant bacterial subsurface isolates were Gram-positive. It has been proposed that cell wall components may help deflect near-UV photons, resulting in a lower dose actually absorbed by target molecules in the cell. Therefore, it is possible that the thicker cell walls present in Gram-positive bacteria screen a larger portion of UV light and that the amount of UV light reaching cellular DNA is decreased.

This section discusses first the unique repair mechanisms of the highly radiation-resistant organism *Deinococcus radiodurans*, a non-sporulating organism. These methods may include making efficient use of redundant genetic information in a manner that other organisms do not, the possession of chromosomes existing in pairs that are aligned relative to one another that can serve as a template for DNA repair, and synthesis of an induced protein that stops DNA degradation during repair. This is followed by a discussion of the various strategies (such as pigmentation) for protection against radiation damage that have been observed in a variety of microbes other than *Deinococcus radiodurans*.

3.5.2 *Deinococcus radiodurans*

Radiation resistance has probably been most widely studied with the microorganism *Deinococcus radiodurans* because this bacterium (formerly *Micrococcus radiodurans*) is unique in that it is extremely resistant to the lethal and mutagenic effects of ionizing radiation and numerous other agents that damage DNA. Full survival of *D. radiodurans* has been reported at ionizing radiation exposures ranging from 5-15 kGy, depending on the bacterial culture conditions. It has now been established that this bacterium does indeed sustain the extreme level of DNA damage expected at such high radiation exposures, but that it has an extremely efficient DNA repair mechanism (Minton 1994, 1996; Daly and Minton 1995).

Deinococcus radiodurans contains four chromosomes per cell during the stationary phase and up to ten chromosomes per cell during the exponential phase. However, high chromosome multiplicity is not uncommon in prokaryotes and is not necessarily associated with extreme tolerance to ionizing radiation or other forms of DNA damage. Following ionizing radiation, *D. radiodurans* can repair more than 100 double strand breaks per chromosome without lethality or mutagenesis in as little as 12 to 24 hours, while other organisms are unable to repair more than a few ionizing radiation-induced double strand breaks per chromosome. *Deinococcus radiodurans*' powerful repair system can assemble intact chromosomes from the hundreds of fragments remaining after a 10 kGy dose. Clearly, *D. radiodurans* must use its redundant

genetic information in a manner that other organisms do not. In *D. radiodurans* chromosomes may exist in pairs that are aligned relative to one another by socalled Holliday junctions and, therefore, a chromosomal fragment in *D. radiodurans* can always find an intact homologous neighbour to serve as repair template. DNA degradation occurs at DNA breaks during normal non-severe DNA damage repair in cells. It is possible that following DNA damage, during the repair phase, synthesis of an induced protein occurs in *D. radiodurans* that is required to stop DNA degradation at the appropriate time during repair. (Minton 1994, 1996; Daly and Minton 1995).

This extreme radiation resistance of *D. radiodurans* and the other members of the Deinococcaceae cannot have occurred as an adaptation to ionizing radiation because there is no apparent selective advantage to being ionizing radiation resistant on earth because such high natural radiation fluxes simply do not occur naturally, not even in the early days of the earths formation (Daly and Minton 1995). The highest reported absorbed dose rate in a natural environment is only about 175 mGy/y (measured in Th rich monazite sands in Brazil (Minton 1996)). Desiccation of microorganisms is known to induce DNA strand break damage. Desiccation experiments have shown that the number of double strand breaks in *D. radiodurans* was dependent on the duration of desiccation, and that the number of breaks increased by periodic partial rehydration. About 60 double strand breaks could be induced through desiccation in *D. radiodurans*. Therefore, the radiation resistance of *D. radiodurans* may be a serendipitous result of its ability to repair its DNA after severe dehydration (Minton 1996, Mattimore and Battista 1996). This adaptation to dehydration is quite different from spore-forming organisms such as *Bacillus subtilis* in which a multiplicity of strategies are employed to minimize DNA damage. Thus, the efficient repair system in *D. radiodurans* might be best thought of as a mechanism to heal DNA fragmentation, whatever its cause.

3.5.3 Other Protective Strategies Against Radiation

Sensitivity to radiation varies depending on culture conditions. The nature of protection could be some metabolic byproduct present in the cell that acts as an internal protective agent. Post-irradiation culture conditions also have a large influence on the radiation sensitivity and post-irradiation treatment can have quite different effects on survival and mutation. For instance, the addition of certain factors (such as glutamic acid, guanine and uracil) to the medium seems to promote increased survival and a decrease in the rates of mutation (De Serres 1961). A study by Krabbenhoft et al. (1967) provided evidence that the growth medium caused cellular biochemical alterations that afforded protection against radiation damage, whereas the study by Peak et al. (1995) confirmed the protection against neutron damage by ethanol in phage plasmid DNA. Dewey (1963) studied the effect of adding ascorbic acid during irradiation. Results showed that it is a powerful chemical protector of *Serratia marcescens* under anaerobic conditions.

Reeve et al. (1990) studied natural lipids from *Deinococcus radiodurans* to determine their potential function as UV screening agents. Isolated lipids from *Deinococcus radiodurans* were reconstituted into dioleoyl phosphatidyl choline (DOPC) liposomes (vesicles) and assayed for their ability to protect cells of *E. coli* against killing by UV light. To rule out the possibility that UV protection might be due to liposome-dependent light scattering, they showed that DOPC

liposomes without lipids had little effect on the sensitivity of *E. coli*. In contrast, the incorporation of various lipids from *Deinococcus radiodurans* into DOPC liposomes was found in many cases to provide protection against UV killing of *E. coli*. By far the most protection was afforded by the lipid Vitamin MK8. While it is possible that combinations of lipids might act synergistically to provide greater UV protection, this is unlikely because total *D. radiodurans* lipids in DOPC liposomes gave considerably less UV protection than Vitamin MK8. Although they conclude that Vitamin K is not likely to account for the extraordinarily high degree of UV resistance of *D. radiodurans*, Vitamin K does show characteristics worthy of its consideration as a UV screening agent.

3.5.4 Pigments

The literature reviewed suggests that pigments play a role in radiation damage resistance. However, the exact nature of that role has not yet been resolved. Mathews and Krinsky (1965) studied the relationship between carotenoid pigments and resistance to radiation in non-photosynthetic bacteria because extremely radiation-resistant microorganisms are highly pigmented which could indicate that carotenoid pigments play a role in protection against radiation damage. However, their experimental results suggested that the protective action of carotenoid pigments is effective only when the bacteria are exposed to visible light and a photosensitizer, whether exogenous or endogenous, and that this protective mechanism is ineffective at shorter wavelengths They concluded that the effects of carotenoid pigments in radiation damage is limited to protecting cells against the lethal effects of visible light and is without effect against ionizing radiation. Krabbenhoft at al. (1967) unsuccessfully attempted to correlate the presence of carotenoid type pigments in *Micrococcus* (now *Deinococcus*) *radiodurans* with radiation resistance. Gascón et al. (1995) concluded that the possession of pigments did not seem to have an important effect on the sensitivity of *Rhodobacter sphaeroides* to UV radiation.

In contrast, in their studies with bacterial isolates from the USDOE Subsurface Microbial Culture Collection, subjected to UV and gamma irradiation, Arrage et al. (1993a,b) found that aerobic Gram-positive pigmented organisms were significantly more resistant than microaerophilic Gram-negative organisms to both radiation treatments. They conclude that pigments have been implicated in near-UV radiation and free radical protection, and that the presence of cartenoids may protect against free-radical-induced cell membrane damage.

Moseley (1963) studied the variation in X-ray resistance of *Micrococcus* (now *Deinococcus*) *radiodurans* and some of its less pigmented mutants. Several spontaneous mutants which had less pigment than the wild type were isolated from *M. radiodurans*. One which had no pigment was obtained by X-irradiation. The resistance to X-rays of these strains was quite different from that of the wild type. The shoulder of the survival curve became smaller with the decrease of pigment content of the cells, and disappeared completely for the mutant with no pigment. This study concluded that it appears that carotenoid pigment or metabolic reactions leading to its formation contribute considerably to the resistance of *M. radiodurans* to X-rays although even the non-pigmented mutant was very resistant to X-irradiation compared with other vegetative bacteria.

Often bacteria isolated from extreme environments do contain pigments. An example are the halophiles. Evaporation pools for the commercial production of salt from the sea (marine salterns) are often red in colour due to the carotenoid pigment of extreme halophilic organisms (Larsen 1981). However, the above review of literature showed a non-consensus about the role of pigments in radiation resistance in general.

3.5.6 Spores and Radiation

Farkas (1994) studied the tolerance of spores to ionizing radiation and the mechanisms of inactivation, injury and repair. Notwithstanding the occurrence of highly radiation-resistant specific non-sporing bacteria (such as *D. radiodurans*), spores are in general considerably more resistant to radiation (by a factor of about 5 to 15) than are vegetative cells of the same strain. This difference is, however, much smaller than the difference in heat resistance between vegetative cells and spores. For instance, for the genus *Bacillus* there is a difference of a factor 1000 between the heat resistance of spores of the most sensitive and the most resistant species, whereas such spores do not differ more than about a factor 4 in radiation resistance. There is also considerable difference between different strains of some species.

Radiation inactivation of both spores and vegetative cells appears to be related primarily to radiation damage to DNA, particularly the formation of single- and double-strand breaks. There is no direct correlation between the radiation resistance and heat resistance of bacterial spores, contrary to what is observed for vegetative cells. It would appear that spore DNA is not intrinsically radiation resistant, but has resistance imposed on it by compositional and structural factors within the spore, i.e., the relatively dehydrated state of the spore core and the specific conformation of the spore DNA may be responsible for its resistance.

In spores, damage to DNA may be a major cause of radiation inactivation but other components may also be involved in radiation damage. Because of the cryptobiotic nature of dormant spores, it is generally considered that metabolic DNA repair does not occur to any significant extent in them. Repair enzymes, however, may be present in an inactive state in dormant spores and may be activated during germination. It was observed in the spores of a radiation-resistant strain of *Clostridium botulinum* that rejoining of DNA single-strand breaks occurred under anaerobic conditions during or immediately after irradiation. These observations were attributed to the effect of the polynucleotide ligase enzyme, which does not require a substrate other than the damaged DNA. This type of spore repair can be inhibited by various chelators and is almost completely eliminated by the presence of oxygen. Recovery of spores of *Cl. botulinum* in the exponential portion of the survival curve seemed to require excision-resynthesis DNA repair after germination, and may depend on the synthesis of new enzymes (Farkas 1994).

The environmental factors, such as temperature or sporulation medium, prevailing during spore formation, may have an effect on radiation resistance. During irradiation, the radiation resistance of bacterial spores is much less sensitive to environmental factors than is the case for vegetative cells. Nevertheless, the effects of temperature and freezing may be considerable. For instance, the maximum kill of irradiated spores has often been observed to occur at 0°C and below freezing, survival increases because ice traps radiation-induced harmful free radicals. At high,

but sublethal temperatures (50-70°C), radiation resistance may be higher than at or below room temperature, probably due to enhanced annealing of free radicals at higher temperatures. The effect of pH on radiation-sensitive spores seems to increase when temperature is reduced to the freezing point or below. Radiation resistance of spores increases with decreasing water activity (a_W), but the effect of a_W on radiation resistance is less than the effect of a_W on heat resistance. Addition of radio-protective substances to the culture environment affects survival in a variety of ways (Farkas 1994).

In addition to inactivation of spores by damaging DNA, radiation can have other effects, such as the stimulation of spore germination at sublethal doses of ionizing radiation. As in heat-induced injury, radiation injury is expressed as an increase in the sensitivity of some spores to some secondary stress. For instance, bacterial spores that survived irradiation showed a decrease in heat resistance. Pre-irradiation before heat treatment induces more drastic changes in spores than the reverse order of the same treatments, and this radiation-induced heat sensitivity of spores persists for a holding period of at least several weeks at room temperature in aqueous suspensions (Shamsuzzaman et al. 1990). Simultaneous application of heat and radiation may cause synergistic destruction of microorganisms. Radiation-induced breaks in, or decarboxylation of, cortex peptidoglycan may be responsible for the heat-sensitization of bacterial spores by ionizing radiation, including the weakening of osmoregulatory or core-dehydrating mechanisms. When gamma-irradiated spores of *Cl. Perfringens* were heated in the presence of increasing concentrations of glycerol and sucrose, the heat sensitivity induced by irradiation decreased progressively (Farkas 1994).

3.5.7 Summary

Microbes have a number of defense mechanisms against radiation damage, including cell wall structure (gram positive cell walls offer more protection), pigments (exact role not resolved), spores (not as effective as for heat damage), heat-shock proteins and protection by other molecules. Cells generally have a DNA repair system that requires template instruction. This repair system, however, is not effective for severe radiation damage, when the SOS repair system is activated. The SOS system involves DNA repair in the absence of template instruction, which increases the possibility of mutations.

Deinococcus radiodurans is extremely resistant to the lethal and mutagenic effects of ionizing radiation (and other agents that cause damage to DNA). This organism has a highly effective DNA repair system and uses its genetic information in a unique manner. Its resistance to radiation damage may be a serendipitous result of its ability to repair DNA after severe dehydration.

3.6 RESISTANT STRUCTURES

The vegetative cells of many bacteria can undergo some degree of differentiation into structurally and physiologically distinct forms, classified variously as endospores, exospores, myxospores, cysts, akinetes and resting stages. Although very diverse, common features of these cell forms are that they possess thickened, structurally characteristic extracellular cell wall layers and are

desiccation resistant. These forms develop, however, during the time the parent vegetative cell is growing in an aqueous environment. Therefore, desiccation and drying are not cues for the development of spores and other structures that happen to be resistant.

3.6.1 Spores

A spore is very different from a vegetative cell and has a much more complex structure in that it has many layers. The outermost layer is the exosporium, a thin, delicate covering made of protein. Within this is the spore coat, composed of layers of spore-specific proteins. Below the spore coat is the cortex, which consists of loosely cross-linked peptidoglycan, and inside the cortex is the core or spore protoplast, which contains the usual cell wall, cytoplasmic membrane, cytoplasm, nucleoid etc. Thus the spore differs structurally from the vegetative cell primarily in the structures outside the cell wall. Dipicolinic acid is found in the core of all endospores, but not in vegetative cells. Spores are also high in calcium ions, most of which are complexed with dipicolinic acid. This complex represents about 10% of the dry weight of the spore. The core of a mature endospore contains only 10-30% of the water content of the vegetative cell, and has the consistency of a gel. The pH of the core cytoplasm is about one unit lower than that of the vegetative cell and contains high levels of core-specific proteins, i.e., small acid-soluble spore proteins (SASPs) (Madigan et al. 2000). These proteins are made in the forespore late in sporulation in amounts sufficient to cover the spore chromosome completely but are degraded in the first minutes of spore germination (i.e., the SASPs function as carbon and energy source during germination). Fairhead et al. (1994) found that binding of α, β-type SASP to spore DNA was a significant factor in spore resistance to freeze drying and concluded that the presence of high levels of these unique DNA-binding proteins in spores of *Bacillus subtilis* as well as *Clostridium* species likely plays a major role in the extreme resistance of such spores to a variety of harsh conditions and thus to their long-term survival both in the laboratory and in natural environments. In contrast, Farkas (1994) concluded that neither α, β-type nor γ-type SASP appear to be involved in gamma-radiation resistance in spores, although they are involved in protection against drying and heat.

3.6.2 Cell Shapes and Other Strategies

The shapes and forms of bacterial colonies may play an important role in determining the extent to which cells evade damage from drying, oxygen and other perturbants. For a given volume, a sphere presents the minimum surface area to the vapour phase, thus retarding the net rate of evaporation. Also, the water present in the colony, usually the interstitial component present in the extracellular wall layers, reduces the net diffusion of gases (oxygen) by 4 orders of magnitude. In view of the destructive effects of oxygen during desiccation, the genes involved in oxygen-scavenging mechanisms are likely to be important in the tolerance of bacterial cells to air drying.

Bacteria of the genus *Arthrobacter* are extremely common in soils and often constitute more than one half of the total bacterial populations. The low minimum growth rate, the accumulation of a large amount of reserve material, the rapid and drastic decrease in endogenous metabolism and the long survival times during starvation, the high resistance to desiccation in soil and the small

spheroidal shape of cells under conditions of nutrient depletion might be the basis for the ecological prominence of *Arthrobacter* in arid soils (Cacciari and Lippi 1986). Chen and Alexander (1973) studied survival of *Arthrobacter* as a function of a_w and found that the minimum a_w for growth was 0.940. Mugnier and Jung (1985) studied the survival of *Arthrobacter* trapped in biopolymer gels as a function of a_w. The number of viable cells remained constant for storage periods of > 3 years at 28 C at a_w of 0.069. Most bacteria cannot grow below water potential < 15 bar (a_w <0.99) but according to Biederbeck et al. (1977), growth of *Arthrobacter oxidans* could take place at water potentials as low as -1300 bar (a_w <0.4). Desiccation tolerance seems to be an important factor in the numerical predominance of *Arthrobacter* species in soils. In addition to its ability to synthesize large amounts of indigenous glycogen-like substances, *Arthrobacter* has the ability to produce extracellular polysaccharides (EPS).

3.6.3 Glasses

Bacterial vegetative cells may accumulate significant amounts of a diverse collection of solutes. Some of these solutes (succrose, trehalose) can behave as aqueous glasses, some of which are stable at 90°C. The thermal transition characteristic of glasses is such that they could provide protection between 0°K and 90°C. EPS may also have these properties, but whether glasses could form in complex biological systems is unclear. In the glassy state, reactions are slowed to periods that are more than sufficient for the times some bacteria may remain viable in the desiccated state. All water in a bacterial glass would be immobilized and the properties of this extremely crowded cytoplasmic glass would appear to be consistent with the requirements for long-term stability. However, the existence of bacterial glasses has not been confirmed yet.

It has also been suggested that spores consist primarily of glasses. Sapru and Labuza (1993) used polymer glass-transition theory to gain information about a possible general mechanism to explain the high heat resistance of bacterial spores. In a glassy state the configuration of vital macromolecules and supramolecular assemblies in the spore protoplast would change extremely slowly when heated. The temperature dependence for heat inactivation rates above the glass-transition temperature was shown to be free-volume dependent and described by the kinetics commonly observed for glassy polymers. Glass-transition temperatures for various spores, predicted by nonlinear regression analysis of their heat inactivation rates at different temperatures, increased with increasing heat resistance as expected.

3.6.4 Summary

The vegetative cells of many bacteria can undergo some degree of differentiation into structurally and physiologically distinct forms which provide increased tolerance to adverse conditions. A common feature of these growth forms is that they possess thickened, structurally characteristic extracellular cell wall layers. The shapes and forms of bacterial colonies may also play an important role in determining the extent to which cells evade damage from perturbants. Compatible solute production and EPS may possible induce glassy states in bacteria, which may afford protection against high temperature.

3.7 MUTATION

Genetic recombination is the process by which genetic elements contained in two separate genomes are brought together in one unit; through this mechanism new genotypes can arise even in the absence of mutation. Mutation is the inherited change in the base sequence of nucleic acid (DNA) comprising the genome of an organism. A strain carrying such a change is called a mutant. A mutant will by definition differ from its parental strain in genotype, the precise sequence of nucleotides in DNA. But in addition, the visible properties of the mutant, its phenotype, may also be altered relative to the parent strain, usually the wild type strain. Mutant derivatives can be obtained from either wild type strains or from a strain derived from the wild type, for example, another mutant (Brock and Madigan 1991). In most cases the changes in the base sequence of the DNA lead to changes in the organism that are harmful but occasionally beneficial changes occur.

Mutations can either be spontaneous or induced. Spontaneous mutations in any gene occur about once in every 10^6 to 10^{10} replications. There are wide variations in the natural rates at which various types of mutations occur. *Point mutations* are changes of single bases that may or may not lead to changes in protein because of the fact that the genetic code is degenerate, meaning that there is no one-to-one correspondence between word and code (there are several codes for one amino acid). Point mutations are usually reversible through further mutations. A *silent mutation* is a point mutation that does not lead to a change in the protein. A *missence mutation* results in a change in the protein, which may or may not result in loss of activity of the protein. In a *nonsense mutation*, the point mutation causes the formation of a stop codon, which causes premature termination of the translation, leading to an incomplete protein, that is almost certainly not functional.

Deletions are due to elimination of portions of the DNA of a gene, either small or large. If the deleted segment is large, restoration can only occur through genetic recombination. *Insertions* occur when a new base or several bases are added to the DNA of the gene. Generally, insertions do not occur by simple copy errors as do deletions, but arise from mistakes that occur during genetic recombination. The genetic code is read from one end in consecutive blocks of three bases and any deletion or insertion of a base results in a reading-frame shift, which causes the translation of the gene to be completely upset. Partial restoration resulting in active proteins is possible through insertion of another base near the one deleted. Nonsense mutations near the beginning of translation (the 5' end) will have much more severe effects than nonsense mutations farther down the chain. This is called polarity. Because of polarity, nonsense mutations at the 3' end will generally have no effect on genes translated at the 5' end. Many but not all mutations are reversible. *Suppressor mutations* are new mutations that suppress the effect of the original mutation and restore the original phenotype. *Genetic transformation* is a process by which free DNA is incorporated into a recipient (competent) cell and brings about genetic change (Brock and Madigan 1991).

Rates of mutations vary widely, but may be significantly increased by the use of mutagenic treatments, such as chemicals, UV radiation and ionizing radiation. Many kinds of mutations arise as a result of faulty repair of damage induced in DNA by mutagens. A complex cellular

mechanism, the SOS regulatory system, is activated as a result of DNA damage. This initiates a number of DNA repair processes, but since they occur in the absence of template instruction, many errors and hence mutations may occur (Brock and Madigan 1991).

Traditional assumptions are that spontaneous mutations arise randomly, and multiple mutations are the result of completely independent events. Hall (1988) questioned this, because it was found that spontaneous double mutants occurred orders of magnitude more frequently than expected on the basis of independent mutations. Therefore, mutation rates may be highly variable and subject, like other cellular processes, to modulation by normally encountered environmental factors. There seems to be an entire class of mutations that occur only when they are advantageous to the cell. Cairns et al. (1988) also discussed the question of random versus directed mutation and described some experiments suggesting that cells may have mechanisms for choosing which mutations will occur (i.e., directed mutation under the control of genes). They suggested that both may be occurring in the same strain. For instance, the work by Hall (1977) showed that *E. coli* (lacking lactose fermentation) had to mutate to acquire lactose fermentation. Many mutations occurred during growth in yeast extract or M9 glycerol prior to plating on minimal lactose plates, but some happened on the plates in the presence of lactose, which put pressure on the cells to become lactose fermentation positive (*lac+*). Hall (1977) proved that the accumulation of late *lac+* mutants occurred only in the presence of lactose.

This *E. coli* experiment suggested that populations of bacteria, in stationary phase, have some way of producing (or selectively retaining) only the most appropriate mutations in response to selection. It may be due to the activation of cryptic genes in *E. coli*. Cryptic genes are genes that are present but not readily expressed. Bacteria apparently have an extensive armoury of such cryptic genes that can be called upon for the metabolism of unusual substrates. The mechanism of activation varies. In some cases it occurs simply by the movement of an insertion sequence into a position upstream of the cryptic gene, but in others it may require several changes in base sequence (each with perhaps a frequency of less than 10^{-8}). It is difficult to imagine how bacteria are able to solve complex problems like these - and do so without at the same time accumulating a large number of neutral or deleterious mutations, unless they have access to some reversible process of trial and error.

3.7.1 Summary

Mutation is the inherited change in the base sequence of DNA, and may be spontaneous or induced, often as a result of faulty DNA repair. A mutant differs by definition from its parent in genotype but may or may not differ in phenotype. In most cases mutations lead to changes in the organism that are harmful but occasionally beneficial changes occur. It has been suggested that bacteria in stationary phase may be able to retain only the advantageous mutations, as a result of the activation of cryptic genes.

3.8 EXOPOLYSACCHARIDES (EPS) AND BIOFILMS

In environments with very dilute nutrient concentrations (such as most deep subsurface environments), the local enrichment of nutrients on adsorptive surfaces increases their microbial utilization. Microbes preferentially grow on these surfaces in biofilms which provides them with

a better chance of surviving the nutrient-poor conditions in that environment. Bacteria growing in a wide variety of environments have been shown to be surrounded by a fibrous, anionic, exopolysaccharide (EPS) matrix which acts as an ion-exchange resin, attracting and concentrating charged nutrients and organics. This sessile biofilm mode of growth may protect adherent cells from environmental toxins.

Many bacteria respond to desiccation by channeling energy and nutrients into the production and secretion of copious amounts of EPS that have a high viscosity and tend to be hygroscopic, often contain more water than the bulk environment and may decrease the degree of water loss from the cells. It seems that the cryoprotective effect of saccharides is a general characteristic and not limited to mono- or disaccharides and that carbohydrates in general and EPS in particular may function as very efficient, protective agents if they are appropriately attached to the cell walls or membranes. Functions of EPS in addition to protection against desiccation may include anchorage to the substrate, protection against phagocytic predation, masking of antibody recognition, prevention of lysis by other bacteria and viruses and protection against oxygen damage during desiccation.

The synthesis of EPS in bacteria is not only complex but requires the coordinated expression of sets of genes that respond to changes in the water potential of the cell and its environment. It seems likely that EPS synthesis represents a focal point of the ability of some bacteria to express desiccation tolerance. Potts (1994) provides a set of essential features of a protective extracellular biopolymer, which include properties such as high water retention, complex structure resulting in a poor substrate for utilization by competitors, toxic to prevent grazing by eukaryotes, and no interference with cell functions and other activities.

3.8.1 Summary

In dilute environment, bacteria may attach to surfaces where they survive better in biofilms. Bacteria growing in a wide variety of environments are often surrounded by a fibrous EPS matrix. Functions of EPS include concentrating nutrients and organics, protection against desiccation, anchorage to the substrate, protection against phagocytic predation, masking of antibody recognition, prevention of lysis by other bacteria and viruses and protection against oxygen damage during desiccation.

3.9 CROSS-PROTECTION RESULTING FROM ADAPTIVE STRATEGIES

This chapter has discussed the microbial capabilities and adaptive strategies employed by microorganisms as a response to a variety of (extreme) individual environmental stresses. However, it is often the case that several stresses occur simultaneously (or consecutively). It is apparent from the literature that some of the adaptation and survival strategies employed by microorganisms may have synergetic protection against multiple stress factors. An important example is the observation that production of heat shock proteins as a result of heat shock appears to provide protection against radiation stresses (Mitchel and Morrison 1982a,b, Fujikawa and Ohta 1994, Lage and Menezes 1994). Details of cross-protection occurrences relevant to this review and the Yucca Mountain environment are discussed in Chapter 5 (Section 5.7).

4. THE YUCCA MOUNTAIN ENVIRONMENT

The Yucca Mountain (YM) environment and the potential perturbations that may take place as a result of the construction and operation of a high level radioactive waste repository are the subject of a large number of reports (e.g., Hardin 1998, Wilder 1996, Wilder 1993). This chapter is limited to a discussion of the particulars of the YM environment and how it is expected to change over time, as they pertain to the potential for microbial occurrence, activity and survival in a YM repository.

The Topopah Spring Tuff geological unit of YM has been selected for appraisal as a potential nuclear waste repository site. This horizon is in the unsaturated or vadose zone, about 300 m below the surface and at least 200 m above the water table. This horizon, consisting of a welded devitrified ash flow tuff, is porous and fractured and the pores are partially filled with water. It is estimated that the region is >90% saturated. The remaining pore space is filled with gas that has essentially an atmospheric composition, but is perhaps somewhat richer in CO_2. The water is believed to be a $NaHCO_3$ groundwater of near drinking water quality with a near-neutral pH. Since this horizon is above the water table and porous, even under natural conditions intermittent periods of wetting and drying as a result of surface water infiltration (precipitation) are expected. In addition, the waste packages to be emplaced within such a repository will produce heat from the decay of radionuclides in the waste and this heat is expected to drive away a substantial amount of water in parts of the YM horizon, which may then condense in locations further away from the heat source. The evaporating water is expected to leave evaporite deposits closer to the near field and condense in locations further away from the heat source. Above the repository horizon such water may be able to re-circulate, increasing the evaporite deposit. On rewetting, the evaporites may dissolve to form aqueous solutions of substantially higher ionic strength. These intermittent wetting and drying periods and the redistribution of moisture as a result of heat will probably result in considerable variations in the salt content of the pore water at the YM site, and this could, therefore, possibly exert a fluctuating osmotic stress on microorganisms at these locations.

Although the geological horizon selected as a potential site for a high level radioactive waste repository is described as a single unit, it is by no means physically or chemically homogeneous. The rock itself is a welded tuff, which might at the resolution of models be considered homogeneous, but vugs (openings caused by expanding gases during the original formation of the rock), fractures, and fracture fillings give it a heterogeneous character. In addition, the physical openings of the repository and the chemical and physical properties of the materials that would be selected add an additional degree of heterogeneity. The construction and operation of a nuclear waste repository in this horizon will result in a number of physical changes to the environment that will have considerable consequences for the occurrence, survival and growth of microorganisms in this region.

Projections of temperature changes as a result of the emplaced waste packages are very uncertain at present because the precise thermal loading conditions in a future repository have not been defined. However, temperatures in the near field are expected to initially increase, over a period of decades, to as much as 200°C, with subsequent cooling after about a 200 year period as

radioactive decay progresses. For all loading conditions that appear physically possible at present, the temperature in the vicinity of the waste packages significantly exceeds 100°C for more than 100 years.

Temperature changes are expected to modify the chemistry of water in a repository environment over time, due to the dissolution and precipitation of solid phases in both the rock and introduced materials, and computer modelling codes are available to evaluate many long-term abiotic geochemical reactions. Temperature changes and high temperatures will also affect the microbial population naturally present in the rock and/or introduced with repository construction and materials emplacement. The present understanding of microbial effects in a repository is not complete and a better understanding of expected biological changes and reactions as a result of temperature is crucial in order to develop a more complete and satisfactory scenario of the biological processes that may affect the performance of the repository.

The most extreme gradients in pH and chemical composition are expected to be due to repository components, both the mechanical stabilization and transportation components as well as the waste packages themselves. As discussed above the chemical properties will vary with time. In addition, construction materials will vary in physical properties such as porosity, permeability and a_w through time.

Depending on the design of the waste package, i.e. thin-walled or thicker-walled, and depending on the age of the waste, it is possible that a certain amount of radiation will emanate from the waste packages emplaced in a YM repository. If the waste packages are designed such that they are not self-shielding, radiation effects will occur but they will be felt only close to the packages because of the efficient shielding of radiation by potential surrounding materials such as backfill, concrete and rock. Therefore, any radiation effects should occur only in the near field environment of a YM repository containing non-self-shielding containers.

Microbes have been found in freshly excavated tunnel rock from YM (Amy et al. 1992, Haldeman and Amy 1993). These microbes must have been present in the rock before excavation because they were found throughout the rock samples and not in the surface layers only. Communities that have not expressed themselves locally have the potential of expressing themselves if conditions change to support their growth. For example, although cryptoendolithic communities would not be found in the YM subsurface (they rely on photosynthesis), they could possibly form in the YM tunnel walls over time during the operational phase of the repository (artificial light, drying conditions as a result of tunnel ventilation) because generally, porous rock with a certain minimum density can be inhabited by cryptoendoliths.

The factors of most importance to microbial activity at YM are water, temperature, pH, nutrition and radiation, and these will be discussed in relation to prevailing and expected conditions at YM in this chapter.

4.1 WATER AVAILABILITY

Water is essential to microbial activity and it is generally agreed that if water is not available, microbial activity will not occur. Water will be available at YM intermittently and it is available

to some extent in the rock. Although rock saturation is routinely measured in rocks, other factors such as water potential and water activity more appropriately describe the availability of water to microorganisms. However, the present YM environment is not characterized in this manner and the degree to which it will change over time is not presently known. Therefore, the following sections describe the relationship between relative humidity, water potential and water activity, water retention by porous materials and methods for in situ measurement of water potential.

4.1.1 Relationship Between Relative Humidity, Water Potential and Water Activity

Water in an unsaturated environment and in living cells is normally subject to certain forces that lower its potential energy relative to free water in the reference state. Since pure, free water is usually assigned a water potential of zero, the potential energy of water in unsaturated systems is always negative. Water flows spontaneously from high to low potentials and the availability of water for physiological processes decreases as the potential is lowered. The following is a summary of the theory and measurement of water potential as explained in the articles by Papendick and Campbell (1980), Brown (1976,1990) and Potts (1994).

The total water potential (ψ) can be expressed as a sum of components identifiable with the forces that retain, or act on, the water and affect its energy state:

$$\psi = \psi_\pi + \psi_m + \psi_g - \psi_p + \psi_\Omega \qquad [4.1]$$

Where :

- ψ_π = the osmotic potential due to solutes in the water (always negative)
- ψ_m = the matric potential which includes both adsorption and capillary effects of the solid phase (always negative)
- ψ_g = the gravitational potential, proportional to elevation differences from the reference (negative or positive, depending on the reference level)
- ψ_p = the pressure potential resulting from external gas or hydraulic pressure applied to the water (negative or positive, depending on the reference pressure)
- ψ_Ω = the overburden potential caused by weight from overlying matter on water present in a nonrigid porous body (always positive)

In most soil systems, matric and osmotic components contribute more significantly to the water potential than the others and exert a greater effect on water flow and availability for physiological processes. The matric potential is the largest component in most unsaturated soils and the osmotic potential is significant in saline soils. Differences in water potential across small distances such as the thickness of a cell wall can exist only if resistance to flow is very high. The water potential of a microbial cell in soil is, therefore, likely to be in near equilibrium with that of its immediate environment, even though components of the water potential may differ substantially over short distances.

Water potential can be expressed in thermodynamic terms:

$$\psi = (\mu_w - \mu_w^\circ)/V_w \qquad [4.2]$$

in which

μ_w = the chemical potential of the water in a particular system
μ_w° = the chemical potential of pure free water (μ_w°) at the same temperature
V_w = the partial molal volume of water (1.8×10^{-5} m^3/mole at 4°C)

The thermodynamic expressions relating to water potential for equilibrium conditions between the liquid and vapour phases and for water in aqueous solutions are:

$$\psi = RT/V_w \times \ln h = RT/V_w \times \ln a_w = RT/V_w \times \ln \varphi N_w \qquad [4.3]$$

in which

R	=	universal gas constant (8.31×10^{-5} m^3 bar/mole.K; 1 bar = 10^5 N or Pa/m^2)
V_w	=	the partial molal volume of water (1.8×10^{-5} m^3/mole at 4°C)
R/V_w	=	0.461 MPa K^{-1} when expressing ψ in MPa or 4.61 when expressing ψ in bar
T	=	Kelvin or absolute temperature
h	=	equilibrium relative humidity (a fraction)
a_w	=	water activity (a fraction)
φ	=	activity coefficient
N_w	=	mole fraction of water

An approximate relationship between water potential (in bars) and relative humidity at 20°C is:

$$\psi = 1350 \times (h-1) \qquad [4.4]$$

A change in relative humidity of 0.01 is, therefore, equivalent to a change in water potential of about 14 bars. Growth of microorganisms often responds to changes in water potentials of several bars or less, sometimes even to fractions of bars, corresponding to relative humidity changes of 0.001 or less. Microbial activity has been reported at humidities below 0.7 (water potential of about -500 bar). Where ambient humidity is above 0.7, it may be possible to measure the activity of certain microorganisms even in air dry soil.

Microbiologists have tended to discuss microbial water relations in term of water activity, a_w. Water (or solvent) activity is numerically equal to the vapour pressure of the solution relative to that of the pure solvent:

where

P and P_0 are the vapour pressures of the solution and solvent and
n_w = the number of moles of water (55.51 molar)
n_i = the number of moles of all solutes

For a 1 molar solution, $P/P_0 = 0.9823$ and such a solution has a water activity of 0.9823 and will equilibrate with an atmosphere of 98.23% relative humidity.

In an ideal solution, a_w is independent of temperature. The effect of temperature is small for dilute non-ideal solutions and for concentrated, non-ideal solutions is significant to the extent that it affects the activity coefficient of solvent or solute. Table 4.1 (after Brown (1976) and Pedersen and Karlsson (1995)) shows a list of approximate limiting water activities for microbial growth.

TABLE 4.1

APPROXIMATE LIMITING WATER ACTIVITIES FOR MICROBIAL GROWTH

Water Activity (a_w)	Material	Bacteria	Yeasts	Fungi
1.000	pure water	*Caulobacter, Spirillum*		
0.995	human blood	*Streptococcus, Escherichia*		
0.990	groundwater (500 m)	*Bacillus, Pseudomonas* sulphate-reducing bacteria		
0.98	sea water	*Pseudomonas, Vibrio* sulphate-reducing bacteria		
0.95	bread	most gram-positive rods	Basidiomycetous yeasts	Basidiomycetes
0.90	maple syrup, ham	most gram-positive cocci *Lactobacillus, Bacillus*	Ascomycetous yeasts	*Fusarium Mucorales*
0.85	salami	*Staphylococcus*	*Saccharomyces rouxii* (in salt) *Debaromyces* (in salt)	
0.80	fruit cake, jams		*Saccharomyces* bailii (in sugars)	*Penicillum*
0.75	salt lake, salt fish	Halophiles		*Wallemia, Aspergillus*
0.70	cereals, candy dried fruit			Xerophilics *Chrysosporum*
0.65				*Eurotium*
0.60			*Saccharomyces rouxii* (in sugars)	*Xeromyces bisporus*

4.1.2 Water Retention by Porous Materials

Water in porous media (such as YM Tuff) is retained largely by matric forces in pores and interconnecting pore necks, as lenses at points of contact between mineral and/or organic particles, and as films on particle surfaces. At saturation water content, all pores are filled with liquid water and the matric potential ψ_m is zero. As water is removed by processes such as drainage, the largest pores empty first (ψ_m is lower in the smaller pores than the larger ones). The water content of porous media at a given matric potential depends, therefore, on the total pore volume that is interconnected and on the size distribution of the pores, which in turn are related to the size of the constituent particles. For instance, coarse-textured soils low in organic matter typically range in porosity from 0.3 to 0.4, whereas the porosity of clay and organic-rich soils may exceed 0.6.

Typical water content - water potential relationships for porous materials are of the general form:

$$\psi = a\,\theta^{-b} \qquad [4.6]$$

in which

θ = water content
a and b = constants for a given porous material

Studies of the response of microorganisms to water require both the water content and water potential of the system under study. At water potentials < -1 bar, the water content - water potential relationship of soil is dominated by surface adsorption effects. When the thickness of the water film is reduced to 6 to 8 molecular layers of water, plants are virtually unable to extract the water and growth ceases. For a clay soil with a specific surface area of 220 m^2/g, and a water content of 0.22 m^3/m^3 , the water, if spread evenly, would cover this surface with a thickness of only 0.8 nm.

Most soils contain a wide range of particle sizes (from sand, 2 mm to <0.002 mm for clay). The individual grains are packed or arranged in different ways resulting in a broad distribution of pore sizes. The capillary rise equation relates the matric potential of water in a capillary to the radius of curvature r (μm) of the meniscus:

$$\psi_m = 2\sigma/r \qquad [4.7]$$

in which

σ = surface tension (72.7 $g.sec^2$ or 0.727 bar . μm at 20°C)

The capillary rise equation can be used to estimate the pore size distribution in a porous matrix such as soil. A decrease in matric potential from ψ_{m1} to ψ_{m2} will result in release of water from pores of an effective diameter ranging from d_1 to d_2 (this approximation is only valid at high matric potentials, i.e., >1 bar). When a saturated soil is allowed to drain, a certain amount of tension or 'pull' must be applied to the water before the largest pores will begin to empty. The

matric potential at which the pores of a saturated soil first begin to drain is called the air entry potential.

Desiccation plays a determinative role in the ecophysiology of bacterial communities found in aerophytic environments, on and inside rocks, on and in soils, in crusts and accretions, in soils and sediments, in the phyllosphere, in dusts and aerosols and on the skins of animals and humans. At high matric potentials water retention in soils and sediments is dependent on the capilliary effects and is therefore strongly influenced by soil structure. At lower potentials the effect of structure is much less pronounced and the soils texture and specific surface are more important. A soil water potential of 0.1 bar is normally associated with water saturation of soil capillaries of < 30 μm in diameter, 0.3 bars with capillaries < 4 μm and at potentials < 5 bar the soil water is thought to be distributed as a film only a few water molecules thick (Potts 1994).

Table 4.2 (from Papendick and Campbell 1980) shows the relationship between water potential, relative humidity (or a_w), and effective pore diameter in soil at 20°C, calculated with the capillary rise equation.

TABLE 4. 2

RELATIONSHIP BETWEEN WATER POTENTIAL, RELATIVE HUMIDITY (OR WATER ACTIVITY a_w) AND EFFECTIVE PORE DIAMETER IN SOIL AT 20°C

Water Potential (Bar)	Relative Humidity	Effective Pore Diameter (μm)
-0.001	1.0000	2908
-0.002	1.0000	1454
-0.005	1.0000	582
-0.1	1.0000	291
-0.02	1.0000	145
-0.05	1.0000	58.2
-0.1	0.9999	29.1
-0.2	0.9999	14.5
-0.5	0.9996	5.82
-1	0.9993	2.91
-2	0.9985	1.45
-5	0.9963	0.582
-10	0.9926	0.291
-20	0.9853	0.145
-50	0.9637	0.058
-100	0.9286	0.029
-200	0.8624	-
-500	0.6906	-
-1000	0.4769	-
-2000	0.2274	-
-5000	0.0247	-

If the effective pore diameter distribution in Topopah Spring Tuff is known or could be determined, it may be possible to calculate a 'composite' water potential and hence an a_w value, which could then possibly be used as an 'on-off switch' in modelling attempts of microbial activity in the YM environment. Due to the heterogeneity of the tuff unit, it is expected that several water potential values may be more useful than one average value.

4.1.3 Methods for Measuring Water Potential

It is important to note that although water potential is one of the most important physical properties of soil, measurements relevant to YM are lacking, to some extent because it is difficult and tedious to measure reliably. A number of methods are briefly reviewed by Gee et al. (1992). One method involves equilibrating samples over salt solutions (of known water potentials) and then weigh the samples periodically until a constant weight (or water content) has been reached, which can take weeks to months. Another method involves measuring wetbulb depression of water in equilibrium with the sample, using thermocouple psychrometers (Rawlins and Campbell 1986). Depending on the type of psychrometer used, water potentials that can be accurately obtained have limitations in certain ranges. This has made such measurements somewhat uncertain. Measurement of water potentials of relatively dry samples has also been difficult. Gee et al. (1992) also presented a method using a Decagon water activity meter, (Decagon Devices, Pullman, WA), that was originally developed to measure a_w values in food and fibre samples, but performs just as well on soil samples. This meter was used successfully by Stroes-Gascoyne et al. (1997) on samples of compacted buffer material designed for a Canadian nuclear fuel waste disposal vault. The concept of a_w was also used by Stroes-Gascoyne et al. (1996) in evaluating the results from the buffer/container test in which the occurrence of viable bacteria in (heated) compacted buffer material was studied as a function of temperature and moisture content. In this study viable bacteria could no longer be cultured in buffer samples with a moisture content below 15% which converted to a water activity of 0.96.

The water activity meter may be an excellent tool to create a similar correlation between moisture content in YM samples and the ability to sustain viable and active bacteria in YM tuff. In fact, some data are already available. Kieft et al. (1993) studied microbial abundance and activities in relation to water potential in the vadose zones of arid and semiarid sites. Samples taken from volcanic tuff from the Nevada Test Site, where YM is located, were included in this study. Water potentials ranged from -36 to -62 MPa in samples of vitric tuff which corresponds to approximate a_w values of 0.75 and 0.65, and viable plate counts were generally below the detection limit. Water activity (calculated as a function of moisture content, itself a function of precipitation and subsequent water transport through tuff) could possibly be used in a model to be the 'on-off switch' for microbial activity.

4.2 TEMPERATURE

The waste packages to be emplaced within a YM repository would produce heat from the decay of radionuclides in the waste. Projections of temperature are very uncertain at present because the precise thermal loading conditions in a future repository have not been defined. However, temperatures in the near field are expected to initially increase, over a period of decades, to as much as 200°C, with subsequent cooling after about a 200 year period as radioactive decay

progresses. For all loading conditions that appear physically possible at present, the temperature in the vicinity of the waste packages significantly exceeds 100° C for more than 100 years.

4.3 pH

For the conditions in a proposed YM repository, acid pH values could potentially occur as a result of microbial activity, which generally has a pH decreasing effect because of the metabolic byproducts (organic and inorganic acids) that are formed, or as a result of the direct thermal degradation of materials such as PVC (which is a standard pipe material). Under suitable conditions (i.e., presence of O_2 and availability of reduced S and Fe , such as in pyrite), *Thiobacilli* may drastically lower the pH of the environment.

Depending on the final repository design, the use of concrete in the repository (e.g., concrete inverts on which the waste packages would be placed; concrete liners with which the drifts will be lined), could be considerable. Areas with high (concrete-induced) pH values may be quite common and heterogeneously distributed in a repository setting (Meike 1997). The chemical degradation is dependent on the original formulation and pre-treatment and the thermal-, gas chemistry-, and hydrological history of the environment. That degradation history will determine longevity of the high pH. This effect is discussed in detail elsewhere with respect to modelling and experimental studies (Meike 1997).

4.4 NUTRIENT AVAILABILITY

The movement of nutrient and metabolic by-products in and out of an ecosystem is controlled by factors such as the flow characteristics of the system, porosity, solubility, diffusion, viscosity, and specific gravity. Ecosystems with extensive flow or turbulence such as rivers and oceans have considerable movement of materials. Soil systems which are almost static with respect to flow depend on diffusion to move materials. In terrestrial ecosystems diffusion of materials is a function of porosity. Diffusion of materials occurs through the pores and exchange rates between material in the interstitial spaces and external sources affect diffusion rates and the availability of materials essential for microbial growth and activity. Movement of nutrients is further affected by sorption onto inorganic components in the environment which can reduce availability of nutrients in general, with a resulting overall decrease in microbial productivity. If the substance is toxic, the sorption of the toxic compound onto inorganic components would tend to have the opposite effect (increase productivity). Because of adsorption, even excessive levels of microbial inhibitors fail to completely suppress microbial activity in soil.

The present environment at YM is oligotrophic with respect to the supply of nutrients available for bacterial growth. Bacteria in oligotrophic environments often survive in a state of "suspended animation", in biofilms, are small, and may have generation times of tens to hundreds of years (Fredrickson and Onstott 1996). However, even in such an environment, their influence on the geochemical conditions could still be significant on a geological time scale. Furthermore, the establishment of a repository may change the nutrient status of the originally oligotrophic environment through the introduction of nutrients as a result of repository excavation, construction, operation and emplacement of the waste (e.g., Stroes-Gascoyne and Gascoyne 1998, Meike 1994, Meike and Wittwer 1994). In the proposed repository, the

complete assessment of nutrient availability is complicated by the degradation of construction materials over time. The massive amounts of iron and iron alloys, concrete and polymers that are part of the construction design or implicit necessities of construction, will degrade and the nutritive constituents will become available to microbes over time. This degradation is difficult to assess for two reasons. One is the period of time over which the repository is intended to function and the other is the potential chemical reactions and phase changes that occur at the elevated temperatures that the repository will experience.

Various construction components (e.g. polymers and concrete) are expected to degas hydrocarbon and water vapor, the character of which in addition to the availability due to condensation or diffusion away from the local environment will depend on the thermal perturbation.

4.5 RADIATION

A high-level nuclear waste repository at the YM site would be designed to store many containers of waste. Microbes inside the waste package would be subject to radiation from the encapsulated waste. However, depending on the design of the package, i.e, thin-walled or thicker-walled, the age of the waste, and the state of the package through time, the environment outside the waste package may not experience radiation. If the package is designed to have a thin-walled container, or should a container breach occur, it is possible that a certain amount of radiation will emanate from the waste package into the immediate surroundings. In that case, microbes living near the waste containers may receive a continuous dose of radiation but these effects will be felt only very close to the containers due to shielding by surrounding materials such as backfill, concrete and rock. For instance, King and Stroes-Gascoyne (1995) calculated that radiation effects in the Canadian repository concept likely would extend only about 40 cm into the buffer surrounding thin-walled waste containers.

Arrage et al. (1993a,b) subjected six bacterial isolates from the USDOE Subsurface Microbial Culture Collection to UV- and γ-radiation. The study included three aerobic Gram-positive strands and three microaerophilic Gram-negative strains. All bacteria were tested for radiation resistance during the stationary phase of growth since cells are most resistant to radiation at this stage. They found that the aerobic Gram-positive organisms were significantly more resistant than the microaerophilic Gram-negative organisms to both radiation treatments.

The occurrence of indigenous microorganisms has been studied at the Nevada Test Site (e.g., Amy et al. 1992, Haldeman and Amy 1993). Amy et al. (1992) studied almost 50 endolithic aerobic isolates from the Nevada test site and found that 89% were Gram-negative and 65% were pigmented, which may afford some protection against radiation damage (Section 3.5.2).

Pitonzo et al. (1999a) studied the radiation resistance of native endolithic microorganisms present in rock obtained from YM, using γ-radiation and pulverized but otherwise unamended rock samples. Cumulative radiation doses ranged from 0 to 9.34 kGy. Radiation-resistant microorganisms in the rock samples became viable but not culturable (VBNC) after a cumulative dose of 2.33 kGy. VBNC microorganisms lose the ability to grow on media on which they have been routinely cultured in response to the stress imposed (radiation) but they can still be detected

using direct fluorescent microscopy technique and the respiring cell count procedure (Rodriguez et al. 1992). The metabolic capability of the irradiated organisms was reduced considerably. However, this work showed that microbes in YM samples can survive γ-radiation in VBNC state. In a subsequent study, Pitonzo et al. (1999b) stored the irradiated rock samples for 2 months at 4°C, in an attempt to resuscitate the VBNC microbes to a culturable state. Culturable bacteria that had previously been nonculturable were found at all radiation doses in the samples after cold storage, but in numbers that were orders of magnitude lower than the culturable organisms in the unirradiated samples. The number of colony types also decreased from 26 to 10, and metabolic capacity only recovered partially. These results show that microbes can survive considerable radiation doses in a VBNC state, but will recover some of their previous culturable and metabolic capabilities, once the radiation stress is removed. For a YM repository this could imply that radiation will likely curtail activity and metabolic capability of microbes (indigenous or otherwise) present, but that part of this activity can be restored once radiation fields have decayed away to lower values.

4.6 REDOX CONDITIONS

It is likely that very large quantities of Fe alloys will be introduced into the proposed repository as waste packaging material and/or mechanical support. However, unless the repository environment becomes airtight, the oxidation of the iron will occur at a rate that is much slower than the exchange of oxygen with the atmosphere and will not affect the chemical composition of the atmosphere in the drifts (Meike and Glassley 1997).

It is expected that the proposed repository will be aerobic during most of the lifetime of interest. Microbial populations would be expected to succeed one another in order to take advantage of the developing environment. It is difficult to measure Eh in natural habitats and there is considerable heterogeneity, both on larger and smaller scale. The Eh at YM is expected to be oxidizing, but may vary from oxidizing to reducing on a microscale (i.e., so-called micro-environments), and microbial processes will vary accordingly and locally.

Even when the repository is predominantly aerobic, anaerobic microbial activity will probably occur locally. Should thermal conditions produce a vapor seal around the repository drifts, then anaerobes might be expected, especially if other non-biotic processes, such as metal oxidation serve to increase oxygen consumption inside the seal. However, locally, pockets of anaerobes will probably exist, even in a predominantly aerobic environment.

4.7 PRESSURE

At YM, some subsurface microorganisms may be subjected to a lithostatic pressure. However, the pressure in the drifts and fractures will be at atmospheric pressure during most of the repository lifetime. It is possible, during the thermal pulse, that a condensation front may temporarily seal the drift environment and during that period the drift environment may experience pressures that exceed one atmosphere. The extant population will be adapted to this but any introduced organisms may be partially and temporarily rendered less active during adaptation to the higher pressures. It is likely that pressure has only a small influence on the overall microbial activity in the repository drifts. Other factors such as nutrient availability,

water activity and temperature are likely much more important in controlling microbial activity in and around a repository. However, the possible significance of pressure, especially for hyperthermophiles that survive high pressures is within sealed waste containers. In this case it is important to determine whether radiation is sufficient to deter microbial activity within the containers.

4.8 DESIGN OPTIONS

As discussed in detail in other reports (e.g. Meike 1996, Meike 1998a,b) construction materials and materials brought into the proposed repository as a result of repository construction and operation may provide moisture, nutrients and substrates to microbes. By far the largest volume of material, outside of the waste packages themselves will be the mechanical support for the waste emplacement drifts. At times during the recent history of the project, three means of mechanical support have been considered either alone or in combination. They are: steel sets and rock bolts, concrete liners and backfill.

Several backfill materials have been considered. Some backfill materials such as iron filings, calcium carbonate, or apatite have been intended to have a chemical function, such as to modify pH, redox state or sorb radionuclides. Others, such as crushed excavated rock are intended purely as mechanical support or to modify the hydrological behavior near the waste packages. The chemical, physical and hydrological implications of each of these possibilities as they degrade over time has been discussed in detail (e.g. Meike 1996, Meike 1998a,b, Meike and Glassley 1997).

Salient features of the construction environment may be (depending on the final design) high iron content from waste packages and steel sets, high pH and increased carbonate from the concrete (depending on the formulation and pre-treatment), perturbed (crushed and additional moisture) crushed rock in direct contact with the waste packages, and in general an environment far more chemically and hydrologically heterogeneous than the natural one.

4.9 SUMMARY

The environment of a potential repository at YM would be severely modified by the presence of such a repository. Heat emanating from the waste packages will drastically (at least initially) alter the moisture distribution and salt concentrations. Emplacement of potential large quantities of concrete would affect the pH of the environment considerably. Radiation effects would occur near waste packages if they were of a thin-walled design. Nutrients introduction would occur from the emplacement and degradation of construction materials. Emplacement of large quantities of iron could potentially affect the redox environment if some isolation took place and if reduction of O_2 by iron would be faster than replenishing of the aerobic atmosphere in the repository. Pressure effects are not expected to occur to any appreciative extent.

Design features of the repository can potentially have large effects on microbial activity. Salient features of the construction environment may be high iron content from waste packages and steel sets, high pH and increased carbonate from the concrete, perturbed crushed rock in direct contact

with the waste packages, and in general an environment far more chemically and hydrologically heterogeneous than the natural one.

5. MICROBIAL RESPONSES TO SPECIFIC STRESSES RELEVANT TO THE YUCCA MOUNTAIN ENVIRONMENT

In this chapter, those specific stresses are discussed that are important with respect to defining and bounding the expected microbial responses to the Yucca Mountain environment, both during the excavation and construction phases of the repository and after waste emplacement and subsequent closure of the repository. The material in this chapter consists primarily of mechanistic details and case studies which will provide guidelines for, and in some cases first estimates of, limits and rates to be used in future microbial models of a perturbed environment at Yucca Mountain. Mostly the same order as in Chapter 3 (strategies for adaptation and survival) is followed, with water, temperature, pH, nutrients and radiation being of particular importance. Cross-protection and negative effects as a result of adaptation strategies are also discussed.

5.1 WATER

As discussed in Chapters 2 and 3, available water is of utmost importance for microbial activity and survival. The geological horizon at Yucca Mountain in which a potential repository for high level nuclear waste would be located is well above the water table. The rock contains water, but is not saturated and, therefore, a limited amount of water would be available for microbial activity. Sporadic rainfall will occasionally replenish the amount of water available. Emplaced waste packages, however, will be hot for hundreds of years and this will cause redistribution of water in the rock. Therefore, both the indigenous as well as the introduced microbial population will be subject to water stress and desiccation, both because of the inherent conditions at the site andalso because of the nature of the disturbance of the site due to the repository. In the next sections, desiccation and desiccation tolerance in microbes will be discussed in more detail.

5.1.1 Desiccation and Desiccation Tolerance

Laboratory studies of desiccation tolerance in bacteria have focused on aerobes because desiccation in nature usually involves air drying and, consequently, damage by reactive oxygen. Anaerobes are probably no more susceptible to air drying than aerobes because while oxygen may lead to a cessation of growth of strict anaerobes, their spore-forming representatives appear to include some of the most desiccation tolerant of bacteria (Potts 1994). The real barrier to complete understanding of desiccation tolerance is an inability to fully understand the complexity of the state of dried (and wet) cytoplasm. Desiccation tolerance is a manifestation of the unique functions of water in biological systems and the basis for many of those functions remains obscure.

Desiccation is likely to play a determinative role in the ecophysiology of bacterial communities that grow in aerophytic environments (the deserts of hot and cold climates), on and inside of rocks, on and in soils and sediments, in the phyllosphere, and in crusts and accretions, in dusts and aerosols and on the skins of animals and humans.

At high matric potentials, water retention in soils and sediments is dependent on the capillary effects and is therefore strongly influenced by soil structure. At lower potentials the effect of structure is much less pronounced and the soils texture and specific surface are more important. A soil water potential of 0.1 bar is normally associated with water saturation of soil capillaries of < 30 µm in diameter, 0.3 bars with capillaries < 4 µm and at potentials < 5 bar the soil water is thought to be distributed as a film only a few water molecules thick (Potts 1994). However, just a small amount of water is apparently enough for some types of bacteria to survive.

Results from studies with drying and salting (sugaring) of cells emphasize the critical role of bound water. Only a small amount of free cytoplasmic water is required for cell growth and the different growth rates can be accommodated within a wide range of free water values. The real water stress, therefore, is the perturbation of free water such that cytoplasmatic water becomes equal or less than bound water. For enzymatic activity, only a monolayer coverage of a protein with water molecules is required for activity. In the air-dried cell, however, even monolayer aggregations of water molecules on proteins have been perturbed and diminished.

Desiccation studies have been performed in a wide range of environments. Considerable work has been done in the food and medical field on the effects of freeze-drying on cell survival. Freeze-drying in the presence of a socalled cryoprotection agent (such as skim milk, honey, alginate, etc.) results in different levels of cell surface stability and survival, indicating that such agents have to be chosen properly. Ohtomo et al. (1988) found that freeze-drying inhibited capsule and slime production in *Staphylococcus aureus* which consequently brought about changes in the outermost cell surface.

When dried at relative humidities of 40 and 30% respectively, bacterial cells contain around 0.1 g of H_2O and 0.03 g of H_2O per g dry weight, respectively. The lower value is comparable to those measured for other anhydrobiotic cell types such as plant seeds which have water contents of about 0.02 g/g under extreme desiccation. The water content of bacterial spores is lower than for their corresponding vegetative cells but they contain too much water to belong to the class of anhydrobiotic cells and must, therefore, belong to the physiological group of cells that responds to water deficit by osmotic adjustment, i.e., by the use of compatible solutes (Potts 1994).

5.1.1.1 Cell Wall Modifications as a Result of Desiccation

Water plays a critical role in the membrane function and protein regulation in cells and dehydration not only influences the structure of proteins but also their function.

Removal of water profoundly alters the physical properties of membrane phospholipids, leading to destructive events such as fusion, liquid crystalline to gel phase transitions and elevation of permeability (leakage). In heterogeneous mixtures such as those found in biological membranes, phase transitions lead to lateral phase separations of membrane constituents. Certain sugars are

capable of preventing damage from dehydration not only by inhibiting fusion between adjacent vesicles during drying but also by maintaining the lipids in a fluid state in the absence of water. As a result, the changes in permeability and lateral phase separations that would usually accompany dehydration are absent (Crowe et al. 1987).

Bacteria are known to alter their membrane fatty acid components in response to environmental stress, thereby generating characteristic phospholipid fatty acid (PLFA) stress signatures. Kieft et al. (1994) subjected two subsurface isolates (a Gram-negative *Pseudomonas aureofaciens* strain and a Gram-positive *Arthrobacter protophormia* strain) to starvation under dry and moist conditions. Final water potentials were -7.5 MPa for *Pseudomonas aureofaciens* and -15 MPa for *Arthrobacter protophormia.* The numbers of culturable cells of both bacterial strains declined to below the detection limit within both the moist and dried nutrient-deprived conditions, while total cell counts and total PLFA levels remained relatively constant. The dried starved *Pseudomonas aureofaciens* cells showed indeed changes in PLFA profiles that are typically associated with stressed Gram-negative cells, i.e., increased ratios of saturated to unsaturated fatty acids, increased ratios of trans- to cis-monoenoic fatty acids and increased ratios of cyclopropyl fatty acids to their monoenoic precursors. The moist starved *Pseudomonas aureofaciens* cells did not show these changes and the PLFA profiles of *Arthrobacter protophormia* changed very little under either starvation or desiccation. PLFA profiles are a powerful tool for interpreting the physiological status of subsurface microbial communities at Yucca Mountain, and some have already been obtained by Haldeman and Amy (1993) in their study of bacterial heterogeneity in deep subsurface samples taken from tunnels at Rainier Mesa, Nevada Test Site.

5.1.1.2 Locations of Desiccation Damage in Bacterial Cells and Spores

Dehydration and rehydration reactions play crucial roles in the physiological processes of a cell, i.e., in the structure and functioning of macromolecules, but the system is very complex and many questions remain unanswered, e.g., how many water molecules are required to sustain the translational efficiency of a ribosome, the fidelity of RNA polymerase or the secretion of a protein as it crosses a membrane (Potts 1994). The inherent structural organization inside air-dried cells is the most critical but the least understood feature of desiccation tolerance.

Monolayer coverage of proteins by water occurs at levels of 0.3 to 0.4 g H_2O per g dry weight in cells, and 0.05 g/g is needed to hydrate charged and polar groups of protein and to form clusters of water. Acids, polar side chains or peptide-NH bonds are not saturated below 0.1 g/g. Thus, the low water content of dehydrated cells causes changes in the protein distribution, ordering and structure. Although far less is understood of the mechanism by which they do so, some of the same sugars that appear to protect membranes are also effective at preserving structure and function of labile proteins in the absence of water, an effect that is remarkably enhanced by the addition of small amounts of transition metals. (The effect of certain sugars was discussed in Chapter 3, Section 3.1.4). Potts (1994) discusses the work by Webb and coworkers (references 401-409 in Potts (1994)) with dried cells in aerosols. A two-phase death rate was found which was explained in terms of the removal of water molecules first from the -N, =N-H or -OH groups of protein molecules and then, upon further drying of the cells, from the =C=O or =P=O groups.

Dose et al. (1991) observed that DNA in vacuum-dried spores of *Bacillus subtilis* was damaged to a very substantial degree by processes leading to DNA-strand breaks (about 50 strand breaks per genome after 3 weeks of vacuum exposure). Similar effects occurred in *Deionococcus radiodurans* and the data suggested that the steady increase in DNA-strand breaks during long-term exposure of organisms to extreme dryness may be a general phenomenon that would finally limit survival over geological periods of time. If this is the case, microbial life on Mars may not be possible, and by the same token it may be severely restricted in a nuclear waste repository that is designed to be hot and dry.

The data obtained by Dose et al. (1991) allowed them to distinguish an initial phase of general cell and DNA damage (due to removal of liquid and hydrate water) from a continuous phase characterized by progressive DNA damage (i.e., strand breaks). A substantial amount of DNA-double-strand breaks occurred. Exposure to vacuum was not required to induce DNA-strand breaks in cells and exposure of *B. subtilis* spores to a dry atmosphere (70% relative humidity, 20°C) yielded about the same decrease in highly polymeric DNA and viability as vacuum exposure for 1 to 3 days. The effects also depended on strains, and the differences in resistance to dryness may in part be related to differences in the capability to repair DNA. They suggested that the possible mechanism of the formation of dryness-induced DNA-strand breaks is the production of covalent DNA-protein cross-links, which may play a crucial role in the induction of strand breaks.

Further detail on desiccation damage to proteins, nucleic acids, lipids and membranes and mechanisms of damage can be found in the extensive discussion by Potts (1994).

5.1.1.3 Responses to Desiccation and Mechanisms of Tolerance

It is difficult to compare the sensitivities of different groups or genera of bacteria because of the many different techniques used to grow and dry cells and the inherent ability of some cells to form resting stages, cysts and spores. However, a few general observations can be made. The rate at which cells are dried is critical to cell survival. Potts (1994) showed the times of survival following air drying of representatives of the major groups of prokaryotes, which indicated that all bacteria have a capacity to tolerate drying to greater or lesser extents and that the range of time during which bacteria may remain viable in the air-dried stage is extreme.

Studies suggest that cells in the stationary phase are structurally, physiologically and functionally distinct from those in the log phase (Potts 1994). Stationary-phase cells are generally more resistant to desiccation than log-phase cells. Reasons for this are uncertain. Potts (1994) discusses how cyclopropane fatty acids accumulate in stationary-phase cells, but it is unclear how they may protect the cell from desiccation. Lievense and van't Riet (1994) observed an increase in ATP levels of yeast cells in the stationary growth phase when they were dried that did not occur in log-phase cells. The accumulated ATP may serve as an energy source during rehydration of the cells which could result in a higher survival rate.

Rapid changes in the environmental water potential are more lethal than are low water potentials per se. Rapid desiccation prevents enough time for survival mechanisms to kick in, such as adjustment of internal water potential either by passive water loss or accumulation and/or synthesis of intracellular compatible solutes (Chapter 3, Section 3.1.4). Resistant structures such as spores and cysts, while not induced by desiccation, also provide a mechanism of tolerance. With respect to protein stability, extracellular proteins or cell surface-associated proteins must be faced with more drastic perturbations upon drying than their cytoplasmatic counterparts. However, no general strategies for stabilization of extracellular proteins have been established for any of the stress parameters in nature. So-called water stress proteins have been proposed but their existence not proven.

5.1.1.4 Metabolism and Recovery from Dessication

One feature of the recovery of desiccated cells upon rehydration is a very rapid onset of cellular metabolism in conjunction with a stepwise and stringent recovery of metabolic processes. Some investigators suggest that desiccated cells have evolved such that they make good use of enzyme organization with a limited amount of water.

Kieft et al. (1987) found that a rapid increase in water potential of a desiccated soil released a measurable amount of the soil microbial biomass C. Van Gestel et al. (1992) also showed that drying and wetting of soils caused flushes of C and N mineralization due to enhanced availability of decomposable organic compounds. This biomass is likely partially released by transport of organic intracellular compatible solutes which were accumulated by the bacteria during the slow desiccation prior to rapid rewetting, and partially from dead cells that did not survive the desiccation. Rapid water potential increase may, therefore, be a potent catalyst for the turnover of C as well as N, P and other nutrients in terrestrial ecosystems, including at Yucca Mountain.

The indirect effects of desiccation are decreased solute diffusion and decreased cell mobility, both of which contribute to microbial starvation. Solute-synthesis imposed carbon- and energy demands, and accumulation (transport into cell), also requires energy. Rosacker and Kieft (1990) measured the adenylate energy charge AEC = ([ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP]) in moistened and subsequently dried soil samples. AEC values of 0.8-0.9 are indicative of actively growing cells, while AEC values of 0.5 to 0.7 represent dormant cells which are incapable of biosynthesis and values < 0.4 are thought to occur only in dead and dying cells. They also suggested that RNA catabolism in response to starvation upon desiccation may be a major source of AMP in the cells, which was then used as an energy source or as a substrate for intracellular solute synthesis. Their main conclusion was that the rate of soil drying is at least as important as the final extent of drying in determining microbial response. Gradual drying caused a transient AMP increase which may signal starvation-induced endogenous metabolism. The AEC indicator could possibly be used to determine relative humidity boundary conditions for microbial communities of interest to Yucca Mountain (indigenous and introduced) if detection limits for ATP, ADP and AMP were sufficiently low.

5.1.1.5 Effects of Desiccation on Decomposition Processes

The waste package in a Yucca Mountain drift may be surrounded by backfill material, which may be composed of crushed tuff excavated from the repository or crushed tuff mixed with other materials (such as bentonite) intended to modify the chemical and/or hydrological environment (Chapter 4, Section 4.7). If the final design includes a backfill material to be in direct contact with the waste package, then microbial activity could occur close to the package. This activity would need to be considered in relation to possible microbially influenced corrosion of the waste package material, and radionuclide transport mediated by microbes. However, the backfill immediately adjacent to the package would experience the most serious effects of heat, dehydration and possibly radiation, and the impact of these effects on the possibility of microbial activity in backfill needs to be considered.

The effect of water potential on decomposition processes (in soils) has been reviewed by Sommers et al. (1980). The basic decomposition processes involve sequential microbial conversions of reduced organic C compounds to an oxidized end product, principally CO_2, under aerobic conditions and to a variety of incompletely oxidized C compounds in O_2 limited environments. Decomposition rate is influenced by temperature, O_2 levels, water potential, pH, inorganic nutrients and the C:N ratio of the material. With decreasing water potentials, the activity of bacteria will become limited, because of a decrease in the proportion of water-filled pores which results in reduced mobility of bacteria and a limited availability of substrates (reduced diffusion of solutes).

In general, there appears to be a two-phase process affecting microbial decomposition processes in soils. There is an initial rapid decrease in decomposition within the -0.3 to -10 bar range followed by another region where decomposition decreases linearly with decreasing water potential. The role of bacteria is probably minimal once soils attain water potentials of -15 bars or lower ($a_w < 0.985$) resulting in actinomycetes and fungi being the major decomposers in situ soils. Actinomycetes and many of the fungi are capable of surviving at very low water potentials (-40 to -100 bars), but may be metabolically inactive. In laboratory cultures bacteria can often metabolize at -20 to -100 bars but in these systems supply of nutrients is often abundant whereas in situ soils the substrate molecules have to diffuse to the cells, or alternatively the bacteria have to move to the substrate, processes that are both affected by water potential.

Water potential also influences decomposition processes at high soil water contents. Where saturated conditions exist and water flux is low, O_2 can be depleted and anaerobic conditions can develop (because O_2 diffusion in water is much lower than in air). Immediately after the onset of anaerobiosis, facultative anaerobes oxidize organic C to primarily CO_2 whereas equivalent amounts of CH_4 and CO_2 are produced at subsequently lower O_2 tensions, under appropriate substrate and pH conditions. At intermediate O_2 concentrations, fermentation reactions may result in the production of organic acids, alcohols and other partially oxidized organic C compounds with the eventual production of CO_2 and CH_4 as the system becomes fully anaerobic. The following sequence of electron acceptors is typically used with increasingly negative redox potential following the onset of anaerobic conditions in soils containing a supply of oxidizable organic C: 1.) O_2, 2.) NO_3^-, 3.) Mn^{4+} (MnO_2), 4.) Fe^{3+} (in Fe_2O_3 and other iron oxides), 5.) SO_4^{2-}, 6.) H_2 and 7.) CO_2 (Stumm and Morgan 1981).

5.1.1.6 Exopolysaccharide (EPS) Production as a Result of Desiccation

Roberson and Firestone (1992) studied the relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. under starvation conditions. They found that water availability strongly controlled the production and consumption of protein and polysaccharides by the bacteria. Wetting caused an initial decrease in the amount of polysaccharides in all cultures, possibly as a result of consumption of polysaccharides by bacteria growing in response to the increase in water availability. The concurrent increase in protein concentration suggests that some of the polysaccharide carbon may have been used for protein production. Conversely, in the desiccated treatment group, the amount of polysaccharides increased while the amount of protein decreased implying that protein and possibly other cellular components as well are used for polysaccharide production in response to desiccation. Carbon appeared to be shuttled between protein and polysaccharides as the water status of the cultures changed. The lack of an external source of available C (starvation conditions) may be similar to the situation in soil in which the pool of available C is often small and microbial biomass released for 'recycling' after wetting of dry soil can be an important portion of the C available to the microbial community. In addition EPS may, by maintaining a high water content, also increase diffusional availability of nutrients to soil bacteria. This situation may be very applicable to the microbial populations at Yucca Mountain which will undergo many cycles of desiccation and rewetting with associated C recycling. This may be of importance to colloid generation in a Yucca Mountain repository.

Ophir and Gutnick (1994) also discussed the production of EPS in protecting cells from desiccation. They studied mucoid and nonmucoid strains of a number of bacteria and found much higher survival rates for the mucoid strains. In mixed colonies, containing both mucoid and non-mucoid strains, the survival of non-mucoid strains did not increase, suggesting that the EPS must be attached correctly to the cell wall so that only the biopolymer-producing organism is protected. An alternative explanation is that in the developing colony, the two strains were actually physically separated as suggested by the observation of distinct pockets of mucoid and non-mucoid cell aggregates. Trevors et al. (1993) studied the survival of encapsulated cells of *Pseudomonas fluorescens* in soils. Encapsulation of cells was done with alginate enhanced with skim milk or skim milk and bentonite clay. Both treatments significantly enhanced survival in soils. This suggests that a protective cover for microbes may be non-specific.

5.1.1.7 Motility and Its Limits

Motility of microbes may be important with respect to radionuclide migration because of the sorption capacity of microbes for radionuclides. The physical configuration of water in soil which relates directly to water potential may influence the motility of microorganisms (as well as diffusion of gases, nutrients and exudates to and away from biological activity sites), especially those that lack a hyphal system to bridge air space. The movement of motile bacteria may also be limited if the water-filled pores or pore necks in the soil are too small to permit their passage. For example, for maximum movement and dispersion in soil, fungi of the genus *Phytophtora* require water-filled pores of at least 40 to 60 μm in equivalent diameter, whereas the bacterium

Pseudomonas aeruginosa requires water-filled pores of 1 to 1.5 μm in diameter to move in soil. The spread or dispersion of a specific organism in soil at a given water potential may vary considerably with soil type because of differences in numbers of pore sizes over the range favorable for its movement. In typical soils, if the matric water potential is less than -0.1 MPa, then the solute diffusion rate is <50% of the rate under saturated conditions, and microbial movement is negligible below approximately -0.1 MPa ($a_w < 0.9999$, Table 4.2) (Kieft et al. 1993, Griffin 1981). However, pore size is not the only restriction to movement of organisms. The air-water interface itself can hold the organism down like a rubber membrane (Papendick and Campbell 1980).

Colloidal migration may also occur, because bacteria, EPS and spores (potentially all with sorbed radionuclides) can migrate as passive particles in water-filled fractures. Such a mechanism can be treated as migration of organic colloid particles in modelling efforts.

5.1.2 Osmoregulation

Cells do not need to be dehydrated to experience water stress. They may be placed under water stress through dispersal under hyper- or hypo-osmotic conditions, which results from differences in solute concentrations on opposite sides of the semi-permeable cell membrane. High salt concentrations in the environment affect, in addition to osmotic pressure, the denaturation of proteins which are essential for enzymatic activity. Osmotic effects on bacterial survival and growth can be expected at YM and bacteria strategies to cope with these effects (such as the production of osmoprotectants) have been discussed in Chapter 3. More detail on osmoregulation is given here, because the YM environment will be subject to water redistributions which may also cause a redistribution of salts, and hence affects osmotic pressure for microorganisms.

Cells have a certain osmotic pressure which has to be balanced with the osmotic pressure of the external environment. Osmotic stress involves an increase, or decrease, in the osmotic strength of the external environment of an organism, and osmotic regulation or osmoregulation encompasses the active processes carried out by organisms to cope with osmotic stress (Csonka 1989). There are two types of osmoregulatory phenomena: the long-term or steady-state responses that are manifested during the growth of organisms at a constant molarity, and the short-term or transient responses that occur soon after changes in the external osmolarity. Hyperosmotic shock refers to an increase in the external osmolarity whereas hypoosmotic shock is the opposite (Csonka and Hanson 1991).

Upon hyperosmotic stress, passive alteration of the cell volume (shrinkage of the cytoplasm volume) occurs to equalize the water activity inside and outside the cell. This process can have negative consequences because it increases the concentrations of all intracellular molecules which may be inhibitory to cellular processes. If the shrinkage is considerable, plasmolysis may occur. Sudden plasmolysis results in the inhibition of a variety of physiological processes, ranging from nutrient uptake to DNA replication, and is accompanied by an increase in ATP levels of the cells, possibly resulting from the inhibition of macromolecular biosynthesis (Csonka 1989). Active adaptation by a cell to hyperosmotic stress involves increasing the concentration of compatible solutes. They do not, as a rule, cross cell membranes rapidly without

the aid of transport systems. For the most part they do not carry a net electrical charge near pH 7, which aids in their non-reactive character. However, K^+ ions and glutamate are noteworthy exceptions and these solutes may not offer as effective protection against hyperosmotic stress as some of the uncharged solutes (Csonka 1989).

Osmoprotectants (Chapter 3, Section 3.1.4) are compatible solutes (occurring naturally in the environment or added to a growth medium) that can moderate the effects of high osmolarity (and stimulate growth) when they are present extracellularly, suggesting that they can accumulate to high concentrations by membrane transport but not by de novo synthesis. Because some compatible solutes would be N and C sources, their catabolism needs to be regulated to prevent their degradation as long as they are needed as osmotic balancers. High osmolarity apparently suppresses the enzymes that normally would be involved in the catabolism of these substances under low osmotic conditions.

A decrease in extracellular osmolarity results in an influx of water into the cells, which leads to an increase in turgor pressure in cells with inelastic walls. However, bacterial cell walls can withstand pressures of 10 MPa without rupturing and an increase in turgor pressure may not be all that damaging. Nevertheless, bacteria possess four active processes for dissipating excessive turgor by decreasing the concentrations of compatible solutes: dilution by growth, catabolism to osmotically inert molecules (e.g., H_2O, CO_2), polymerization, and excretion. Literature is scant on these processes (Csonka and Hanson 1991). It is also possible that suspension of cells into media of low osmolarity may cause a generalized leakiness of the membranes without causing cell death, such that the loss of intracellular compatible solutes upon hypoosmotic shock occurs passively.

Compatible solutes accumulated as a result of an osmotic response apparently also affect other biochemical processes such as temperature sensitivity and may shift the high temperature limit of growth of many bacteria. Despite higher concentrations of these compatible solutes in bacterial cells as a result of higher external osmolarity, their concentration is often small compared to the concentrations of K^+ and Cl^-, the major osmolytes in certain organisms. Some cytoplasmic compatible solutes may have other functions as well. For instance trehalose, which is used as a compatible solute in many bacteria, is synthesized constitutively in archaebacteria and in the spores of eubacteria, and it has also been proposed to act as a stabilizer of membranes under desiccation.

Most bacteria can accumulate several compatible solutes but seem to prefer some over others. Several observations suggest that in most eubacteria, glycine betaine is preferred over all compatible solutes. This substance is a more potent alleviator of osmotic stress than most other osmoprotectants and it also suppresses the accumulation of other compatible solutes in several species of bacteria (Csonka and Hanson 1991). However, other environmental factors can affect accumulation of compatible solutes, e.g., N limitation in a high osmolarity medium resulted in the preferential synthesis of trehalose over N-containing compatible solutes such as betaine. Often, a shift in osmolarity causes initially a rapid uptake of K^+ and synthesis of glutamate, followed by the synthesis of another solute and excretion of K^+ and glutamate. The osmoprotecting effect of solutes apparently depends not only on their ability to replace K^+ and glutamate but also on other factors such as their interaction with macromolecules.

5.1.2.1 Osmoregulatory Signals

The response of microorganisms to changes in the external osmolarity can be divided into three phases. First there is rapid shrinkage or swelling of the cytoplasmic volume as a result of efflux or influx of water as a result of hyper- or hypoosmotic shock. This is followed by biochemical readjustment of the cells to restore turgor or volumes to levels compatible with growth. The growth of bacteria in media of high osmolarity results in the increased transport or synthesis of a few compatible solutes and an enhanced transcription of a limited amount of genes that encode proteins involved in osmotic stress tolerance. Finally, growth is resumed under the new conditions. However, characterization of the primary signals and the regulation of the osmolarity of the periplasm and the cytoplasm of bacteria remain partially unknown areas in the field of osmoregulation (Csonka 1989 and Csonka and Hanson 1991).

Only a few studies have examined the transient responses to osmotic shifts in bacteria, because the transient time frame is so short (seconds or minutes after exposure to the new osmolarity), and it is much easier to study long-term responses after cells have completed osmotic adaptation. However, even the understanding of the long-term osmoregulatory signals in cells is still unsatisfactory (Csonka and Hanson 1991). For instance, a permease system for proline and glycine betaine (both compatible solutes) is synthesized at a nearly constitutive level in *E. coli* but its activity is stimulated in cells growing exponentially in media of high osmolarity. What stimulates this system is not clear. Research has suggested that it is not turgor pressure of the cell but more likely the membrane tension or some related parameter. But even after the cells have completed osmotic adjustment through the increase in compatible solute concentrations, this permease system remains in an active state, even though the cell membrane has returned to its original condition. It requires invoking a persistent osmoregulatory signal to explain this. Csonka and Hanson (1991) discuss various models for this but none are entirely satisfactory at present. Possible signals include isotropic pressure changes, pressure differentials across the peptidoglycan complex, changes in the internal or external solute levels or water activities, and changes (stretch) in the inner membrane area.

The K^+ level of the cells could be the primary signal for the regulation of some or perhaps all of the cellular processes that are under osmotic control, and the role of K^+ may be direct or indirect. Unclear is what provides the signal for accumulation of the anions to balance the K^+ ions. It is possible that transient fluctuations in the turgor may be sensed by membrane-bound proteins that monitor the structure of the membrane. The turgor pressure of bacterial cells is difficult to measure but can be calculated from the water activities of crude cell extracts, from the threshold osmolarities that induce plasmolysis, or from measuring the threshold external pressure that is required to collapse the vacuoles as a function of the osmolarity of the medium (only applicable to organisms containing gas vacuoles). Turgor pressure of Gram-positive bacteria (15 to 20 atm) is considerably larger than that for Gram-negative bacteria (0.8-5 atm). However, the possibility that the periplasmic space of Gram-negative bacteria is iso-osmotic with the cytoplasm challenges the notion that turgor pressure can regulate cellular processes.

There are very rapid fluctuations in the cellular volume of bacteria during plasmolysis, and, therefore, changes in the concentrations of some metabolites could be an alternative signal in addition to turgor changes for the initiation of the processes of osmotic adaptation. Alterations in

the intracellular volume may be detected by cytoplasmatic proteins that respond to the concentrations of key signal molecules.

Ion channels or transport systems that are activated by deformation of membranes as a result of fluctuations in pressure may be another type of regulation. A further conceivable signal for the osmotic control of some cellular processes could be the osmolarity or water potential of the cytoplasm. A full characterization of the signals that regulate the osmolarity of cytoplasm and periplasm in bacteria remains elusive (Csonka 1989).

5.1.2.2 Osmolarity Effects on Expression of Genes

Changes in extracellular osmolarity generally bring about changes in the expression of only a few genes, which mostly encode for proteins involved in the synthesis or transport of compatible solutes (e.g., so-called channel proteins or porins). For instance, in Enterobacteriaceae only about 20 genes appear to be subject to osmotic control at the transcription level, and this effect may be indirect (i.e., controlled by another aspect of cell physiology affected by osmolarity). Expression of a number of genes in enteric bacteria is discussed in detail by Csonka (1989). Regulatory proteins often occur in two components, with one protein being the sensor of some environmental signal and the other protein being the signal transducer that regulates response. Although for some specific genes (for details see Csonka and Hanson 1991) and references therein) the signal transduction pathway has been well-characterized with respect to the structures and interactions of its components, generally it has been difficult to determine what the signal is and how it is sensed. Isotropic pressure differentials and wall or membrane stretch are less likely signals than the levels of specific solutes, or the cytoplasmic, periplasmic or extracellular water activity a_w, but all may play a role for different organisms and genes.

There are a number of proteins whose structures are abnormally sensitive to the solute composition of the cytoplasm, which is in part determined by the external osmolarity. Such proteins are nonfunctional when the cells are grown in media of low osmotic strength but regain at least partial activity when the cells are grown in media of elevated osmolarity. These mutations result in a so-called osmoremedial phenotype which is associated with slight alterations in the amino acid sequence of the affected proteins, as indicated by the observation that many temperature-sensitive mutations are also osmoremedial (Csonka 1989). Mutations which block the pathway for trehalose synthesis result in sensitivity to osmotic stress.

5.1.2.3 Halobacteriaceae

Bacteria growing best at salt concentrations between 20% (wt/vol) and saturation (about 30% wt/vol) are often referred to as extreme halophilic bacteria or extreme halophiles. A variety of other bacteria have been described as halophilic, but their requirement for salt is more modest. Because of the expected desiccation and redistribution of moisture and salts, Halobacteriaceae and other salt-tolerant microorganisms are expected to play a role in the microbiology of a Yucca Mountain repository. However, due to the length of the material discussed in Chapter 5, the discussion on Halobacteriaceae and other salt tolerant groups of microbes can be found in Appendix B. In addition to Halobacteriaceae and their physiology, the specific responses to high salt environments on non-Halobacteriaceae are also discussed in

Appendix B, including Haloanaerobes, Lipolytic bacteria, Diazotrophic bacteria, Chemolithotrophs, Arthrobacters and Rhizobia.

5.2 TEMPERATURE

Temperature is one of the most important environmental factors for microbial survival, growth and activity. High temperatures (possibly in excess of 200°C) are expected in the YM environment for several hundreds of years. While the limit of biological activity lies possibly around 110 to 130°C and 200°C would be far in excess of this, spores may survive and also, there would be many temperature gradients at YM with a range of high but tolerable temperatures. Upper, lower, and optimum temperatures were discussed in Chapter 3, as well as mechanisms of heat resistance in thermophiles and hyperthermophiles (i.e., heat shock proteins). This section gives more detail on the natural habitats and metabolism observed in thermophiles, with applicability to the YM site.

5.2.1 Habitats of Thermophiles and Hyperthermophiles

The main natural extreme environments, characterized by high temperature that can be colonized by microorganisms are (Kristjansson and Hreggvidsson 1995, Aragno 1981, Stetter 1995):

A. Geothermal Environments:

- freshwater alkaline hot springs and ponds
- heated soils, e.g., acidic solfatara fields
- anaerobic geothermal mud and soils
- geothermally heated deep oil reservoirs
- 'black smokers'
- active seamounts

B. Sunheated Substrates

- rock, soil, mud
- shallow water

C. Organic Materials Heated by the Energy Dissipated by Aerobic Decomposers

- self-heated hay
- compost, manure
- coal refuse piles

D. Environments with Increased Temperatures as a Result of Human Activity

- hot water heaters
- cooling waters

The list of habitats illustrates that thermophiles can be found in natural and man-made environments. Natural habitats are usually associated with active volcanism. On land, volcanic exhalations heat up soils and surface waters, forming sulfur-containing acidic fields (pH 0.5-6) and neutral to slightly alkaline hotsprings. Other environments are smoldering coal refuse piles (acidic pH) and hot outflows from geothermal power plants. The salinity of terrestrial hyperthermal environments is usually low, but much higher in submarine active volcanic areas because of seawater (Stetter 1995).

The Yucca Mountain environment would possibly be best described as a hybrid of the first and the last category would differ from these other environments because of its non-aqueous characteristics and limited water availability. Its characteristics are quite unique and do not fully resemble any of the environments included in the above categories. Its closest analogue are possibly geothermally-heated soils in solfatara fields, but these soils are usually dense, moist and reduced by sulfide. The Yucca Mountain environment is expected to be mainly oxidizing, fairly permeable and unsaturated.

Temperature, pH and salinity interact very strongly and extreme environments need to be described in terms of all three parameters. A 'normal' environment has temperatures in the range of 4 – 40°C, a pH of 5 to 8.5, and a salinity between that of freshwater and that of seawater. The main characteristics of the most studied natural extreme environments are shown in Table 5.1 (Kristjansson and Hreggvidsson 1995).

TABLE 5.1

THE MAIN CHARACTERISTICS OF THE MOST STUDIED NATURAL EXTREME ENVIRONMENTS

Habitat	Temperature (°C)	pH	Salt* (%w/v)
Freshwater alkaline hot springs	>60	>7	<6
Acidic solfatara fields	>60	<3	<6
Anaerobic geothermal mud and soil	>60	5 to 7	<6
Acidic sulphur and pyrite areas	<50	<3	<6
Carbonate springs and alkaline soil	<50	>8	<6
Soda lakes**	<50	>9	>10
Highly saline lakes	<50	5 to 8	>10

* Salt solution can be composed to considerable extent by salts other than NaCl.

* * The soda lakes in Oman were studied as a natural analogue for the effects of concrete in a repository and have pH values ranging to pH 12 (Section 5.3.1.2).

An extremophilic organism is one that can live outside of the 'normal' range of at least one of the environmental factors (live, not just survive), and whose optimal growth conditions are found outside of 'normal' environments. Away from 'normal' environments, species diversity decreases and environmental stress increases. Also, environmental stress factors are usually additive when applied simultaneously, an increase in one increases an organisms susceptibility to another. It is generally observed that species diversity declines as the temperature of the habitat increases and that solar and self-heated environments contain a greater bacterial diversity than geothermally-heated environments. In particular both mesophilic and thermophilic types are often recovered from the former (Kristjansson and Hreggvidsson 1995).

One would assume that thermophiles live in warm environments, but often thermophilic sporeformers have been isolated from rather cool environments. The question is then whether this microorganism was active in this cold environment or merely survived in sporeform. At Yucca Mountain, the expected rapid increase in temperature (after waste package emplacement) may enhance spore formation which enhances survival, but spores themselves are not active in any environment until they germinate and become vegetative. Finding thermophyllic isolates in high-temperature environments does, therefore, by itself not shed light on the actual in situ activity, because the organism could have been present as a dormant spore.

5.2.2 Metabolism of Thermophiles and Hyperthermophiles

Kelly and Adams (1994) and Schonheit and Schafer (1995) have reviewed the metabolism of hyperthermophilic organisms. Hyperthermophiles are characterized by a temperature optimum for growth between 80 and 110°C. The highest temperatures known so far are marine hyperthermophiles. They are considered to represent the most ancient phenotype of living organisms and thus their metabolic design might reflect the situation at an early stage of evolution. Their modes of metabolism are diverse and include chemolithoautotrophy and chemoorganoheterotrophy. No extant phototrophic hyperthermophiles are known. Most of the currently known species (47 species of hyperthermophilic Archaea and Bacteria are known (Stetter 1992)) are strict anaerobic, reduce elemental sulfur (S^0) with H_2 to H_2S (this seems to have replaced O_2 respiration) and are obligate heterotrophs, utilizing mostly peptides instead of carbohydrates (i.e., obligate proteolytic). Growth is generally only obtained on complex proteinaceous substrates and media typically containing one or more of yeast, bacterial or meat extracts, peptone or tryptone. The actual growth substrates in their natural environment are unclear but are probably organics in sediments. Metabolic products typically include acetate, isovalerate and isobutyrate, indicating fermentative-type metabolisms. Few are saccarolytic and they have a very limited substrate range (i.e., they cannot use monosaccharides). Other than proteinaceous substrates and certain sugars, pyruvate is the only other carbon source some hyperthermophiles can use.

5.2.2.1 Autotrophic CO_2 Fixation

There are only a few known autotrophic hyperthermophiles, and autotrophic CO_2 fixation proceeds via the reductive citric acid cycle, considered to be one of the first metabolic cycles, and via the reductive acetyl-CoA/carbon monoxide dehydrogenase pathway. The Calvin cycle

has not been found in hyperthermophiles (or any Archaea) (Schonheit and Schafer 1995, Kelly and Adams 1994).

5.2.2.2 Catabolism of Peptides

Organotrophic metabolism mainly involves peptides as substrates, which are either oxidized to CO_2 by external electron acceptors or fermented to acetate and other products. Both intra- and extracellular enzymes (protease activity) are needed to break proteins into peptides and amino acids. The primary step in the utilization of amino acids as both carbon and nitrogen sources is assumed to be an aminotransferase-type reaction. Peptide fermentation involves transaminases and glutamate dehydrogenase, together with several unusual ferredoxin-linked oxidoreductases not found in mesophilic organisms (Kelly and Adams 1994).

5.2.2.3 Catabolism of Carbohydrates

Sugar catabolism in hyperthermophiles involves non-phosphorylated versions of the Entner-Doudoroff pathway and modified versions of the Embden-Meyerhof pathway. The classical Embden-Meyerhof pathway is present in hyperthermophilic bacteria (*Thermotoga*) but not in Archaea. All hyperthermophiles (and Archaea) tested so far utilize pyruvate:ferredoxin oxidoreductase for acetyl-CoA formation from pyruvate. Acetyl-CoA oxidation in anaerobic sulphur-reducing and aerobic hyperthermophiles proceeds via the citric acid cycle; in the hyperthermophilic sulphate reducer *Archaeoglobus* an oxidative acetyl-CoA/carbon monoxide dehydrogenase pathway is operative. Acetate formation from acetyl-CoA in Archaea, including hyperthermophiles is catalysed by acetyl-CoA synthease (ADP-forming), a novel prokaryotic enzyme involved in energy conservation. In bacteria, including the hyperthermophile *Thermotoga*, acetyl-CoA conversion to acetate involves two enzymes, phosphate acetyltransferase and acetate kinase. A scheme for electron flow during the oxidation of carbohydrates and peptides and the reduction of S° has been proposed, but the mechanisms by which S° reduction is coupled to energy conservation in obligate (and facultative) S° reducing hyperthermophiles is not known (Schonheit and Schafer 1995, Kelly and Adams 1994).

5.2.2.4 Energy Yielding Reactions

The lithotrophic energy metabolism in (hyper)thermophiles is mostly anaerobic or microaerophilic, and based on the oxidation of H_2 or S coupled to the reduction of S, SO_4^{2-}, CO_2 and NO^{3-} but rarely to O_2. The substrates are derived from volcanic activities in hyperthermophilic habitats. The lithotrophic energy metabolism of hyperthermophiles appears to be similar to that of mesophiles. The following are modes of lithotrophic energy metabolism of hyperthermophiles (Schonheit and Schafer 1995):

S-reduction:	$H_2 + S \rightarrow H_2S$
SO_4^{2-} reduction:	$4H_2 + SO_4^{2-} + 2H^+ \rightarrow H_2S + 4H_2O$
$S_2O_3^{2-}$ reduction:	$4H_2 + S_2O_3^{2-} + 2H^+ \rightarrow 2\,H_2S + 3H_2O$
SO_3^{2-} reduction:	$3H_2 + SO_3^{2-} + 2H^+ \rightarrow H_2S + 3H_2O$
CO_2 reduction:	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ (methanogenesis)
O_2 reduction:	

H_2 as electron donor (Knallgas reaction): $2H_2 + O_2 \rightarrow 2H_2O$
Sulphur as electron donor (sulphur oxidation): $2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$
Thiosulphate as electron donor: $S_2O_3^{2-} + 2H^+ + 2O_2 + 3H_2O \rightarrow 2H_2SO_4 + 2H_2O$
Tetrathionate as electron donor: $S_4O_6^{2-} + 3.5O_2 + 3H_2O \rightarrow 4\ SO_4^{2-} + 6H^+$
Pyrite as electron donor: $FeS_2 + 3.5O_2 + H_2O \rightarrow FeSO_4 + H_2SO_4$
NO_3- reduction:
H_2 as electron donor: $5H_2 + 2NO_3^- + 2H^+ \rightarrow N_2 + 6H_2O$
Sulphur as electron donor: $5S + 6NO_3^- + 6H^+ + 2H_2O \rightarrow 5H_2SO_4 + 3N_2$
Thiosulphate as electron donor: $5\ S_2O_3^{2-} + 18H^+ + 8NO_3^- + H_2O \rightarrow 10H_2SO_4 + 4N_2$

The metabolic ability to gain energy by methane formation is restricted to Archaea. They include mesophilic, moderately thermophilic and hyperthermophilic species. The latter are all obligate lithoautotrophic growing on CO_2 and H_2 as sole carbon and energy sources. Methanol and acetate-utilizing hyperthermophilic methanogens are not yet known (Schonheit and Schafer 1995).

The development of an active thermophilic microbial population at Yucca Mountain could either occur via adaptation (mutation) of mesophiles, or by activation of dormant (hyper)thermophiles. Both processes may occur, although the former would probably be less successful because the heating rate (in the order of decades) may be too fast for genetic adaptation (which could require several mutations to occur simultaneously). It seems more likely that population shifts would occur because of dormant forms of (hyper)thermophiles being present and becoming active as temperatures increase.

5.3 pH

Each microorganism has a pH range within which growth is possible, and each usually has a well-defined pH optimum. In Chapter 3, microbial strategies were discussed for adaptation to pH ranges outside of the 'normal' pH range which for most natural environments lies between pH 5 and 9. Since pH excursions would be expected in a potential Yucca Mountain repository, especially towards higher pH regimes because of the likely presence of concrete, this section discusses the occurrence and habitats of alkalphiles and acidophiles, as well as some further detail on alkaline tolerance and adaptation in view of the expected pH regime at YM.

5.3.1 Occurrence and Habitats of Acidophiles and Alkalophiles

Microbial communities that were developed on glass slides suspended in acid polluted (pH 2.9) and non-polluted (pH 6.5) but otherwise chemically similar waters, showed evidence of stress when suspended at the opposite station (Mills and Mallory 1987). Glucose incorporation was inhibited in both translocated communities, but the inhibition was not as severe and recovery of activity was faster for the acid-developed community as compared to the circumneutral community. The communities contained a substantially different set of members with little overlap. The range of pH values at which the members of the acid-developed community could function suggested that the members of that community were generalists, as opposed to narrowly constrained members of the community of the circumneutral station. The organisms from the

acid site were more general in their abilities as compared with their neutral counter parts. These results support the concept that communities developed in extreme environments tend to be generalists, whereas those from mesic environments, due to higher levels of competition present, tend to be specialists, capable of fewer functions but better at those than the generalist. Generalists would be capable of a wider range of function and perhaps have a wider tolerance range for environmental variables as well. The Yucca Mountain repository environment may, therefore, host mostly generalists, because of expected extremes in environmental conditions such as temperature, desiccation and pH deviations.

Most of the alkalophiles (optimum pH > 10.0, Krulwich and Guffanti 1989) that have been isolated and studied to date are aerobic procaryotes and most of the current knowledge concerning alkalophiles has been derived mainly from studies performed with aerobic (or facultative anaerobic) microorganisms. A few strictly anaerobic strains that are at least alkaline-tolerant (e.g., certain *Clostridium* and *Methanobacterium* strains) are known (Krulwich and Guffanti 1989) and only a few obligate anaerobic alkalophile organisms have been described, i.e., *Clostridium paradoxum* and *Clostridium thermoalcaliphilum*, both isolated from a sewage plant and both thermophilic organisms in addition to being obligate anaerobic alkalophiles (Li et al. 1994).

As discussed in Chapter 3, a sodium-proton antiporter appears to be required for homeostasis in alkaline media. According to Bingham et al. (1990) the mechanism of adjustment to a pH change might also involve differential gene expression, as in the bacterial response systems for heat shock, osmotic shock and nutrient starvation. So far, there are few reports of pH-regulated gene expression and the work by Bingham et al. (1990) is the first report of a gene in *E. coli* showing induction in the extreme alkaline range of growth.

Microbes play an important role in soil pH and vice versa. The pH influences the activity of soil microbes and also determines phosphorus availability in soil because microbes use acid production to solubilize insoluble phosphate (Gaind and Gaur 1989). Labile organic phosphorus compounds are mineralized in soils by microbial enzymes collectively called phosphatases. The sources of extracellular enzymes are from the many and varied organisms found in the soil and soil pH determines extracellular phosphatase activity that catalyzes the hydrolysis of esters and anhydrides of phosphoric acid. Phosphomono-esterases and to a lesser extent phosphodiesterases are the most widely studied of the soil phosphatases. Both acid and alkaline phosphomonoesterases occur in soils and some with a neutral pH optimum also occur. Phosphodiesterase activity in soil is usually optimum at pH values ranging from 9 to 11. Acid phosphatases are produced by bacteria, fungi, yeasts, protozoa, mycorrhizal fungi and plant roots. Alkaline phosphatases are produced by bacteria, fungi and earth worms (Herbien and Neal 1990).

5.3.1.1 (Obligate) Acidophiles

Although most bacteria grow best at neutral pH, acidophilic bacteria exist and some are even obligate acidophilic, unable to grow at all at neutral pH. Probably the most important factor for obligate acidophily is the plasma membrane, which actually dissolves when the pH is raised to neutral. The cells lyse, suggesting that high concentrations of hydrogen ions are needed for

membrane stability (Brock and Madigan 1991). Obligate acidophilic bacteria include several species of the eubacterial genus *Thiobacillus* and several genera of archaebacteria, including *Sulfolobus* and *Thermoplasma*. *Thiobacillus* and *Sulfolobus* oxidize sulfide minerals and produce sulphuric acid, thereby creating their own pH environment. Reactions for sulfur oxidizing bacteria are:

$H_2S + 2O_2 \text{ ----> } SO_4^{2-} + 2H^+$

$S^0 + H_2O + 11/2\ O_2 \text{ ----> } SO_4^{2-} + 2H^+$

$S_2O_3^{2-} + H_2O + 2O_2 \text{ ----> } 2SO_4^{2-} + 2H^+$

Some sulfur bacteria may bring pH down to <1. When growing autotrophically, the sulphur bacteria use the Calvin cycle to fix CO_2. Some are able to grow using organic compounds and a few are able to grow anaerobically on reduced sulfur compounds using NO_3^- as electron acceptor.

Most iron oxidizing bacteria also oxidize sulfur and are obligate acidophiles (Brock and Madigan 1991). Aerobic oxidation of Fe^{2+} to Fe^{3+} is an (low) energy yielding reaction for a few bacteria. They must oxidize large amounts of Fe^{2+} to get sufficient energy. Fe^{2+} is only stable at low pH, which explains the acidophilic character of iron oxidizers. *Thiobacillus ferrooxidans* grows autotrophically on either ferrous iron or reduced sulfur compounds. Another iron-oxidizing bacterium is *Sulfolobus* which lives in hot, acid springs at temperatures up to the boiling point of water. *Sulfolobus* can oxidize ferrous iron, but also sulphide, elemental S and a variety of organic compounds. It can therefore grow either lithotrophically or organotrophically. In *Thiobacillus* ferrooxidans the internal pH is about 6, yet its preferred environment is near pH 2. This pH difference across the cytoplasmic membrane represents a membrane electrochemical gradient that can play a role in ATP synthesis. However, to maintain a neutral pH environment, protons entering the cell through the proton translocating ATPase (driving the phosphorylation of ADP in the process) must be consumed and this occurs in the reaction:

$2\ Fe^{2+} + 1/2\ O_2 + 2H^+ \text{ ----> } 2Fe^{3+} + H_2O$

Thus, although energy conservation in *Thiobacillus ferrooxidans* results from a classical chemiosmotic ATPase reaction which couples the entry of protons to the synthesis of ATP, the proton gradient in *Thiobacillus ferrooxidans* is not established as a result of electron transport but instead is a simple consequence of the natural habitat of the organism.

One of the most common forms of iron and sulphur in nature is pyrite, FeS_2. The bacterial oxidation of pyrite is of great significance in the development of acidic conditions in mines and mine drainage, by the following reactions:

$FeS_2 + 3\ 1/2\ O_2 + H_2O \text{ ----> } Fe^{2+} + 2SO_4^{2-} + 2H^+$

The resulting acidic conditions stabilize Fe^{2+} ions, which are then oxidized to Fe^{3}+ ions by Thiobacillus ferrooxidans. The Fe^{3+} ions can then spontaneously react with more pyrite:

$FeS_2 + 14\ Fe^{3+} + 8H_2O$ ----> $15\ Fe^{2+} + 2SO_4^{2-} + 16H^+$

This continues with the Fe^{2+} ions being oxidized to Fe^{3+} ions again and so on. Acid mine drainage occurs because of the above cycle. The rate limiting step, the oxidation of Fe^{2+} ions to Fe^{3+} ions occurs at acid pH only in the presence of bacteria that require O_2. This explains how coal deposits are stable until mined, upon which a problem develops unless the rock does not contain pyrite. The above processes are made use of in so-called microbial leaching or biomining of low grade Cu (and other metal) ores, using piles or dumps in which the following reactions occur:

$Cu_2S + O_2$ ----> $CuS + Cu^{2+} + H_2O$

$CuS + O_2$ ----> $Cu^{2+} + SO_4^{2-}$

$CuS + Fe^{3+} + H_2O$ ----> $Cu^{2+} + Fe^{2+} + SO_4^{2-}$

$Fe^0 + Cu^{2+}$ ----> $Cu^0 + Fe^{2+}$

$Fe^{2+} + O_2$ ----> (*Thiobacillus ferrooxidans*) Fe^{3+}, which oxidizes more CuS when fed back into the leaching pile.

The dominant bacteria in acid mine drainage are acidophilic autotrophs but some heterotrophic bacteria grow in acid mine drainage and coal spoils (Mills and Mallory 1987). Although adapted to life in acidic conditions many of the heterotroph strains characterized in acid mine drainage had in fact a pH optimum near neutrality. Therefore, acid mine drainage seems to lower diversity but not the physiological potential of the microbial community (Mills and Mallory 1987).

A study by Jerez et al. (1988) found that, when cultures of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* were shifted from 30 to 41°C, cells reduced general protein synthesis with the concomitant increase of a specific group of heat shock proteins. A pH shift from 3.5 to 1.5 also elicited an apparent heat shock-like response in these bacteria. This work confirms again the existence of cross protection (Section 5.7). Whether these organisms would be prevalent at Yucca Mountain depends on the availability of pyrite, sulphur and O_2 in the environment. Cross protection, however, is an important consideration for microbial populations at Yucca Mountain. These populations will be subjected to heat, desiccation and nutrient deprivation, and all these factors may induce a low or high pH tolerance and adaptation.

5.3.1.2 (Obligate) Alkalophiles

Most bacteria thrive in neutral range environments and, generally, a pH greater than 10 should inhibit many microbes, but there are alkali tolerant and alkalophilic organisms belonging to a number of genera, some being inhabitants of normal soil and water environments. Alkaline-tolerant organisms show optimal growth in the pH range 7.0 - 9.0 but cannot grow above pH 9.5, and alkalophilic organisms show optimal growth between pH 10.0 and 12.0. The extreme

alkalophiles can be subdivided further into facultative alkalophiles, which show optimal growth at 10.0 or above but which can grow well in neutral pH range, and obligate alkalophiles which show optimal growth above pH 10.0 but cannot grow below pH 8.5 - 9.0 (Krulwich and Guffanti 1989). Amongst obligate alkalophiles, *Bacillus* species appear the most common and are easily recovered from most soils, presumably including those in the vicinity of a waste disposal site. For instance, Tiwari (1993) reported that the soil pH in the vicinity of a cement factory increased, with resulting shifts in the dominant microbial species in those soils, especially in the fungal flora.

Alkalophilic bacteria form a diverse group, ranging from eubacteria to archaebacteria (see for instance Table 1 in Krulwich and Guffanti 1989). Both Gram-negative and -positive non-spore-formers have been described and strains of Gram-positive endosporeformers, particularly *Bacillus* strains are well-represented. Most of the alkalophilic organisms are aerobic or facultative anaerobic, but a few strictly anaerobic strains that are at least alkaline-tolerant (e.g., certain *Clostridium* and *Methanobacterium* strains) are known.

Bath et al. (1987) have studied alkaline springs in Oman because the hydrogeochemistry and microbiology of these springs could be considered an analogue for the cement-induced high pH porewaters expected to develop in certain types of radioactive waste repositories. Their work is important for the Yucca Mountain environment because concrete structures may form an important part of the repository, and high porewater pH values may be expected. The natural bacterial populations indigenous to the alkaline springs in Oman would provide an indication of what microbial activity might be available in the cement-influenced (reducing) environment of a repository.

The study by Bath et al. (1987) included many microbial enrichments from the alkaline spring waters which had pH values ranging from 11.2 to 11.4. No nitrifying, denitrifying, sulphur oxidizing and methanogenic bacteria were found. A wide range of colony types were isolated from aerobic and anaerobic heterotrophic plates incubated at 30°C. Most of the isolates were alkalotolerant, with very few, under strict definition, being alkalophilic and the two isolates judged to be alkalophilic were strict aerobes (*Caulobacter* and *Flavobacterium* sp.). They also found some photosynthetic microorganisms, but those species would not be relevant for the repository environment at Yucca Mountain. An alkalophilic facultative anaerobe isolate was identified as a *Bacillus* species.

Identification of isolates showed that the bacterial population of the alkaline spring waters was similar to those of less extreme environments, with *Bacillus* and *Clostridium* species dominating. Most isolates had a relatively wide pH range for growth, from pH 6.9 to beyond 10 and in some cases beyond 11. Sulphate reducing bacteria (SRB) isolated were likely lactate oxidizing SRB of the genera *Desulfovibrio* or *Desulfotomaculum*. Gradient pH agar plates were used to grow SRB and it appeared that SRB could grow to at least pH 10.2 in mixed cultures. In single cultures maximum growth occurred between pH 8.5 and 9.5, likely because SRB are community organisms, gaining nutritional requirements from the products of other bacteria.

In nutrient status, the alkaline spring waters were predominantly reducing, oligotrophic, containing little nitrogen and negligible phosphorus. They were also low in sulphate but other forms (more reduced) of sulphur may have been available (e.g., thiosulphate) for SRB. The counts of bacteria present were low (10^1 to 10^3 /mL). According to Brock (1966), 10^6 bacteria/mL would be required for any significant environmental effect to be seen as a result of microbial activity. However, over the geological time scales considered in repository performance, low reaction rates due to small microbial populations may still be important. Bath et al. (1987) concluded that the high pH (pH 11.2 - 11.4) in these alkaline spring waters was not a limiting factor for microbial growth but rather the lack of nutrients were, especially with regard to carbon, nitrogen and phosphorus. Phosphorus limitation is serious because this element is involved in energy transfer in microbes and its deficiency is more likely to limit productivity than any other material except water. Restricted soluble nutrient supply could be compensated for by rapid turnover (cryptic growth), but this was not thought to be the case in these springs.

The most interesting microbial findings in these alkaline spring waters with relevance to cementitious repository environments were Clostridia and SRB. There is a common association between Clostridia and SRB in less extreme environments. Some *Clostridium* species can convert CO_2 to acetate which may act as substrate for SRB. However, autotrophic strains of SRB exist, which would not depend on fermenters for organic carbon substrate supply. Concrete porewater could, depending on the formulation, have a pH of 13 - 11 for thousands of years, after which it would decrease to 10.5 and lower, due to leaching of calcium hydroxide. SRB can participate in steel corrosion and they may also affect concrete integrity by the production of H_2S. However, despite finding SRB at relatively high pH in alkaline spring waters, this does not prove that they could survive the higher pH of concrete pore water and derive energy from steel canister corrosion in a repository.

Marine environments abound in alkaline-tolerant bacteria belonging to many genera, with smaller numbers of true alkalophiles which usually belong to the major alkalophile genus *Bacillus*, but although most extreme alkalophiles are *Bacillus* species, extensive studies of highly alkaline saline lakes have yielded only one possible member of this group. There may be special biological problems associated with the combined stresses of halophilicity and alkalophily (Krulwich and Guffanti 1989). Highly saline waters could possibly occur locally at the Yucca Mountain site, as a result of evaporation and redissolution phenomena.

Most truly alkalophilic organisms have been isolated from very specific man-made or natural environments, or from enrichment culturing of soil. Such enrichments have yielded eubacterial alkalophiles (usually *Bacillus*), facultative alkalophiles (genus *Flavobacterium*) and alkalophilic actinomycetes. Notably, the alkalophilic actinomycetes and many of the alkalophilic *Bacillus* species have been isolated from soils that are not particularly alkaline. Although the organisms are more prevalent in alkaline soils, true alkalophiles have been isolated even from acidic soils. Presumably there are microenvironments that allow the growth of even extreme, obligate alkalophilic organisms (Krulwich and Guffanti 1989). This is important with respect to the Yucca Mountain environment, because it is likely that alkalophilic microorganisms will be present naturally which could then more easily adapt to, and thrive in, a cement-induced high pH environment.

5.3.1.3 Concrete Environments

Of particular interest for the Yucca Mountain environment is the potential attack of microbes on concrete materials under both oxidizing and reducing conditions. Experimental evidence of microbial degradation of concrete under anaerobic conditions is very limited and the effect of SRB on concretes has not been studied widely. Moosavi et al. (1985) studied corrosion of reinforced concrete exposed to various environments, including SRB cultures and found that the biogenic sulphide permeated through the concrete, albeit slowly, and subsequently pitting corrosion of the reinforcement occurred. Since a sulphide film on steel would be more voluminous than the protective oxide film, spalling may also occur in reinforced concrete.

Rogers et al. (1993) discussed biological activity as the causative factor in failure of concrete in sanitary sewers. Three groups of organisms have been identified that are destructive to concrete in sewers and elsewhere, i.e., sulphur oxidizers, nitrifiers and heterotrophs. Of the sulfur oxidizers, the genus *Thiobacillus* is generally identified in sulfuric acid attack of concrete structures. They are rod-shaped, Gram-negative, chemoautotrophs that obtain energy by oxidizing reduced, inorganic sulphur sources such as elemental S, thiosulphate and polythionates. Species involved are *T. neapolitanus*, *T. intermedius*, *T. novellus*, and *T. thooxidans*. All need S, O_2, CO_2 and sufficient moisture for the reaction: $S + 1\ 1/2\ O_2 + H_2O$ ---> H_2SO_4. While many *Thiobaccilli* are acidophiles, others prefer near neutral or even alkaline conditions as high as pH 9. Their temperature range is 20 – 50°C ,with extremes on either end.

Of the nitrifiers, the species *Nitrosomonas* and *Nitrobacter* are chemoautotrophs (carbon source is carbon dioxide, carbonates or bicarbonate). They oxidize inorganic nitrogen compounds (e.g., ammonium) for energy while making nitric acid and have fairly neutral pH requirements. They are often implicated in the deterioration of buildings made of concrete or sandstone (Diercks et al. 1991).

Heterotrophs oxidize reduced organic carbon to CO_2 and/or organic acids and include a wide variety of organisms such as fungi and aerobic and anaerobic bacteria. Their progressive growth could lower the pH of a cement-induced high pH environment making conditions more favourable for acidophilic autotrophs such as *Thiobaccilli*. They could also cause an increase in radionuclide solubility because of organic acid production. However, these acids may be further used by other bacteria and may, therefore, not be available for solubilization reactions. Heterotrophs have not been studied specifically in the context of concrete corrosion, but Rogers et al. (1993) reported that at least 42 microbiological species have been identified in environments associated with concrete corrosion, further suggesting that there are sources in addition to autotrophs influencing concrete corrosion.

In sewer pipes, CO_2 and H_2S, formed by heterotrophs and SRB, condense on the cement surface with water vapor forming weak acidic conditions. The condensates react to form carbonates and thionates which lower the surface pH to below 11, followed by bacterial growth of Thiobacilli species that are weakly acidophilic. Once their acid production has lowered the pH to below 5, *T. thiooxidans* begins to grow (Rogers et al. 1993). This describes an ecological process spanning large ranges in pH, in which the high cement pH is modified by Thiobaccilli such that

obligate acidophiles can grow in what essentially is a high pH dominated environment. This illustrates the extent of microenvironment formation possible with microbial activity. It also illustrates that pyrite containing aggregate should not be used for concrete or that concrete structures should not be built on or in contact with pyrite containing rock. Both may be considerations to keep in mind for the Yucca Mountain environment.

The above shows how acid producing bacteria may modify high pH environments. A study by Kalin and McCready (1991) shows that alkali generating microbes can be used to treat acid mine drainage by adding organic matter (straw and saw dust) to various types of acid drainage. Alkali generating populations identified included iron reducers, sulphate reducers and ammonifiers. In coal acid mine drainage amended with organic matter, the microbial alkali generation is dominated by ammonifiers ($N_2 + 6H^+ + 6e$ ----> $2NH_3$). Concentrations of heavy metals in the acid drainage waters decreased with concurrent increases in pH (3.2 to 6.5) in localized areas of the test cells.

5.4 NUTRIENTS

The undisturbed environment at YM is expected to be oligotrophic with respect to the supply of nutrients available for bacterial growth. Bacteria in oligotrophic environments have a number of strategies to survive in such a situation, as discussed in Chapter 2. These include survival in nearly total metabolic arrest, miniaturization of cells, changes in macromolecular quantities and cellular densities, enhancement of cell membrane transport mechanisms and changes in genetic expression (Amy 1997). Cells in natural settings, by the nature of their surroundings, eventually reach a state of starvation-survival under most conditions (Poindexter 1981). This is the case in both aquatic (including marine) and soil environments and in the deep subsurface where total counts generally exceed culturable counts by several orders of magnitude. If lack of culturability is an indicator of dormancy, this would suggest that most subsurface environments contain dormant (or dead?) organisms (Amy 1997).

Viable but nonculturable (VBNC) cells appear to have to undergo some sort of resuscitation to become culturable again, which, depending on the organism, can be accomplished through treatments such as low temperature, warming, short growth period on non-selective medium followed by replicate plating on selective medium, ionic shock, and distilled water. Haldeman et al. (1994) noted an increase in the viable biomass and a decrease in species diversity in groundwater samples with increased storage time (at 4°C) and attributed this to the resuscitation of previously non-culturable biomass.

There are probably many other as yet untested resuscitation ways (Amy 1997) and even in subsurface environments dominated by dormant bacteria, the potential for resuscitation of indigenous bacteria exists. This potential has great implications, especially in the field of bioremediation. The potential introduction of large amounts of microbial nutrients and energy sources during the excavation and construction of a YM repository will almost certainly affect the viability of the indigenous population, as well as the populations that will be introduced with the repository materials.

5.4.1 Nutrient Limitation and Supply in a Repository

Much of the literature available regarding the impact of nutrient supply and regulation on multi-species populations comes from the studies of consortia for remediation and production purposes. Because of this, much of the literature is oriented toward the design of a particular process and is conducted on pre-selected populations. However, some useful information can be gleaned from this literature (e.g., Hazen 1997) but is not further discussed here.

In a high-level nuclear waste repository setting, some nutrients would be available for microbial activity from the groundwater and rock, but considerably more would be introduced by excavation processes, the waste packages and the engineered barrier materials emplaced in such a repository. For instance, Stroes-Gascoyne (1989) calculated limiting nutrients and resulting microbial population sizes for a Canadian repository based on in situ available nutrients plus those introduced through the emplacement of waste containers, buffer and backfill materials. This methodology (McKinley et al. 1985) results in the calculation of maximum population sizes based on the limiting nutrient (and energy constraints). However, this is of limited use because the calculated population sizes are likely much larger than the ones encountered in reality (not all nutrients are readily available), and moreover, it is not easy to relate a certain population size to chemical effects, although Brock (1966) has stated that 10^6 bacteria/mL would be required to see any significant environmental effect as a result of microbial activity. McKinley and Hagenlocher (1993) further developed such limiting nutrient and energy constraint calculations to produce a biomass production rate which was then converted to organic complexants to assess the impact of this biomass on radionuclide mobility in a Swiss repository.

Introduction of nutrients during construction and operation of a repository can also add to the nutrient supply. For instance, explosives may be encountered as residue in a geological repository, depending on the mining techniques that are used. Stroes-Gascoyne et al. (1996a) and Stroes-Gascoyne and Gascoyne (1998) determined the potential introduction of large amounts of easily usable nutrients into a Canadian nuclear fuel waste repository in granite as a result of excavation with explosives (ammonium nitrate and diesel fuel). Large amounts were mainly associated with the excavated rock, and leachates from this rubble enhanced bacterial population by as much as two orders of magnitude.

Explosives have also become an important subject in bioremedial study. Boopathy et al. (1994) determined that carbon source was significant to the rate of TNT bioremediation. Molasses was the most effective carbon source of those attempted, which included succinate, citrate, acetate, glucose, sucrose and malic acid. But all of the rates were on the order of 1-10 ppm/h. For bioremediation purposes, such a distinction will be economically important. However, in a repository environment, where organic carbon sources (such as diesel fuel) may be controlled and limited (Meike 1998b), such ranges of rates might be used for a first order approximation of time to degradation of complex compounds. It is possible that the finer distinctions of carbon source may disappear, or may be simplified because the more general trends are all that can be resolved on the geological time scale over which changes in a repository need to be predicted.

Meike et al. (1999) performed long term (~one year) nutrient limitation studies using a somewhat concentrated (100x J-13) water in which the major nutrients were systematically limited and

microbial species and communities found in Topopah Spring Tuff. Results from these experiments suggested that the dominant microbe population is determined by the available nutrients and that the resultant chemical effect is population specific. This study also documented oscillatory chemical behaviour.

5.4.2 Rates of Metabolism

Specific consortia studies for bioremediative purposes provide first estimates of optimum rates. Such parameters may be useful for determining whether certain microbial activities are at all significant. For example, Lackey et al. (1994) have studied the degradation of organic waste mixtures by consortia of native organisms. They determined that pulse feeding the bioreactors made biodegradation most efficient. This is related to the fact that the microbial population is maintained at a sub-optimal nutritive status, and is, therefore, ready to make efficient use of nutrient supply when it is provided.

Dawes (1976) reviewed the literature concerning the metabolism of prokaryotes as it relates to starvation and survival. He emphasized that survival mechanisms vary from organism to organism, but that in general, microorganisms that are adapted to low nutrient environments are better equipped for long-term survival than organisms accustomed to regular moderate and high nutrient conditions or feast and famine conditions (such as those that might exist in the mouth or gut of a higher organism). To some extent, this may suggest that those organisms that are already existing in the repository are those that would remain over the long run. However the question remains which organisms will be able to take advantage of the introduced nutrients, and how increased microbial activity during those brief periods would influence the chemistry and hydrology of a YM repository environment.

Another question remaining is how suitable laboratory-derived reaction rates would be in a model for in situ microbial activity at YM.

5.4.3 Use of the Nutrient-Based Growth Models

McKinley and Hagenlocher (1993) calculated microbial biomass production based on a mass balance of the four most important microbial growth elements (C, N, P and S) and energy constraints in a Swiss repository. They showed that biomass production was limited by energy availability. They further assumed that all biomass was converted to large complexing molecules, in order to assess the effect of this biomass on radionuclide release and mobility. Their calculation showed that at worst, radionuclide complexation was enhanced by a factor of two, which falls easily within the safety margins of a Swiss repository. Stroes-Gascoyne (1989) used this approach to calculate maximum biomass production in a Canadian repository, and the MING (Microbial Impacts to the Near-field Geochemistry) code (TSPA 1998) is also based on the Swiss mass and energy balance concept, for the YM site. The MING code takes into account the availability of nutrients in a given repository/drift environment, the energy necessary to convert those nutrients to microbes, the level of oxygen in the repository, as well as the temperature and moisture content of the repository atmosphere. Materials identified in the proposed repository design are decomposed into their basic elements over time and their contributions to the nutrient cycle of microbes are included with those nutrients available in the

groundwater passing through the repository. The MING code and its associated conceptual model for microbial activity (Meike 1998a) attempts to quantify the overall global bulk effect of microbes on the near field geochemical environment. Results obtained with this model indicate that the estimates of microbe masses growing in the potential repository system suggest that effects on the near field geochemical environment would be negligible, but that microbially influenced corrosion (MIC) and other localized microbial attack of materials cannot be precluded. Consideration of microbes as colloids should also be given consideration in future work while it was also concluded that there is some potential for additional ligands generated locally (TSPA 1998).

In Section 2.1.1, expressions were discussed that can be used to describe microbial substrate transformation. The Monod equation best describes typical microbial kinetics. This equation formed the basis of a model developed by Humphreys et al. (1995) for intermediate and low level radioactive waste at the UK Drigg site that predicts in detail the extent of microbial transformation of cellulose and the concentrations of various metabolic byproducts that can affect radionuclide mobility. King et al. (1999) also used Monod expressions in a mathematical model developed to predict the extent of sulphate reduction by SRB in a Canadian repository. The model is based on a series of mass balance equations that describe the kinetics of sulphate reduction by two types of SRB (one organotrophic, one chemoheterotrophic), the growth and death of SRB, the supply and consumption of nutrients (acetate and hydrogen) and reactants (sulphate), and the consumption of sulphide by precipitation with aqueous Fe(II) or by (Cu) container corrosion. Simulations with this model showed that the amount of sulphate was limiting the SRB activity in time and that sulphide precipitated as FeS rather than causing corrosion. The latter was confirmed recently by results from the isothermal test at AECL's Underground Research Laboratory. Buffer (50% bentonite/50% silica sand) was buried for six years in a granite borehole, covered with a concrete cap. Upon excavation, viable SRB were found and the concentration of sulphide had increased slightly over the six-year period (Stroes-Gascoyne and Hamon 2000). However, the rate of sulphate reduction derived from this test suggested a much lower in situ SRB activity (and sulphate conversion) than assumed in the model by King et al. (1999).

This illustrates that assumptions necessary to develop and run microbial models need to be tested as close as possible to realistic repository conditions. Sometimes observations from natural analogue sites can be useful in achieving this. An example is the determination of microbial activity in clay-based buffer and backfill environments. Considerations were that pore throats in compacted or high clay content environments are smaller than average microbial dimensions. Laboratory studies showed that, depending on clay content, microbes could not migrate into compacted clay and in some instances could not even survive (Stroes-Gascoyne et al. 1997, Motamedi et al. 1996). Observations from the natural environment supported these considerations and findings: clay is not an optimum environment for microbes and their diversity and numbers are low when clay content is high in sediments (Chapelle 1993). Very few viable bacteria were found deep in the Boom clay deposit (Merceron 1994). Another example is shown at Pocos de Caldas in Brazil where the movement of uranium across a redox front was influenced by S cycle organisms (West et al. 1992).

5.5 RADIATION

The spectrum of electromagnetic radiation is continuous from extremely energetic short wavelength gamma rays to long wavelength low energy radio waves. Microbes at Yucca Mountain could potentially be affected by ionizing radiation from the radioactive waste in the repository and, depending on waste package design, exposure could vary from negligible to 0.06 Gy/min. (Pitonzo et al. 1999a). Any influence of non-ionizing (UV) radiation may occur only as long as the repository is open and illuminated by electric light, which could, for instance on wet surfaces, enhance the growth of phototrophic organisms and as such introduce organic material into tunnels and drifts of the repository.

Studies to determine the effects of radiation on microbes have been carried out with different types of radiation, i.e., gamma, ultraviolet (UV), electron beam, neutrons and microwaves. Often these studies have focused on vegetative cells of specific (non-sporing) organisms such as the highly radiation-resistant *Deinococcus radiodurans* or the extensively studied *Escherichia coli.* Natural populations in soils have also been studied (e.g., Popenoe and Eno 1962, Stotzky and Mortensen 1959), as well as spores (e.g., Farkas 1994).

Several forms of radiation are highly mutagenic. Mutagenic radiation falls into two categories, ionizing and non-ionizing. Although both kinds of radiation are used in microbial genetics, non-ionizing radiation such as UV has found the widest use, often because it is easier to work with UV and the effects are similar to those of gamma rays. In addition, many of the molecular mechanisms that repair UV damage are identical to, or overlap with, those involved in the repair of ionizing radiation damage. Similarly, although microwave radiation is not of concern in the Yucca Mountain environment and studies of the effects of microwave radiation on bacterial cells would be of most interest to the food industry, they may nevertheless reveal effects that are common to other types of radiation. Appendix C briefly reviews the kinds of damage each of these radiation types can cause in the bacterial cell.

5.5.1 Positive Effects of Radiation

Hormesis is the stimulation by sub-harmful doses of any agent. The hypothesis predicts that minute doses of ionizing radiation will benefit growth, development, nutrient utilization, reproduction, resistance to radiation and infection, and lifespan (Luckey 1982). Concepts of mechanisms of radiation hormesis should take into consideration that mutation is not a major factor since effects are rarely seen in progeny of irradiated individuals; that most effects are probably due to the indirect action of radiation upon biological fluids to produce free radicals; and that the complex mechanisms possible in mammals do not exclude equivalent responses in simpler organisms.

According to the review by Luckey (1982), about 70 reports pertaining to microorganisms provide ample evidence that radiation hormesis occurs independently of complex multicellular systems. Data from protozoan studies support the concept that ionizing radiation may be essential for optimum physiological performance (Luckey 1982).

Conter et al. (1984) studied the response of *Synechococcus lividus* to irradiation at a dose rate of 20 mGy/year and found the response to be dependent upon both the age of the inoculated cells and lightning conditions. Incubation of cells in darkness or periodic lighting accentuated the stimulating effect of irradiation on log-phase or transient cells but for stationary cells, radiostimulation decreased when the cells were subjected to darkness or periodic lighting. These results suggested that the radiostimulation could be related to regulation of RNA synthesis through the Pentose Phosphate Pathway in this organism. If radiation dose was small, radiostimulation could potentially occur in a YM repository.

Whether the initial exposure to radiation is large or small, acute or chronic, internal or external, pre-exposure to radiation appears to increase an organism's resistance to subsequent lethal doses of radiation when compared with controls. This is radioresistance and its induction has also been noted for microorganisms which were previously irradiated (e.g., Pollard and Achey 1975, Smith 1973, as quoted by Luckey 1982).

5.5.2 Radiation Studies with Specific Microbes

Many studies have been performed with cultures of the highly radiation-resistant *Deinococcus radiodurans*. Appendix D gives important highlights from a considerable amount of mostly recent studies. Mattimore and Battista (1996) conclude that *D. radiodurans* is an organism that has adapted to dehydration and that its DNA repair ability is a manifestation of that evolutionary process. They believe that *D. radiodurans* is ionizing radiation resistant because it is resistant to desiccation and because desiccation resistance appears to require extensive DNA repair. Many studies on radioresistance have also been carried out with *Escherichia coli*. These are also reviewed in Appendix D because both sets of studies may aid in the understanding of the potential radiation resistance of microbes at the Yucca Mountain site.

5.5.3 Radiation Resistance of Yucca Mountain Isolates

The many studies with *Deinococcus radiodurans* and *E. coli* reviewed in Chapter 3 and Appendix D have shown that there are many mechanisms that may play a role in radiation resistance in microbes. Because the natural population of microbes at Yucca Mountain is expected to be adapted to desiccation, some naturally occurring microorganisms at YM may also be radioresistant to a certain degree. The radioresistance of microbes in rock samples from the YM site was studied by Pitonzo et al. (1999a). Radiation-resistant microorganisms in the rock samples became viable but not culturable (VBNC) after a cumulative dose of 2.33 kGy and their metabolic capability was reduced considerably. In a subsequent study, Pitonzo et al. (1999b) stored the irradiated rock samples for 2 months at 4°C, in an attempt to resuscitate the VBNC microbes to a culturable state. Results showed that microbes can survive considerable radiation doses in a VBNC state, and recovered some of their previous culturable and metabolic capabilities, once the radiation stress was removed. For a YM repository this could imply that radiation will likely curtail activity and metabolic capability of microbes (indigenous or otherwise) present, but that part of this activity can be restored once radiation fields have declined.

5.5.4 Environmental Influence on the Radiosensitivity

A large range in the sensitivity of microbes to radiation has been observed and it is likely that environmental factors determine to a certain (or even a large) extent the effects. Many types of environmental variations appear to have an important influence on the relative radiosensitivity of particular types of cells (De Serres 1961).

5.5.4.1 Oxygen Concentration

It has been shown that there is a marked reduction in radiosensitivity under conditions of anaerobiosis in a variety of microorganisms. A greater resistance to lethal effects is obtained with anaerobiosis even when the cells are frozen. Cells cultured under anaerobic conditions were more radioresistant than cells grown aerobically. Endogenous metabolism in dense cell cultures causes anoxia and a decreased sensitivity. The concentration of oxygen required to produce a marked change in radiosensitivity is very small and nearly the same in a variety of different organisms (De Serres 1961). Antioxidant defense mechanisms such as the scavenging enzymes catalase, superoxide dismutase and peroxidase likely play an important role in the radioresistance of *Deinococcus* species, by preventing the accumulation of reactive oxygen species (Wang and Shellhorn 1995).

5.5.4.2 Culture Conditions

Sensitivity to radiation varies, depending on culture conditions. For *E. coli* it was observed that the cells were most sensitive when grown under aerobic conditions without glucose, whereas cells grown anaerobically with glucose were the least sensitive. The nature of protection could be some metabolic byproduct present in the cell that acts as an internal protective agent. Post-irradiation culture conditions also have a large influence on the radiation sensitivity and post-irradiation treatment can have quite different effects on survival and mutation. The addition of certain factors (such as glutamic acid, guanine and uracil) to the medium seems to promote increased survival and a decrease in the rates of mutation (De Serres 1961).

Krabbenhoft at al. (1967) showed that cultures of *Micrococcus radiodurans* (now *Deinococcus radiodurans)* grown in TGYM medium (tryptone, glucose, yeast extract, DL-methonine) are ten times more radiation resistant than cultures grown in PCNZ medium (plate count agar, containing tryptone, yeast extract and glucose, supplemented with Nzcase, a tryptic digest of casein). It was shown that this was not caused by physiological age differences (cells grow faster and enter death phase earlier in PCNZ medium), relative amounts of DNA, RNA and protein in the cells or the pH of the medium. No change in the tetrad cellular arrangement occurred either and cell mass was similar. Results suggested that the amount of radiation-sensitive sites (for 'hits') had been increased or the number of radioprotective units decreased in cultures grown in PCNZ medium. This study provided evidence that the growth medium caused cellular biochemical alterations, and that precursors of factors related to increased radiation resistance were probably present in PCNZ medium grown cells, but that the factors themselves could not be synthesized in PCNZ medium: Exposure of PCNZ-grown cultures to TGYM medium prior to irradiation restored radiation resistance to that of TGYM medium grown cells.

5.5.4.3 Temperature

Temperature also has an influence on radiation damage. The effects apparently are much less at below freezing temperatures depending on rate of freezing, oxygen content, etc. The effects of temperature seem to vary per species but seem small in the normal growth range temperatures. Post-irradiation temperature is also important but highly dependent on species (De Serres 1961). For instance, the maximum kill of irradiated spores has often been observed to occur at 0°C. At lower temperature, survival increases because ice traps radiation-induced harmful free radicals. At high (but sub-lethal) temperatures (50-70°C), radiation resistance may be higher than at or below room temperature, probably due to enhanced annealing of free radicals at higher temperatures (Farkas 1994). Thayer et al. (1990) found that the temperature of irradiation but not the presence or absence of air significantly influenced the survival of several *Salmonella* species in chicken. These species were strongly protected against irradiation by temperatures below -20°C and sharply increased survival occurred when the samples were irradiated in the frozen state.

The temperature in the Yucca Mountain repository will vary locally, spatially and temporally, but is expected to be very high (>100°C) in most parts for many years. These high temperatures would cease the activity of most if not all microbes and would, therefore, not likely afford protection against radiation damage at that time. However, microbes preconditioned at temperatures during the heating and cooling phases just before and just after the loss of activity could provide some protection against radiation damage that might occur after that preconditioning. The temperature preconditioning could occur at different times and localities in the repository due to its chemical and physical heterogeneity.

5.5.4.4 Water Content

There is a direct relationship between the water content and the radiation sensitivity of a microbial population. The drier the organisms, or their environment, the less sensitive they are (De Serres 1961). In experiments in which various chemicals were used to dehydrate cells of *Saccharomyces cerevisiae* a progressive decrease in radiosensitivity was found with the increase in glycerol concentration. Additional protection was afforded by anoxic conditions in the above cases (De Serres 1961). Ward et al. (1981) irradiated dried sewage sludge and studied the effects on indigenous and inoculated enterics (such as *Klebsiella* sp., *Enterobacter* sp., *Proteus mirabilis, E. coli, Streptococcus faecalis, Salmonella typherium*). They concluded that the rates of bacterial inactivation by ionizing radiation in sludge could be altered by changes in water content but that the amount and direction of alteration varied between bacterial species. In no case was moisture loss found to result in an excessively large D_{10} value (i.e., the total dose of radiation needed to reduce the microbial population an order of magnitude).

Moussa and Diehl (1979) showed that radiation resistance in three *Salmonella* species increased steeply with a reduction in water activity (a_W) from 1.0 to 0.8, less steeply between 0.8 and 0.5 and remained constant below a_W of 0.5.

5.5.4.5 Biological Factors

Alterations in radiosensitivity can be brought about by various environmental factors that modify the intracellular environment indirectly. In addition, there are a number of biological factors that modify the intracellular environment more directly and some of these also have been found to bring about marked changes in the radiosensitivity of certain groups of cells. These factors include correlations with the various phases of the growth (or division) cycle (De Serres 1961). Lag phase cells had an increase in resistance to X-rays, logarithmic phase cells showed a slow but steady decay in resistance with increasing time and the stationary phase was distinguished by a gradual return to the initial sensitivity. Haploid and diploid strains of yeasts reacted differently to radiation and generally there were differences in radiosensitivity amongst different strains of a species.

There is a wide range in the sensitivity of various microorganisms to X-irradiation and also considerable variation in the shape of the survival curves. For instance the survival curve of *E. coli* strain W-1485 was found to have two exponential components, about 66% of the cells were more sensitive than the other 34%. Subculture of either the sensitive or resistant cells always gave cell populations with the same mixture of sensitivities (De Serres 1961).

5.6 PRESSURE

Significant pressure development is not expected at YM and, therefore, pressure effects on microbial activity and growth are not discussed here. However, the effects of pressure on microbial life are discussed in Appendix D, because high pressure has an influence on microbial activity at high temperature, which could potentially be of importance if microbial activity were to occur in the waste package itself.

5.7 SIMULTANEOUS STRESS FACTORS - CROSS-PROTECTION AND NEGATIVE IMPACTS

Adaptation of microbes to a given stress may increase or reduce tolerance to other stresses. An example is cross-protection against environmental stresses such as heat, low pH and oxidation, induced by osmotic shock and nutrient (e.g., carbon) starvation (Gauthier and Clement 1994). A possible reason for this is that starvation induces the formation of certain types of stress proteins which are thought to renature improperly folded, denatured proteins (Foster and Hall 1991).

Cross-protection effects would be important with respect to survival and adaptation of naturally present and introduced microbes in the changing environment induced by the repository at Yucca Mountain. For instance, the groundwater at Yucca Mountain is oligotrophic and the naturally present microbial population may, therefore, be in a starved state, possibly affording some resistance to heat and desiccation which would occur as a result of the construction and operation of a repository. In the following sections, a number of multi-stress case studies with vegetative cells and spores, relevant to the YM environment, are discussed, to illustrate the complexity and potential of microbial adaptation to a variety of concurrent environmental stresses.

5.7.1 Cross-Protection in Vegetative Cells

5.7.1.1 Starvation and Heat Resistance

Starvation-mediated protection of bacteria against heat, oxidative stress, acid stress, autolysis and light has been reported for several species (Jorgensen et al. 1994). The resistance produced by starvation may even be more protective than preadaptation to the particular stress itself. Starvation-associated heat resistance might, in part, be explained by the cross-induction of heat-shock proteins. However, heat shock proteins may not always play a role. Jouper-Jaan et al. (1992) demonstrated increased heat resistance in starved cells of a heat-shock protein-deficient *E. coli* strain. Three bacterial strains were submitted to heat stress after different times of starvation. All three developed starvation-mediated cross protection against heat. While shorter times (2 to 24 hours) of starvation gave near maximal protection, increased protection occurred after prolonged periods (9 d) of non-growth. An obvious de novo protein-synthesis-mediated induction of protection against heat stress during starvation was not found. Starvation-induced cross-protection against heat may be dependent on protein synthesis in the initial phase of starvation while after prolonged starvation the continuous protection offered may not be mediated by de novo protein synthesis.

Preyer and Oliver (1993) studied starvation-induced thermal tolerance as a survival mechanism in a psychrophilic marine bacterium. Results showed that starved cells generally show more resistance to heating than non-starved cells and because starvation is the normal physiological state of copiotrophic, heterotrophic bacteria in oligotrophic marine waters, the data of Preyer and Oliver (1993) suggest that starvation conditions may be a significant factor in providing heat tolerance in psychrophiles.

5.7.1.2 Osmotic Stress and Heat Resistance

Jorgensen et al. (1994) studied the effects of starvation and osmotic stress on the viability and heat resistance of a *Pseudomonas fluorescens* strain. Reduction of 5-cyano-2,4-ditolyl tetrazolium chloride (CTC) was used as a measure of viability in situ without addition of extra nutrients. Carbon-starved cells developed an increased heat resistance and prolonged starvation resulted in further protection. Viable but non-culturable (VBNC) cells were found during heat challenge, implying that culture methods underestimate the recovery potential of these cells. Osmotically stressed *Pseudomonas fluorescens* maintained a high viability, whereas culturability was rapidly lost. In contrast to starved cells, no protection against a subsequent heat challenge was found in osmotically-stressed cells, but an increased salinity of the heating medium alone resulted in elevated heat resistance of non-stressed cells.

5.7.1.3 Heat and Radiation Resistance

Lucht and Stroes-Gascoyne (1996) determined the effects of gamma irradiation at elevated temperatures (up to 90°C) on naturally occurring microorganisms in clay-based engineered barrier materials designed for use in a Canadian nuclear fuel waste disposal vault. Combination treatments of radiation and heat applied simultaneously gave rise to biphasic survival responses which suggested the presence of two population types in the clay-based material. One

population showed a decrease in radiation resistance with an increase in temperature, implying a synergy between heat and radiation effects. The second population showed an increase in radiation resistance with increasing temperature, possibly due to desiccation effects during irradiation.

5.7.1.4 Starvation and Acid (Low pH) Resistance

Cross-protection phenomena occur in many areas of microbiology. For instance, the ability of water-borne enteric pathogens to colonize the very acidic human gut is likely to depend on their structural and physiological state (Gauthier and Clement 1994). They remain infectious even in a dormant, VBNC state. Conditions prevailing in natural aquatic environments are generally harsh for enteric bacteria, but it may be possible that resistance to gastric pH conditions in various enteric bacteria and in natural coliforms from waste water and human feces could be modified by a short incubation in oligotrophic freshwater and seawater. Exposure of stationary phase cells to seawater for 100 minutes induced a high level of acid resistance (down to pH 2.5 for 2 hours) in *E. coli* and *Shigella* strains. Induction of this resistance was extremely efficient, as it increased acid resistant viable cells by orders of magnitude. The effect was considerably more marked in *E. coli* and *Shigella* species than for the other species tested (*Salmonella*, *Klebsiella* and *Yersinia*) which were characterised by a higher intrinsic acid sensitivity. A similar acid resistance was induced in fecal coliforms from wastewater and feces, and acid resistance could also be induced in phosphate buffers, and was only slightly lower than the resistance induced in seawater. It can, therefore, be assumed that the protective effect can develop in any kind of oligotrophic natural water, and that the acquired acid resistance is mainly induced by nutrient starvation, with possibly a slight additional effect due to osmolarity.

5.7.1.5 Starvation and Salinity

Two types of *E. coli*, one adapted to seawater and one not adapted, were exposed to starvation conditions in sterile seawater (Garcia-Lara et al. 1993). The number of viable (culturable) pre-saline adapted microorganisms remained constant during the test. However, the number of culturable non-adapted microorganisms decreased, but the number of cells did not. Both types of microorganisms increased DNA content and thymidine incorporation and maintained their metabolic potential. Scaravaglio et al. (1993) have shown that an *E.coli* is less resistant to low osmotic conditions when starved. This suggests that a somewhat saline environment is conducive to survival.

Thorsen et al. (1992) studied the long-term starvation survival of a fish pathogenic bacterium (*Yersinia ruckeri*) at different salinities. It was found that *Yersinia ruckeri* could survive in unsupplemented water for at least four months at 0 to 20% salinity. At 35% salinity, however, survival of the culture decreased. It was also shown that genome replication was initiated before the onset phase of starvation had been completed.

5.7.2 Cross-Protection in Spores

5.7.2.1 Temperature of Sporulation and Heat Resistance

Temperature limits are often very different for vegetative and spore-bearing growth. In some cases the temperature interval in which completion of the spore-bearing apparatus occurs is much narrower than the interval allowing vegetative growth. Heat resistance of spores is due predominantly to dehydration of the spore protoplast, to an increased concentration of Ca^{2+} and probably also to the structure of the spore membranes and to other (unknown) factors. The heat resistance of spores may be increased by sporulation temperature, and heat shock proteins may be involved. The temperature at which sporulation took place can have a considerable influence on spore heat resistance.

One of the triggers for spore activation is heat. In general, heat activation is a reversible phenomenon and dormancy is restored after a certain delay if the spores are not placed in conditions suitable for germination. Heat induction of spore germination in bacteria is restricted to the endospores produced by Bacillaceae and the requirements for heat activation vary greatly from species to species (Aragno 1981). A large number of hypotheses have been proposed for the heat activation of both bacterial endospores and the spores of fungi. Little is certain about the processes involved but in many cases high temperature is supposed to act primarily on membranes but it is not clear whether proteins or lipids or both are involved. In many fungi, spores require a treatment at low temperature before germination can occur. Contrary to heat activation, cold activation is not reversible and once spores are thus activated they cannot restore dormancy. The mechanisms of cold activation are also poorly understood.

Fungi may be important in the environment of Yucca Mountain because this environment will be unsaturated and the mycelia of fungi may be able to survive such an environment better than bacteria because of their ability to form spores and their ability to form mycelia with which they can bridge over dry spots in a heterogeneous environment. Temperature may induce developmental changes in many fungi (e.g., mycelial or yeast-like growth forms). The mechanisms of such biphasic growth patterns remains largely unexplained but may involve a cell wall composition modification in the transition from mycelial to yeast-like cells. The former contain mostly β-1,3-glucans, which give the hyphal walls a more rigid fibrous structure than the walls of the yeast-like phase cells containing mainly short-chained α-1,4-glucans (Aragno 1981).

Sedlak et al. (1993) studied the effects of heat shock applied early in sporulation on the heat resistance of *Bacillus megaterium* spores. Cells of *Bacillus megaterium* were heat shocked at 45°C for 30 minutes during various sporulation stages and then shifted back to a temperature permissive for sporulation. This induced the synthesis of heat shock proteins in the sporangia and delayed inactivation of the spores at 85°C. Heat shock proteins could be detected in the spores.

De Pieri and Ludlow (1992) studied the relationship between *Bacillus sphaericus* spore heat resistance and sporulation temperature. *Bacillus sphaericus* 9602 was grown in batch culture at various temperatures. At 10°C and 12°C the maximum sporulation yield was <10% while at 15,

20 and 30°C a sporulation yield of > 95% was achieved. However, at 40°C, *Bacillus sphaericus* grew only vegetatively. The heat resistance of spores grown at 15°C and 20°C was significantly higher than for those grown at 30°C. Such effects can be misleading, because in laboratory studies, temperatures of 30°C would be used to obtain a high spore yield, but the resulting spores would not have realistic heat resistance compared to those grown at more moderate temperatures as would be the case in many natural environments.

Beaman et al. (1988) concluded from their studies that heat shock affects permeability and resistance of *Bacillus stearothermophilus* spores. Partially activated spores were much more resistant to heat than the original dormant spores even though both had the same spore protoplast density. Heat shock induced a higher level of resistance in the partly activated spores. This might be explained if heat shock caused expansion of the cortical peptidoglycan against the intact coat with a resulting lower water content in the protoplast (and higher water content in the cortex). Also, elevation of temperature during sporulation causes increased heat resistance in the resulting spores. An alternative explanation is the selection, by heat shock, of a pre-existing super-resistant sub-population in an originally heterogeneous population of dormant spores.

These studies show that heat resistance of spores will be affected by the temperature at which they form in their environment. At Yucca Mountain, sporulation of the natural population may occur as conditions become less favourable for vegetative mesophilic cells, probably at slightly-raised temperatures. These spores will be more heat resistant than their vegetative parent cells but whether they can withstand the expected >100°C temperatures at Yucca Mountain is not known. It is likely that only a select few spore types (i.e., those from thermophilic vegetative cells, the super-resistant sub-population) will retain germination power after exposure to temperatures in excess of 100°C for a considerable length of time, as will be the case in the Yucca Mountain repository.

5.7.2.2 Relative Humidity and Heat Resistance

The Yucca Mountain environment will increase drastically in temperature after waste emplacement. An increase in temperature will reduce the relative humidity or water activity in this environment. A reduced water activity will have a negative effect on most vegetative cells and may also affect the viability (germination power) of spores.

Beaman et al. (1989) found that the low heat resistance of *Bacillus sphaericus* spores correlated with high protoplast water content. Pfeifer and Kessler (1994) studied the effect of relative humidity of hot air on the heat resistance of *Bacillus cereus* spores. A distinct maximum of heat resistance was found at 40% relative humidity for *Bacillus cereus* spores. At 122°C the inactivation rate constants at 40% relative humidity were five orders of magnitude smaller than at 100% relative humidity and two orders of magnitude smaller than at 1% relative humidity. At relative humidities of more than 40% the inactivation rate constants were strongly temperature dependent, whereas at lower relative humidities they were less temperature dependent. The occurrence of a maximum was ascribed to the existence of two inactivation mechanisms, the first is retarded and the second is accelerated by a reduction in relative humidity. The first mechanism is likely a protein denaturation, whereas the second may be an oxidative process, but no consistent theory could be developed yet (Pfeifer and Kessler 1994).

The Yucca Mountain environment is expected to be mainly oxidizing with a complex moisture content pattern that, in and near the repository, is expected to vary with episodic periods of moisture occurring only locally. Depending on the local and temporal relative humidity patterns, spores may survive high temperatures at different rates in a YM repository.

5.7.2.3 Nutrient Availability and Heat Resistance

Germination of heat-damaged spores will be greatly affected by the environment in which germination is to take place. For instance, Brown and Gaze (1988) evaluated the recovery capacity of heat-treated *Bacillus stearothermophilus* spores in six different growth media. The study was prompted by the fact that paper strips impregnated with spores of known heat resistance have been routinely used to monitor sterilization treatments in hospitals and the bottled fluids industry. The results of Brown and Gaze (1988) showed that the variation between the media was lowest when spores were recovered from unheated strips and increased greatly with the severity of heating. This showed that by choosing a medium which has a reduced recovery capacity it is possible to achieve a misleading indication of the lethal effect of a thermal process.

At Yucca Mountain, the growth environment is naturally oligotrophic, but may be locally varying because of the disturbances brought about by the repository construction processes (i.e., nutrients may be introduced). Whether this will affect spore recovery is not known.

5.7.2.4 pH, Salinity and Heat Resistance

Hutton et al. (1991) found that spores of *Clostridium botulinum* were less heat resistant when heated in a medium of decreased pH. Survival of the spore population decreased by 50% when heated in pH 5.0 buffer compared to pH 7.0 buffer. Addition of NaCl to the recovery medium reduced the number of colony forming units in a population of heated spores. The presence of 2% NaCl decreased survival by 20-40% irrespective of pH of the heating medium. Combined effects of pH and NaCl could be illustrated in 3D histograms.

At Yucca Mountain, neither pH nor salinity of the original groundwater is extreme. However, the addition of concrete may increase pH and the thermal pulse may evaporate water, leaving behind an evaporite crust which could subsequently dissolve and result in high salinity pore water. Therefore, these factors may be important, but inhomogeneous in both a spatial and temporal sense, for spore resistance or recovery at Yucca Mountain.

5.7.2.5 Radiation and Heat Resistance

Shamsuzzaman and Lucht (1993) studied the resistance of *Clostridium sporogenes* spores to radiation and heat in various nonaqueous suspension media. A comparison of the heat D_{10} (10% survival) values of the spores with and without prior irradiation in various media showed that irradiation at 5.0 kGy greatly increased their heat sensitivity. These results may be of importance to the Yucca Mountain situation if the waste packages to be emplaced are not self-shielding. In such a case, radiation would be present immediately upon emplacement of the waste packages, and would affect the microbial population very near to these packages. The subsequent increase

in temperature may then be more detrimental to the spores present than it would have been without the radiation field. If the waste packages are self-shielding, this effect would not be important at Yucca Mountain.

Kim et al. (1987) carried out preliminary studies on the radiation resistance of thermophilic anaerobic spores and on the effect of gamma radiation on their heat resistance. The study included spores of *Desulfotomaculum (Clostridium) nigrificans* and *Clostridium thermosaccharolyticum*. The combination of radiation treatment with a subsequent heat treatment had a synergistic sporicidal effect. The synergistic effect increased with the increasing radiation dose, although the heat sensitization in the dose range used and at the heat levels studied was relatively moderate. It appeared that the radiation resistance of thermophilic anaerobic spores (e.g., of importance to the canning industry) is in the same order of magnitude as that of mesophilic spores with much lower heat resistance. Radiation treatment decreases the heat resistance of surviving cells. Kim et al. (1987) concluded that factors which determine the radiation resistance and the heat resistance of spores are not the same. It may be that radiation treatment decreases the ability of spores to maintain the dehydrated state of their core (expanded cortex theory). It is possible that the higher the heat resistance, the more dehydrated the core of the spores is and the more radiation resistant the biophysical and physiological mechanisms are in maintaining the heat resistance, i.e., the dehydrated state of the core.

5.8. SUMMARY

The material in this chapter consists mainly of mechanistic details of microbial responses to physical stresses relevant to the YM environment. Water stress, as a result of desiccation (high temperatures) and osmotic stress (evaporation) were discussed by means of case studies reported in the literature. It appears that a rapid change in water potential may be more detrimental for microbial activity and survival than a low water potential per se. Since high temperatures will be a fact in a YM repository, the habitats and metabolic pathways (nutritional and energy) of (hyper)thermophiles were discussed. Adaptation of the in situ microbial to high temperatures can occur either via adaptation (mutation) of mesophiles, or activation of dormant (hyper)thermophiles. Both processes may occur although the former would probably be less successful because the heating rate (in the order of decades) may be too fast for genetic adaptation. It seems more likely that a population shift would occur as a result of dormant forms of (hyper)thermophiles becoming active upon temperature increase.

Case studies of the environmental occurrence of acidophiles and alkalophiles were reviewed in Section 5.3. Because of their unique adaptation strategies, microbial life can occur over the pH range of 1 to 12, and except near concrete bulkheads or in the case of concrete inverts or drift liners, quick changes in pH are generally not expected at YM such that the indigenous population can adapt in time. Insufficient nutrient supply can lead to VBNC microorganisms, which are probably not very active in situ but are nevertheless viable and awaiting their niche. Intermittent water flux may stimulate these VBNC organisms. Modelling efforts have used nutrient transformation as basis, but realistic in situ metabolic rates are needed for such models to produce reasonable results. Such rates are difficult to derive from laboratory studies, and natural analogues may be of use here. Radiation, which at low levels can have a stimulating effect on

microbial activity, and lethal effects at high levels, may or may not play a role at YM, depending on the eventual waste package design.

Of most importance to the YM environment are probably the documented cases where the occurrence of simultaneous stress factors result either in enhanced (cross-protection) or reduced resistance of microbial populations to these stresses.

6. IMPLICATIONS FOR THE YUCCA MOUNTAIN ENVIRONMENT AND SOME PARAMETER RANGES FOR FUTURE MODELLING

This chapter discusses the implications that can be deduced from the material discussed in Chapters 1 to 5 for microbial activity in a potential Yucca Mountain repository. Modeling of microbial activity in such a manner that it can be interfaced with inorganic models is the ultimate objective, and therefore, where possible, on/off switches for microbial activity or ranges of parameters that control such activity will be suggested, although in some cases these are not well-defined and/or need to be confirmed by additional laboratory or field studies.

Many of the changes in the repository are expected to occur slowly from a microbial perspective. In addition to the chemical heterogeneity that is expected to be associated with the construction materials and waste package in the emplacement drifts, gradients in moisture and temperature will be created somewhat concentrically from the drifts as a result of the conditions brought about by the waste. Microbes will likely be able to take advantage of the unique niches created by these gradients, and complex microbial communities may, therefore, develop. However, the on/off switches dictated by the changing environment will determine the actual activity of these communities and, therefore, their impact on the geochemical parameters of the environment.

It has been reported repeatedly that adaptation to environmental stresses such as heat shock or nutrient deprivation in microbes occurs through the production of stress proteins which may then protect the organism from other stresses (such as radiation or extreme pH values) in addition to the stress to which they were formed in response. Such cross protection is particularly important at Yucca Mountain, where natural conditions for microbes are generally nutrient poor and locally may be lacking in free water. The construction of a repository and subsequent placement of radioactive waste will alter conditions such that additional stresses (e.g., high temperature, radiation and desiccation) will be present. However, the naturally already stressed population may be able to tolerate the lesser extremes of these added stresses and this should be taken into account when setting on/off switches for microbial activity in the performance assessment models developed for the Yucca Mountain repository.

6.1 WATER AND SALINITY EFFECTS

The Topopah Spring Tuff horizon where a repository would be located at Yucca Mountain is in the unsaturated zone, about 300 m below the surface and at least 200 m above the water table. This horizon is porous and fractured and the pores are partially filled with water. It is estimated that the region is >90% saturated. The remaining pore space is filled with gas of essentially

atomospheric composition, but perhaps somewhat richer in CO_2. The water is believed to be a $NaHCO_3$ groundwater of near drinking water quality with a near-neutral pH. Since this horizon is above the water table and porous, even under natural conditions intermittent periods of wetting and drying as a result of surface water infiltration (precipitation) are expected. In addition, the waste packages to be emplaced within such a repository will produce heat from the decay of radionuclides in the waste and this heat is expected to drive away a substantial amount of water in parts of the YM horizon, which may then condense in locations further away from the heat source. These intermittent wetting and drying periods as a result of sporadic precipitation, and the redistribution of moisture as a result of heat from the waste packages will influence the activity of the microbial population, both introduced and indigenous, in a future YM repository.

Pore water may become more concentrated during periods of evaporation and drying and if this occurs, the microbial population naturally present may be subjected to periods of higher osmotic stress. Bacterial populations native to the Yucca Mountain site have been sampled and studied by Haldeman and Amy (1993), but no specific studies were done to determine their halophilic character. Because of the generally dilute groundwater composition, truly halophilic bacteria probably do not occur in the groundwater at Yucca Mountain, but it is possible that many of the species may have at least mild halotolerant properties because of the intermittent water fluxes at the site. Also, bacteria may be located in the rock under very low water potentials, and, therefore, may be quite halotolerant, because a resistance to low water potentials may also provide resistance to osmotic stress. In addition, the groundwater is quite oligotrophic and bacteria adapted to such environments are generally also likely more resistant to osmotic-, water- and temperature stresses.

Rewetting after drying episodes could (temporarily and locally) increase microbial activity. For instance, Cabrera (1993) conducted a modelling study of the flush of nitrogen mineralization in soils caused by drying and rewetting cycles. Several factors in this study may have contributed to the N flush that followed rewetting dry soil. A significant proportion of the soil microorganisms probably died during drying, and dead microbial cells available for decomposition could have caused part of the N flush. The youthful state of the microbial population that developed after rewetting could also have been responsible for part of the enhanced N mineralizaton. In addition, the N flush might have been partly due to an increase in the availability of organic substrates through desorption from soil surfaces and through an increase in organic surfaces exposed. Similar effects may be also be of importance at Yucca Mountain with respect to the drying and rewetting cycles there. Although the halotolerant character of the native microbial population at Yucca Mountain is not known, it is possible that during drying episodes, many bacteria would accumulate EPS, compatible solutes and osmolytes. A certain percentage would not be successful and die anyhow. Then, upon rewetting, cell material from dead cells, EPS and compatible solutes accumulated in both living and dead cells would be available for survivors and a localized more intense period of bacterial activity may occur. In a repository situation one could speculate that this rapid activity could potentially release radionuclides sorbed to EPS as it is metabolized or that it may change the pH and redox conditions in the environment which could also affect radionuclide mobility.

However, it is also possible that many of the native population are in fact very sensitive to high salt concentrations, because of the oligotrophic character of the groundwater at Yucca Mountain,

and that, therefore, a large percentage would die upon drying as a result of the introduced heat. The synthesis of compatible solutes and osmolytes would likely have to be accomplished largely from organic sources inside the cells because of the oligotrophic character of the groundwater at Yucca Mountain. This increased synthesis might cost considerable energy, which is likely also in short supply. This would certainly be the case for autotrophs (Kieft and Spence 1988). Without further study of the microbial population at Yucca Mountain it is not possible to conclude whether natural drying and rewetting cycles would have very drastic effects on the population in the rock.

Much more drastic effects on the microbial population at Yucca Mountain are expected from the heat of the waste containers emplaced in the repository. The heat is expected to drive most of the water away from the vicinity of the containers to more remote areas where the temperature is such that condensation will occur. During the redistribution of water, the bacteria present will be subject to increasing osmotic stress, compounded by temperature and desiccation effects. It is difficult to separate these effects, but it is likely that most organisms will either die or become VBNC, and almost inactive. Certainly, microbial activity will be greatly reduced in areas adjacent to the waste package, until temperatures are such that liquid water can return. Microbial activity will likely be much more intense at the locations where the evaporated groundwater recondenses, because of an increased availability of liquid water and elevated but not prohibitive temperatures.

Ideally, osmotic levels at which microbial activity ceases in the Yucca Mountain environment are needed, such that they can be used as on/off switches in models that determine the level of microbial activity in various locations in the repository. Tests on Yucca Mountain microbial populations need to be performed to define such levels because the halophilic character of the naturally occurring microbes both in the groundwater and in the rock has yet to be determined.

Table 4.1 showed water potentials at which certain groups of microbes will become inactive (and may even die). At Yucca Mountain, the local water potential, which can be derived spatially as a function of rock properties (e.g., pore size distributions, Table 4.2) and hydrology (periodic precipitation), could be used as an on-off switch for microbiological activity in model calculations. Some of the data required to attempt this may in fact already exist in the work of Kieft et al. (1993), although their work on the microbial abundance and activities in relation to water potential in the vadose zones of arid and semi-arid sites (including samples from the Nevada Test Site) showed only weak correlations between microbial abundance and activity and water content or water potential of the rock. Based on the overriding influence of water on microbial activity and on the data in Table 4.1, a water activity of 0.9 is a reasonable estimate as a cutoff for significant microbial activity. In addition, various rates would need to be estimated for the microbial reactions considered when water activity is >0.9. Such rates have not been investigated in this report, but could be obtained from published studies, or from well-designed laboratory studies that approach realistic in situ conditions as closely as possible. For instance, King et al. (1999) used published rates for sulphate reduction in their model of microbial sulphide production and Cu container corrosion in the backfill of a Canadian waste vault. For fungi, the water potential cutoff for activity should be set lower, at about 0.7.

6.2 TEMPERATURE

The time frame of temperature shifts can be obtained from thermal models that have been developed for the Yucca Mountain repository. The temperature distribution depends on the spatial location of the waste packages, but temperatures in most areas of the repository are expected to be well in excess of 100°C, for thousands of years.

It is not clear whether the hyperthermophiles found in certain geothermal areas of the world are 'ancient' organisms or 'new' adaptations, or, in case of the latter, how long it took to develop these populations. The development of an active thermophilic microbial population at Yucca Mountain could either occur via adaptation (mutation) of mesophiles, or by activation of dormant (hyper)thermophiles. Both processes may occur, although the former would probably be less successful because the heating rate (in the order of decades) may be too fast for genetic adaptation (that could require several mutations to occur simultaneously, which has a low probability of success). Another argument against adaptation through mutation is that the nutrient environment at the Yucca Mountain repository is very oligotrophic, causing inactive or low-activity in situ microbial populations to be present, with slow generation times. For instance, Fredrickson and Onstott (1996) suggest that some subsurface microbial generation times may be in excess of a hundred years. If that is the case at Yucca Mountain, microbial adaptation to the expected temperature range through mutation may not be easily accomplished, and any surviving microbes would probably be dormant and not active.

Mesophiles naturally present or introduced at Yucca Mountain may have an increased heat tolerance because of their generally starved conditions in the oligotrophic Yucca Mountain environment and would probably survive slightly raised temperatures. Some mesophiles may survive the larger temperature increase by sporulation, but would effectively be inactive and not be able to germinate until the temperature decreased. It is unlikely that mesophiles would adapt to temperatures >100°C in a relatively short period of time.

Thermophiles and hyperthermophiles can survive (probably in a dormant state) at lower temperatures in nature for a long time and have been isolated from relatively cold environments in nature. It seems, therefore, more likely that population shifts would occur because of dormant forms of (hyper)thermophiles being present and becoming active as temperatures increase. As discussed, their viability would be restricted by the need for liquid water. Indigenous microbial populations naturally present at Yucca Mountain have been investigated (Amy et al. 1992, Haldeman and Amy 1993, Haldeman et al. 1994) but specific isolations of thermophiles have not been carried out. The presence of dormant thermophiles in the Yucca Mountain environment can, therefore, not be ruled out at present. In addition, non-indigenous species will be introduced during construction, and survival and reactivation of such species, if present, at higher temperatures is a possibility.

Environments that are subject to large temperature variations and/or that are heated at intervals would favour facultative thermophiles or thermophiles with resting stages. Environments that have fairly stable high temperature regimes would favor strict thermophiles without resting stages. However, other characteristics of the hot environments will strongly influence their microbial flora, e.g., the supply of liquid water, carbon and energy sources. In the Yucca

Mountain environment, elevated temperatures will remain for thousands of years, in an environment limited in water availability and nutrients. Thermophiles and hyperthermophiles may be favoured initially but depending on water availability and carbon and energy supply, both are expected to be limited in their activity, their only mode of survival may be in a resting stage and not as actively metabolizing cells.

Many known hyperthermophiles are obligate anaerobes, and use the reduction of S with H_2 for energy instead of O_2 reduction. The Yucca Mountain environment is expected to be largely aerobic which would exclude the active growth of many known anaerobic hyperthermophiles. The concentration of S in the Yucca Mountain rock is low, which would further restrict the activity of these hyperthermophilic organisms, unless S is introduced in construction materials such as polymers or concrete. Most hyperthermophiles are heterotrophic (only a few autotrophic species are currently known) and many are proteolitic. The Yucca Mountain environment is expected to be largely oligotrophic, which can further restrict activity, unless construction and other peripheral materials provide the necessary nutrients.

From the above considerations, it is possible (and perhaps likely) that the initial thermal input to the repository will enhance microbial activity. The dominant organisms and the accompanying dominant activities will shift with time from mesophilically dominated and sporulation of the mesophilic organisms to dominance and reactivation of hitherto dormant thermophiles and hyperthermophiles (if present). It seems unlikely that a thriving and active population of (hyper)thermophiles will exist in Yucca Mountain in those areas that are in excess of 100-130°C because of the lack of liquid water in the absence of pressure and in light of the currently-known limits to life. If the environment is locally pressurized, which could be the case if plugged fractures occurred or within a waste package, activity may occur to about 130°C, but would not likely be prolific if a shortage of required substrates and nutrients (such as S, proteins, etc.) can be guaranteed through the appropriate choices of construction materials. Areas of Yucca Mountain where the temperature is lower than 100°C may be able to support a population of thermophiles and hyperthermophiles but again, this activity would be hampered by less than optimal conditions. In any model predicting the effect of microbially-mediated chemical reactions, these temperature limits would have to be included together with other bounding conditions (such as moisture and nutrients) to determine the location of microbial activity and thus the generation of biomass and microbially mediated geochemistry.

6.3 pH

Most natural environments have pH values between 5 and 9 and microorganisms with pH optima in this range are most common. They possess a physiologically triggered pH homeostasis mechanism, based on proton pumps as well as K^+/H^+ and Na^+/H^+ antiport systems, that maintain a relatively constant pH inside the cell over the broad range of exterior pH values. The pH values expected in a Yucca Mountain repository could range from circum-neutral to extremely alkaline, depending on the amount and locality of concrete used in the drifts.

The Yucca Mountain repository environment could potentially contain micro-environments with acid pH values as a result of microbial activity, which generally has a pH decreasing effect because of metabolic byproducts (organic and inorganic acids), or as a result of PVC

degradation. Under suitable conditions (i.e., presence of O_2 and availability of reduced S and Fe, such as in pyrite), *Thiobacilli* may drastically lower the pH of the environment.

However, areas with high (concrete-induced) pH values may be more common than microenvironments with low pH, because of the potentially considerable use of concrete in the repository (e.g., concrete inverts on which the waste packages might be placed; concrete liners with which the drifts might be lined). Very high pH values may inhibit an unadapted microbial population, particularly if pH values in excess of pH 12 are reached. However, obligate alkalophiles (pH optimum >10.0) have been isolated from a number of natural environments, especially alkaline lakes and springs. Amongst obligate alkalophiles, *Bacillus* species appear most common, and can in fact be isolated from most soils. Therefore, it is possible that the indigenous population at Yucca Mountain may adapt. Also, microbial byproducts may lower the cement-induced high pH effects such that microbial activity is possible. The endemic microbial population in a location close and similar to Yucca Mountain has been investigated (Haldeman and Amy 1993), but not for the occurrence of alkalophiles or acidophiles. Therefore, it cannot be concluded that alkalophiles and acidophiles do exist naturally at Yucca Mountain, but the reviewed literature suggests strongly that tolerance of, and adaptation to, fairly extreme pH values may be expected in the naturally occurring microbial population at Yucca Mountain. For modelling purposes, pH values >1 or <12 could be used as on/off switches for microbial activity in the repository at Yucca Mountain.

6.4 NUTRIENTS AND NUTRIENT AVAILABILITY

As discussed by Kieft et al. (1993), addition of water in some of their vadose samples studied sharply increased the rate of mineralization of added organic substrates, but had no effect in others, suggesting that additional factors (such as N, P) may limit microbial activity. This could also be the case in Yucca Mountain and needs further investigation.

Ecosystems are energy-driven (Morita 1993) and, therefore, the amount of energy present, the quality of the energy, its bioavailability, the turnover rate, and the replenishment of energy must be taken into account, when evaluating in situ activity of microbes. Some of this information could be obtained from laboratory and field experiments, making sure they were performed under realistic conditions. For instance, starvation experiments are sometimes conducted with "gluttonous" bacteria that are accustomed to laboratory media. These are poor representatives of the bacteria that would be encountered in many natural environments, including the Yucca Mountain environment. Bacteria in oligotrophic environments often survive in a state of suspended animation, are small and may have generation times of tens to hundreds of years. However even in such an environment, their influence on the (local) geochemical conditions could still be significant on a geological time scale.

Morita (1993, 1982) summarizes that the normal state for most bacteria in ocean environments is the starvation mode and that the microbes themselves make most ecosystems oligotrophic. The condition results from the fact that there likely are representatives from all physiological types of bacteria in any environment as well as abilities to rapidly utilize various substrates when they become available. A similar situation is likely true of the microbes that will be present in the proposed repository.

Nutrient and energy constraints are expected at Yucca Mountain, in that the environment is expected to be oligotrophic with respect to the supply of nutrients and energy available for microbial growth. Typical organic matter concentrations in the rock and groundwater at Yucca Mountain are low and the extant microbial population is probably in some sort of dynamic equilibrium with these concentrations. Organic C, N, P and S are likely constantly recycled between the planktonic and (probably orders of magnitude larger) sessile population (in biofilms on fracture- and particle surfaces). Part of the dissolved organic matter may be biologically stable, but data on this are missing in general for deep subsurface environments.

Furthermore, the establishment of a repository may change the nutrient status of the originally oligotrophic environment through the introduction of nutrients as a result of repository excavation and operation and emplacement of the waste. This may lead to an overall increase in microbial cell numbers in locations of increased nutrients and an enhancement of microbial effects on repository performance (e.g., corrosion, gas production, radionuclide transport).

The majority of starvation and survival studies have been conducted using marine organisms. As yet there is no evidence that that freshwater or soil organisms will behave in a strikingly different manner. However, as Amy et al (1993) point out, there are few studies of starvation in non-marine microorganisms. Amy et al (1993) have isolated viable microorganisms from 450 m depth at Rainier Mesa, Nevada Test site and found that the starvation behavior of these subsurface isolates appeared to be typical. They demonstrated that 4 of the 5 isolates increased in number at the onset of starvation, and decreased in cell size. This result is similar to that of Nystrom and Kjelleberg (1989) who noted a 4-fold initial increase in cell numbers during the first six hours of a C, N and P starvation regime. Amy et al. (1993) also demonstrated that cell number decreases to a constant level within 60 days. This means that for purpose of the repository model, the process of starvation can be modeled as instantaneous. It is important, however, to quantify the generation of EPS during the transition to metabolic arrest as potential source of colloidal material. Also, although microorganisms will probably exist predominantly in the starved state, this does not necessarily imply that the greatest impact of microorganisms will be when they are in the starved state. Amy and Morita (1983a) have studied recovery from metabolic arrest. The demonstrated recovery time was in the range of days for several weeks of starvation. Cells starved for long periods of (geologic) time have also been cultured in the laboratory (Amy et al. 1993). Therefore, to a good approximation for the resolution of a repository model in geologic time, revival of microbes to an active state can be modeled as instantaneous or "switched on".

The element of time reduces some of the complexity in the repository. Within the resolution of the repository time scale, microbes can be assumed to take "instant" advantage of available nutrients and energy sources. It can also be assumed that stress factors (heat, desiccation etc.) will have immediate impact, e.g., below the a_w threshold there will be no activity and above that level activity is possible. They can be expected to have the necessary time to undergo the phases necessary to survive any stressful event (e.g., heat, nutrient starvation, salinity change). However, in modelling terms they can be described as going "immediately" into metabolic arrest with a first order estimate of 0.3% survival rate.

For the purpose of modelling the following attitude could be adopted. The default condition for microbes is in a starvation state. This is punctuated by periods of growth which are relatively short with respect to the repository lifetime. These periods of growth are limited by the available nutrients and cease when the nutrients are utilized or pass out of the system. It is important to note that, although, the periods of microbial activity may be intermittent, localized and relatively short lived, their impact may be greater than the far longer periods of starvation and survival. Examination of the starvation survival literature suggests that the amount of time required for cells to go into, or come out of, the starvation-survival state is neglegible on the time scale of interest. What remains, then, to sketch out a picture of microbial activity is to identify the kinds of mechanisms that will bring nutrients to microbes in the repository. The following are identified:

1) nutrients that are deposited as part of the original construction;
2) nutrients that are products of the thermal breakdown of construction and natural materials;
3) nutrients that are products of the non-thermally loaded breakdown of construction materials;
4) nutrients that are carried into the repository setting by intermittent water fluxes.

6.5 RADIATION

In a Yucca Mountain repository, hormesis (stimulation by subharmful doses of an agent) effects on microorganisms could possibly occur in those locations where the radiation dose has dropped to extremely low values due to shielding by surrounding materials. However, since conditions in this environment are expected to be very hot and dry for many years, hormesis effects may be obliterated by the detrimental effects of the hot and dry environment. Of more concern is whether microbes can somehow at all survive these harsh conditions (confounded by potentially high radiation fields near the containers), such that they can continue to grow again when conditions improve.

The microbial population within the waste containers will be subjected to gamma radiation. Those in the near field will also be exposed if the waste containers are not self-shielding. Dose may be low, depending on the type of the container and the age of the waste, but the accumulated dose could be significant over time. The effects of a low but continuous dose on microbes are not known, however, the potential for hormesis effects is real.

If radiation fields are present, they will be exerting their damaging effects in the presence of considerable heat and hence desiccation. A number of studies have indicated that resistance to heat or desiccation often affords microbes a certain degree of radiation resistance. This is especially true for the highly radiation resistant organism *Deinococcus radiodurans* but also in other vegetative bacterial cells and in spores.

The study by Amy et al. (1992) isolated approximately 50 bacterial strains from rock and groundwater samples taken at the Nevada Test Site. Sixty-five percent of these isolates contained pigment. The literature reviewed showed a non-consensus about the role of pigments in radiation resistance in general. However, often bacteria isolated from extreme environments do contain pigments. An example are the halophiles. Evaporation pools for the commercial production of salt from the sea (marine salterns) are often red in colour due to the carotenoid

pigment of extreme halophilic organisms (Larsen 1981). Therefore, if waste package design is such that radiation becomes a concern in a YM repository, isolates from the Yucca Mountain site need to be tested for radiation resistance and a correlation with pigmentation may or may not emerge.

Results from a study by Pitonzo et al. (1999a,b) showed that YM isolated microbes could survive considerable radiation doses in a VBNC state, but recovered some of their previous culturable and metabolic capabilities, once the radiation stress was removed. For a YM repository this could imply that radiation to a certain cumulative dose (2.3 kGy as reported by Pitonzo et al. 1999a) will likely curtail activity and metabolic capability of microbes (indigenous or otherwise) present, but that part of this activity can be restored once radiation fields have decayed away to lower values.

6.6 COLLOIDS

In a heterogeneous environment such as an emplacement drift, microbial biomass cannot be expected to adhere to all substrates in the same manner. This means that some designs or locations of a specific design may generate more biocolloids, or conversely, supply a better sorptive biobarrier than other designs or locations. Biocolloids can be mobile, and therefore, transport sorbed radionuclides. A study by Chen et al. (1999) showed mobility and survival of injected bacteria in a heated block of Topopah Spring Tuff, although the relationship between bacterial migration and the movement of water in this thermally perturbed environment was not yet fully understood. Their observations suggested the possibility of rapid bacterial transport in a thermally perturbed repository environment, and therefore, the effects of microbial transport on radionuclide migration need to be incorporated in future models.

The second contribution, specifically to transport of radionuclides, is EPS production. Although the controlling factors for attachment and detachment are not discussed in this report, it is clear that the generation of EPS, and thus the estimate of its mass, is determined by the type of organism, its state of growth and the nutrient conditions. Future modelling efforts could include concentrations of colloids based on the amount of EPS produced.

6.7 ANAEROBIC ENVIRONMENTS

At Yucca Mountain, saturation will only occur intermittently and only locally if at all. Directly after water ingress, microbial activity will likely increase or where inactive, initiate. Locally, given the heterogeneity of the water distribution, the activity could even progress to anaerobisis, depending on how fast the water drains and air is reintroduced. The local a_w values are the key to model microbial activity and all related microbial processes. Because biotic processes will be expected to occur primarily within or beneath films, assumptions would have to be made regarding the onset of anaerobic conditions.

6.8 PRESSURE

The Yucca Mountain environment is very different from some well-known geothermally-heated environments in that it is not a saturated environment, but in fact would be severely restricted in

the availability of moisture because of temperatures expected to be in excess of 100°C and maybe as high as 200°C for as much as a few hundred years. High pressure may be possible locally, should fractures be plugged by precipitated minerals, or inside waste packages. The currently-accepted temperature limit to life is somewhere in the neighbourhood of 130°C and the Yucca Mountain near-field environment may, therefore, be inactive, if temperatures near 200°C are indeed reached. However, temperature profiles are not expected to be uniform in the proposed repository, and it is expected that many parts of the repository environment will in fact experience lower temperatures. If those temperatures are lower than about 130°C, the possibility of life exists. However, liquid water is needed for life and at temperatures in excess of 100°C, pressure is needed to keep water in its liquid form. Because the environment at Yucca Mountain may be quite porous, the required pressure to keep water liquid at temperatures >100°C may not develop. If this is the case, liquid water may not exist at temperatures >100°C at Yucca Mountain. The water would move away as vapor and water temperatures would not exceed 100°C, but dry rock would have a much higher temperature. Viable organisms may, therefore, only be expected in areas with liquid water at Yucca Mountain.

6.9 MODELLING

Concurrent with the presented 1996 literature review in this report, modelling efforts of microbial effects at YM has been ongoing. The Microbial Communities Model (MCM) was developed together with the construction of the Microbial Impacts on the Near-field Geochemistry (MING) code, to quantify bounding assessments of microbial growth, to use as a basis to discuss the potential microbial impacts on the near-field environmental geochemistry (TSPA 1998). The model uses an idealized and simplified approach, similar to that of McKinley et al. (1997). The approach is based on a combined mass balance- and thermodynamic approach, because not all required information for a kinetic approach (mathematically possible) was available or could be obtained within a reasonable time frame. The mass balance approach used instead uses abiotic processes (e.g., corrosion rates, sequential degradation of materials) to determine the rate at which nutrients become available to microorganisms and then assumes that the microorganisms convert those nutrients to the products (i.e., biomasss) instantaneously, using limiting guidelines of energy availability and the availability of all the required nutrients in the proper ratio (i.e., the average composition of microbial material).

The MING output is designed to define only the bulk system redox chemistry of the near-field system given the variable temperature and the pH of the system, it does not reflect the actual chemical speciation or reactions that might occur at a more localized scale. The chemical output is designed to capture molar quantities of the production of byproducts (such as CO_2, H_2S and H^+ ions) over one linear meter of repository drift. Output is also designed to enable evaluation of the quantities of microbes produced, the limits to microbial growth imposed by both nutrients and energy, and the stipulation of which nutrient is limiting growth in the repository.

A number of simplified calculations have been performed with MING to date and results showed a maximum biomass production of 10 to 12 g per year per meter of repository based on the nutrients and energy in the large mass of mild steel that would be emplaced. Biomass production based on the nutrients in the other constituents (concrete, C-22 alloy and J-13 groundwater) varied from 1 to .01 g per year per meter.

McKinley et al. (1997) converted biomass to complexing capacity and showed that the impact on radionuclide complexation was negligible in a Swiss repository. The low biomasss production at YM, calculated globally with MING may also not have a huge impact on radionuclide complexation and hence mobility, but the model cannot discriminate between localized areas of specific or intensified micobial activity, which could be especially important for waste package corrosion. For such specific mechanisms, a kinetically-based model, using Monod expressions may be more useful.

The model for low and intermediate level nuclear waste by Humphreys et al. (1995) uses Monod expressions to calculate gas (e.g., methane) production from cellulose at the Drigg site in the UK. The model by King et al. (1999) for the activity of SRB in compacted bentonite-based buffer material in a Canadian repository and the effects of that activity (i.e., sulphide production) on Cu-container corrosion also used the kinetic Monod equation as a basis, as well as published sulphate reduction rates. These rates were carefully chosen from a number of published rates, to simulate the repository environment as closely as possible. However, the choice of reaction rates is crucial for realistic predictions with such models. For instance, Stroes-Gascoyne and Hamon (2000) found a very low in situ sulphate reduction rate in compacted bentonite-based buffer material that had been buried for 6 years in a granite borehole of realistic dimensions. Only 0.02 to 0.5% of the sulphate initially present was converted to sulphide, whereas in the model, all sulphate was converted to sulphide in 1-2 years. This illustrates the need for realistic choices and measurements of pertinent microbial reaction rates. The model by King et al. (1999) did include bacterial death due to a number of factors including radiation, as well as precipitation of the sulphide with Fe(II) present either in the groundwater or produced by the dissolution of biotite, and the calculations showed that only 0.3% of the sulphide produced actually reached the container. Depending on the input parameters, corrosion rates for a variety of cases could be calculated. In case of radiation emanating from the container at an initial absorbed dose rate of 11 Gy/h (equivalent to the dose rate expected), they found virtually no SRB activity predicted near the container and consequently, no corrosion was predicted for this case.

Such kinetic models may need to be applied for the YM assessment, to model MIC, and other important localized processes. The MING model, although very useful for a broad picture, is not able to give the localized variety in intensity and activity that may be needed for predicting effects such as corrosion. In fact, recommendations from the initial MING runs suggest continued efforts to link biomass production with development of a more sophisticated approach to include specific microbial activity for local waste package corrosion effects. Consideration of microbes as colloids should also be pursued in future work since microbes may act as or can stabilize colloids, as well as produce strong complexing agents of multivalent ions. Both effects can be very localized.

The material in this report can be used in the development of such specific kinetic models for concerns and reactions that warrant a more sophisticated prediction of microbial activity and its effects in certain parts of a YM repository.

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APPENDIX A:

IMPACTS OF MAN MADE MATERIALS ON MICROBIOTA: POSSIBLE RELATIONSHIPS TO THE INTEGRITY OF THE YUCCA MOUNTAIN REPOSITORY ENVIRONMENT – A COLLECTION OF LITERATURE

Collected by D. Haldeman, University of Nevada, 1996

A.1 INTRODUCTION

This data base was created to provide background information concerning the role that microbiota may play in determining the long-term stability of a nuclear waste repository. Microbiota are indigenous to deep subsurface geological strata, and will be introduced during the construction of a repository. Because of their tenacious survival capabilities and their diversity of metabolisms, discerning impacts of man-made materials on microbiota as well as their impacts on the materials and the repository environment, is a complex task. Many factors need to be considered, and this data base provides articles that apply to specific areas of importance. Much of the basis of the literature search was discussed by an interdisciplinary group at a workshop held on April 10-12 1995, in Lafayette, California (Horn and Meike 1995). This Appendix contains a valuable collection of papers on relevant topics and a resource for initial and advanced reading on the subject.

A.2 LITERATURE SEARCH OUTLINE

Part 1 – Specific to the Subsurface – aquifers are mostly excluded

I Subsurface Microbiology

Reviews
Activity
Diversity
Heterogeneity – Distribution
Sampling
Vadose Zone
Mines/Rocks/Caves

Part 2 – General Microbial Ecology – with application to subsurface repository

II Microbial Growth and Responses to Environmental Parameters – Abiotic Factors
Growth Properties (General)

pH
Temperature
Radiation
Radiation (General)
Mutations and Mutants
Osmoregulation
Desiccation

III Survival

Survival
Dormancy

Dormancy (General)
Spores
Resuscitation
Viable But Not Culturable
Cryptic Growth
Injury
Stress Proteins
Bacterial Sizes

IV Diversity of Microbiota

Diversity (General)
SO_4 Reducing Bacteria
Anaerobes
Acetogens
Aeromicro
Vapor Phase
Fe(II) Oxidizing Bacteria
Autotrophs
H_2 Oxidizing Bacteria
Genetic Detection

V Nutrients and Cycling

Cycling (General)
Geo Micro
Limiting Factors

VI In Situ Considerations

Attachment
Biofilms
Polymers
Polysaccharides
Interactions
Interactions Microbe/Microbe
Competition
Consortia
Colonization/Succession

Part 3 – Impacts of Microbiota on Repository Integrity

VII Degradation
Hydrocarbons
Diesel Exhaust

Concrete
Polysaccharides
Miscellaneous
VIII Corrosion

IX Transport of Bacteria, Metals and/or Radionuclides

Transport
Metals
X Waste Disposal

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APPENDIX B:

HALOBACTERIACEAE AND SPECIFIC RESPONSES TO HIGH-SALT ENVIRONMENTS IN NON-HALOBACTERIACEAE

B.1 HALOBACTERIACAEA

Bacteria growing best at salt concentrations between 20% (wt/vol) and saturation (about 30% wt/vol) are often referred to as extreme halophilic bacteria or extreme halophiles. A variety of other bacteria have been described as halophilic but their requirement for salt is more modest than that of the above types and are referred to as moderate halophiles or slight halophiles. Bacteria indigenous to the marine environment could be grouped with the slight halpohiles (Larsen 1981). Halobacteria (archaebacteria that live in extremely saline environments) do not have a detectable turgor pressure, possibly because the osmolarity in their environments does not change drastically. However, the lack of turgor begs the question what provides the force for cell wall expansion in archaebacteria, because their cell walls are much more flexible than those of eubacteria, for which turgor is thought to cause cell wall expansion during growth (Csonka 1989).

The halobacteriaceae are chemoorganotrophic and thus depend on a source of organic material for their growth and development. Most known isolates utilize proteins and amino acids rather than carbohydrates. There are two genera: *Halobacterium* and *Halococccus*. They occur in extremely saline natural waters, in crude solar salt, in proteinaceous products (fish, hides, viscera) heavily salted by the use of crude solar salt, in brines from such products, and in materials (wood, concrete etc.) and tools that have been in contact with solar salt or salted products. Evaporation pools for the commercial production of salt from the sea (marine salterns) are often red in colour due to the carotenoid pigment of extreme halophilic organisms (Larsen 1981).

There is a mass occurrence of halobacteriaceae in Great Salt Lake (its northern basin is virtually saturated NaCl brine), to a level of about 7×10^7 cells/mL and a biomass of about 300 g (wet weight) per m^3. Another ecosystem where they occur en masse is the Dead Sea. The NaCl concentration of the Dead Sea is less than half of that of the northern basin of Great Salt Lake. However, the Dead Sea has a very high content of $MgCl_2$ and a notable content of $CaCl_2$, such that the total concentration of inorganic salt is about equal to that of Great Salt Lake. Dead Sea halobacteriaceae require at least 15% NaCl and grow best at NaCl concentrations between 20% and saturation.

Halobacteriaceae are also prominent members of the biocommunity of the alkaline, extremely saline lakes of the Wadi Natrun, Egypt and Lake Magadi, Kenya. From an ecological point of view, these alkaline lakes are particularly interesting since their saline waters, in contrast to the more neutral salines, contain very little Mg^{2+} (and Ca^{2+}). The response to Mg^{2+} of isolates from these alkaline salines may well differ from the isolates from the non-alkaline saline habitats (Larsen 1981).

In halobacteriaceae, the osmotic problem created by the high salt concentration in the environment is overcome by at least equally high concentrations of salt inside the cells. Internal salt is mainly KCl, but it also contains some NaCl. The proteins of these organisms are characteristically acid in nature. For isolation purposes in the laboratory a medium with 25% wt/vol NaCl is most often used. Ions such as K^+ and Mg^{2+} may partially replace Na^+ and Br^- and

NO_3^- may partially replace Cl^-, but not completely. The requirement for K^+ is similar to that of most other bacteria (2mM). Most isolates prefer amino acids as carbon source and often are unable to use glucose. Maximum growth rates occur between 40-45°C, and no growth occurs below 10°C and above 55°C. The organisms are obligate respiratory, utilizing O_2 as terminal electron acceptor, but some strains are also reported to grow anaerobically on NO_3^-. Most grow slowly even under favorable conditions. Pigment seems to play an ecological role by protecting the organisms against radiation from the sun. Other unusual qualities that seem to be common to the halobacteriaceae are the lack of muramic acid as a component of the cell envelope, and the use of the mevalonate pathway instead of the malonate pathway in the synthesis of phospholipids. The phospholipids are to a large extent composed of phosphoglycerol derivatives containing dihydrophytol chains, ether-linked to the glyceryl moiety, instead of the ester-linked fatty acids commonly found in eubacteria (Larsen 1981).

Moore and McCarthy (1969) assessed the genetic relatedness among various strains of halophilic bacteria by DNA-DNA duplex formation and RNA hybridization. They found that all of the strains of extremely halophilic rods were closely related, and that the extent of divergence of the base sequence is similar for the major and minor DNA components. Parallel experiments with ribosomal RNA revealed a relationship between the extremely halophilic rods and cocci and a more distant relationship to moderate halophiles and to a photosynthetic extreme halophile. The kinetics of DNA renaturation showed that the genome size of the extreme and moderate halophiles is similar to that of E. coli. Moore and McCarthy (1969) concluded that the presence of two DNA components seemed to be characteristic of all the non-photosynthetic strains of extreme halophiles so far examined, whether rods or cocci.

Kushner (1988) discussed the true intracellular ionic environment of halophilic microorganisms. His work suggests that halophilic archaebacteria have a truly salty internal environment, whereas halophilic and salt-tolerant eubacteria may have salty external environments but much less salty internal ones. However, the water and salt in the internal environments of archaebacteria may well have limited freedom.

Generally, the intracellular concentrations of K^+ are up to 2000 times greater than extracellular ones in halophilic archaebacteria. A reasonable estimate for the internal ions of *Halobacterium halobium* and related organisms, growing in 4 M NaCl + 0.01 M KCl, is about 3.5 M KCl, 1.0 M NaCl, and 0.1 M $MgCl_2$ but the ratios of the major cations probably vary greatly during growth. Many of the enzymes of this group of bacteria function well in, or even require, the kinds of ions present in abundance in the cytoplasm. For many enzymes, NaCl and KCl have approximately the same effects; others behave differently in their salt responses. It has not been resolved exactly how halophilic archaebacteria keep such high concentrations of ions (i.e., potassium) in their cells. A high potassium content may have certain energetic advantages (proton motive force) for a cell but it is likely that some sort of internal binding of these ions to macromolecules or structures is necessary to keep internal concentrations high. Certain cytoplasmic proteins (enzymes) in halobacteria have strong salt- as well as strong water-binding capacities but non-halophilic proteins can also bind water tightly. A number of enzymes, in fact, bind enough water in the crystalline form to permit them to function even in anhydrous inorganic solvents (Kushner 1988).

Halophilic and halotolerant eubacteria have not been studied as extensively as halophilic archaebacteria, and there are some paradoxes. A number of intracellular enzymes of halophilic eubacteria appear to function best in the absence of salt and are inhibited by concentrations of salts (usually NaCl or KCl) that may be present in the cytoplasm of these bacteria. However, other intracellular enzymes and some membrane-associated enzymes and exozymes appear to function well in high salt concentrations. It also appears that Cl^- is a generally toxic ion for these and other eubacteria and that one of the functions of the cytoplasmic membrane is to keep Cl^- ions low in the cells. It is possible that the cell-associated cations of some halophiles may be bound to the envelope such that the internal environment of these cells is really low in salts (Kushner 1988). Such cells would be halophilic on the outside but not on the inside. There is good evidence that the envelopes of both halophilic and non-halophilic bacteria can bind substantial amounts of cations. If the cell-associated cations of halophilic and salt-tolerant bacteria are largely bound to the envelope and if relatively few anions are present in the cytoplasm, then compatible solutes possibly supply the high osmotic pressure of the cytoplasm which must balance the external osmotic pressure. However, compatible solute concentrations may not be high enough to balance the external NaCl concentration. The role of macromolecules in providing osmotic pressure may have been unfairly neglected, because osmotic pressure of macromolecules cannot be assumed to vary linearly with concentration as predicted by simple van't Hoff law. Hydrophilic macromolecules in the cell could supply substantial amounts of osmotic pressure. Though it is very difficult to obtain a convincing figure for the contribution made by such molecules in the living cell, it should be noted that oligosaccharides in the periplasmic space of *E. coli* may play an important role in maintaining the osmotic pressure in this compartment.

B.2 SPECIFIC RESPONSES TO HIGH SALT ENVIRONMENTS IN NON- HALOBACTERIACEAE

B.2.1 ARTHROBACTERS

Arthrobacters (one of the main genera in the Coryneform bacteria) are aerobic chemoheterotrophic, Gram-positive soil bacteria that can metabolize a wide range of organic compounds. They are also resistant to desiccation and long-term starvation (which may make them particularly useful for bioremediation in dry desert soils). They have a dimorphic growth cycle in which exponential phase cells appear as irregular bacilli and stationary cells as cocci. This dimorphic cycle is under genetic control but can be modified by nutritional conditions.

Deutch and Perera (1992) concluded that Arthrobacter morphology may be useful in monitoring osmotic stress in microbial communities in terrestrial habitats. Upon exposure to salt, there was a linear decrease in the specific growth rate during the exponential growth phase as the solute concentration (NaCl) was raised, but the arthrobacters were quite tolerant and growth occurred even at 1.5mol/L NaCl. The final yield in the cultures in the stationary phase were not affected by the presence of the added solutes. However, the presence of exogenous solutes had a dramatic effect on the morphology. Clusters of branching myceloid cells rather than the typical bacillary forms predominated during the exponential growth phase. These myceloids did not

undergo complete septation and persisted into the stationary phase. Similar responses were observed with potassium sulphate as the exogenous solute but less dramatic morphological effects were found with added polyethylene glycol or sucrose. The myceloids formed in response to osmotic stress could not be disrupted mechanically but were more sensitive than normal cells to lysozyme, particularly during the stationary phase. Arthrobacters have been previously found to form myceloids when deprived of nutrients such as biotin, vitamin B_{12} or manganese. However, in the presence of 0.9 mol/L NaCl and 1000 fold additions of these nutrients, myceloid cells still formed during the exponential phase and persisted into the stationary phase, suggesting the myceloid cells are induced by the addition of salt, rather that by deprivation of key nutrients. In medium with 0.9 mol/L NaCl, in the presence of osmoprotectants (proline, glutamate, glutamine, glycine betaine, choline, trehalose, gamma-aminobutyric acid), the growth rate was not increased significantly or formation of myceloids prevented during the exponential phase. These results indicate that arthrobacters exhibit characteristic responses to osmotic stress and suggest that these bacteria may contain novel osmoprotective compounds. Formation of myceloid cells in response to osmotic stress may also affect the use of arthrobacter in bioremediation.

B.2.2 DIAZOTROPHIC BACTERIA

Madkour et al. (1990) investigated the mechanism of osmotic adaptation in certain diazotrophic bacteria (e.g., the aerobic, free-living, N_2 fixing *Azotobacter chroococcum*, *Azospirillum brasilense* and *Klebsiella pneumoniae*) that are adversely affected by high osmotic strength (i.e., from soil salinity and/or drought in a desert setting). Natural abundance ^{13}C nuclear magnetic resonance spectroscopy was used to identify all osmolytes accumulated during osmotic stress in the studied bacteria. Results showed that the intracellular accumulation of osmolytes in diazotrophic bacteria can be attributed to (i) enhanced osmolyte uptake from the medium (i.e., uptake of glycine betaine, proline and possibly glutamate), (ii) increased net osmolyte biosynthesis (i.e., trehalose, glutamate and proline), or (iii) both of these mechanisms. The species of osmolyte accumulated and their preference in a given cell were subject to qualitative and quantitative changes depending on the prevailing environmental and nutritional conditions. This study suggested that none of the organisms tested could synthesize glycine betaine, but that they all selected it when it was available in the medium. It was also observed that osmotically stressed cells generally favoured the shift from glutamate to trehalose or proline as the culture aged or as salt levels increased, since the latter osmolytes provided the greater osmotic stress protection needed for long-term adaptation to the new environment. Glutamate appears to be more of a short-time response to osmotic stress. Glutamate is a pivotal metabolite, it can be readily accumulated or disposed of, which allows it to be a reasonable response to sort-term stress. Proline and especially trehalose are specialized metabolites, which likely require the induction of specific systems to synthesize and accumulate these. Also, glutamate is negatively charged at a physiological pH and a cationic counter ion must also be accumulated to maintain electroneutrality. K^+, known to accumulate in stressed enteric bacteria, may act as a counterion to glutamate in the diazotrophic bacteria as well. Trehalose eventually replaces the K^+ and glutamate which accumulate after hyperosmotic shock, in the enteric bacterium *E. coli* and this could also occur in the diazotrophic organisms studied.

B.2.3 CHEMOLITHOTROPHS

Relatively little is known about osmoregulatory mechanisms in chemolithotrophs (compared to what is known about heterotrophs). Kieft and Spence (1988) investigated the osmoregulation and iron oxidation in *Thiobacillus ferrooxidans* under salt stress, in the presence of osmoprotectants. The choice of salt (NaCl, KCl, Na_2SO_4 and K_2SO_4) used as an osmotic stressor had a profound effect on rates of iron oxidation. Salt stress for *Thiobacillus ferrooxidans* was not merely a function of the solute water potential but was heavily influenced by the inhibitory action of specific ions. Anions had the greatest influence, with chloride salts being more inhibitory than sulphate salts. NaCl inhibited the ability of *Thiobacillus ferrooxidans* to oxidize iron and the nearly complete inhibition of iron oxidation in 0.4 M NaCl (which has a water potential of -2.5 MPa, or an a_w of 0.98 (Brown 1990)) suggests that *Thiobacillus ferrooxidans* is a non-halophilic bacterium. As such, the organism may be expected to accumulate free amino acids as a response to salt stress (as generally occurs in many heterotrophic nonhalophilic bacteria). However, exogenously supplied glutamic acid was inhibitory at all salt concentrations tested and potassium glutamate was not accumulated as an osmoprotectant in this organism. Both proline and betaine were osmoprotectants in the presence of sulphate or high concentrations of chloride salt (0.4M) but only proline stimulated iron oxidation in cells exposed to lower concentrations of chloride salts (0.2 and 0.25 M). The generally low salt tolerance of these and other chemolithotrophs could be caused by the fact that the synthesis of organic osmoprotectants is energetically expensive for chemolithotrophs.

B.2.4 RHIZOBIA

Zahran et al. (1994) studied the effects of osmotic and heat stress on lipopolysaccharides (LPS) and proteins of rhizobia (Gram-negative motile rods that fix N_2 in symbiosis with legumes) isolated from the root nodules of leguminous trees grown in semi-arid soils of the Sudan, and of agricultural legumes grown in salt-affected soils of Egypt. Many strains of rhizobia can grow and survive at salt concentrations which are inhibitory to the growth, infection and nodulation of various legumes. The highest salt tolerance recorded in this study was 1.7 M NaCl (10% w/v), but the highest tolerance for sucrose was only 1.0 M which confirms that salt and sucrose affect cells in different ways (Csonka 1989). Salt or sucrose treatment altered the LPS patterns of the majority of the rhizobia studied. Strains that could grow in no more than 1% (w/v) salt were more severely affected than tolerant strains. These sensitive strains generally produced lower amounts of, and different, LPS when under osmotic stress. The salt-tolerant rhizobia also changed the composition of their LPS when stressed in 3% (w/v) NaCl, indicating production of LPS with relatively long chain length. Such long-chain LPS may help protect the cells from the stress. In halophilic rhizobium strains, osmotic stress had little effect on the LPS.

Up to seven heat-shock proteins have been detected in Arctic rhizobia (Cloutier et al. 1990). In the tropical rhizobia studied by Zahran et al. (1994), a 65 kDa protein was detected in all four strains studied and heavily overproduced under heat stress, which needs further study. The change in the patterns of proteins synthesized under osmotic stress indicate that metabolic changes were taking place in response to the stress. The osmotic tolerance in these strains may be correlated with tolerance to drought.

B.2.5 HALOANAEROBES

Rengpipat et al. (1988) stated that the understanding of aerobic halophilic bacteria is based on the examination of species isolated from salted foods, hides, sea water and various sources of saline sediments, but that little is known about haloanaerobes, other than the common occurrence of *Haloanaerobium praevalens* in Great Salt Lake sediments (Zeikus et al. 1983). Rengpipat et al. (1988) isolated a new species *Halobacteroides acetoethylicus* sp. nov. from deep subsurface gas bearing sandstones and brine waters associated with an injection water filter on an offshore oil rigg in the Gulf of Mexico. The organism was a Gram-negative non-sporeforming anaerobic rod which existed singly or in pairs and grew best at 10% NaCl but not above 22% or below 5% NaCl. Its isolation has extended the niche for haloanaerobes from hypersaline lake sediments to the deep subsurface hypersaline waters associated with gas and oil bearing sediments. Differences in species habitats are quite significant for haloanaerobes because hypersaline environments vary greatly in chemical composition.

B.2.6 LIPOLYTIC BACTERIA

Shabtai (1991) isolated and characterized a lipolytic bacterium (*Pseudomonas aeruginosa* YS-7) that was capable of growing in a low-water-content-oil-water emulsion. This species is normally grown in 95% water-based medium with 2-5% glyceride. After inoculation into 99% glyceride with 1% water-based medium, growth occurred until a water content of <0.01% (the reduction in water content resulting mainly from evaporation), at which point growth and respiratory activity ceased. At the final stage of the experiment (water content <0.01%) the bacteria appeared to be aggregated in the oil-rich medium. Until this stage the bacteria were associated with the water droplets in the culture and were not dispersed in the glyceride phase. When 1% water was added to the culture, another growth cycle occurred. This isolate also survived at 85% in cold acetone precipitation, compared with other bacterial strains surviving at just 10-20%. The isolated strain also appeared to be highly osmotolerant, as indicated by its resistance to increased concentrations of salts and other compounds (e.g., hydrolysed glycerol) in the decreasing water phase of the culture. Obviously, at such high concentrations these compounds not only contribute to the reduction in water activity but also exert their osmotic stress on the growing microbial cells. The isolate could also tolerate concentrated amphipatic agents such as detergents or soaps, suggesting it may be endowed with a uniquely stable membrane or certain protective extracellular structures, either of which might protect against or neutralize the amphipatic agents.

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APPENDIX C:

RADIATION TYPES AND DETAILS OF RADIATION RESISTANCE IN *DEINOCOCCUS RADIODURANS* AND *E. COLI*

C.1 RADIATION TYPES

Studies to determine the effects of radiation on microbes have been carried out with different types of radiation, i.e., gamma and ultraviolet (UV) radiation, electron beam, neutrons and microwaves. Often these studies have focused on vegetative cells of specific (non-sporing) organisms such as the highly radiation-resistant *Deinococcus radiodurans* or the extensively studied *Escherichia coli*. Natural populations in soils have also been studied (e.g., Popenoe and Eno 1962, Stotzki and Mortensen 1959), as well as spores (e.g., Farkas 1994). Several forms of radiation are highly mutagenic. Mutagenic radiation falls into two categories, ionizing and non-ionizing. Although both kinds of radiation are used in microbial genetics, non-ionizing radiation such as UV has found the widest use, often because it is easier to work with UV and the effects are similar to those of gamma rays. At the Yucca Mountain site, UV radiation will not occur. However, although the penetration power of UV is much less than that of gamma rays, this is not a problem when studying microbial cells. In addition, many of the molecular mechanisms that repair UV damage are identical to, or overlap with, those involved in the repair of ionizing radiation damage. Similarly, although microwave radiation is not of concern in the Yucca Mountain environment and studies of the effects of microwave radiation on bacterial cells would be of most interest to the food industry, they may nevertheless reveal effects that are common to other types of radiation. The following briefly reviews the kinds of damage each of these radiation types can cause in the bacterial cell.

C.1.1 GAMMA RADIATION

Ionizing radiation is powerful and includes short wavelength rays such as X rays, cosmic rays and gamma rays. This radiation causes water and other substances to ionize and mutagenic effects are brought about indirectly through this ionization. Among the potent chemical species formed by ionizing radiation are chemical free radicals, of which the most important is the hydroxyl radical $OH^{\cdot}$. Free radicals react with, damage and may inactivate macromolecules in the cell. DNA is probably no more sensitive to ionizing radiation than other macromolecules, but since each DNA molecule contains only one copy of most genes, inactivation can lead to a permanent effect (Brock and Madigan 1991). Gamma radiation results in single and double strand breaks in the DNA of an organism. These breaks occur in the deoxyribose phosphate diester backbone of the DNA molecule, and about 15 single breaks occur for each double strand break (Francia et al. 1985). In addition, ionizing radiation also causes damage to heterocyclic bases (breaks in purine and pyrimidine rings), elimination of rings, cross-links between bases and cross-links between bases and amino acids (Francia et al. 1985). Failure to repair strand breakage is the main cause of cell death from ionizing radiation.

C.1.2 UV RADIATION

UV radiation tolerance is mediated by enzymatic repair of DNA damage through several well-documented pathways, although overall UV resistance may also depend on physiological and behavioural traits such as cell morphology, pigmentation and phototaxis. Although many of the genetic mechanisms involved in DNA repair are virtually ubiquitous in bacteria (e.g., the *RecA* and *LexA* genes control the inducible DNA repair in the SOS repair mechanisms) and there is extensive regulatory overlap of DNA repair processes and other stress-induced responses such as

oxidative protection, there is wide variation in the phenotypic expression of UV radiation survival among different bacterial species and strains.

Both UV radiation and ionizing radiation can induce DNA strand breakage and pyrimidine dimer formation. The latter is a state in which two adjacent pyrimidine bases become covalently joined, so that during replication of the DNA the probability of DNA polymerase inserting an incorrect nucleotide at this position is greatly enhanced (Brock and Madigan 1991). The purine and pyrimidine bases of the nucleic acids absorb UV radiation strongly and pyrimidine dimers are the major cause of cell lethality from UV light while failure to repair strand breakage is the main cause of cell death from ionizing radiation. Therefore, the survival kinetics of an organism exposed to each treatment may differ somewhat.

Arrage et al. (1993a,b) subjected six bacterial isolates from the USDOE Subsurface Microbial Culture Collection to UV- and gamma-radiation. The study included three aerobic Gram-positive strands and three microaerophilic Gram-negative strains, as well as three reference bacteria, two *E. coli* strains and *Deinococcus radiodurans* (a Gram-positive and red pigmented organism). All bacteria were tested for UV resistance during the stationary phase of growth since cells are most resistant to radiation at this stage. They found that the aerobic Gram-positive organisms were significantly more resistant than the microaerophilic Gram-negative organisms to both radiation treatments. *D. radiodurans,* an organism that has an exceptionally efficient repair mechanism for radiation-induced DNA lesions, was by far the most resistant. This organism is discussed further in Section C.2.

Contrary to the hypothesis that subsurface bacteria should be sensitive to UV light, the organisms studied by Arrage et al. (1993a,b) exhibited resistance levels as efficient as those of surface bacteria. A total of 31% of the aerobic subsurface isolates were UV resistant, compared with 26% of the surface soil bacteria that were tested. Several aerobic, Gram-positive, pigmented, subsurface isolates exhibited greater resistance to UV light than all of the reference bacterial strains tested except *Deinococcus radiodurans*. None of the microaerophilic Gram-negative non-pigmented subsurface isolates were UV resistant and these isolates exhibited levels of sensitivity similar to those of the Gram-negative reference bacteria *E. coli* B and *Pseudomonas fluorescens*. The aerobic subsurface bacteria resistant to UV light tolerated higher levels of H_2O_2 than the microaerophilic organisms. The conservation of DNA repair pathways in subsurface microorganisms may be important in maintaining DNA integrity and in protecting the organisms against chemical insults, such as oxygen radicals, during periods of slow growth.

There is molecular evidence that suggests that limited movement of microorganisms in the subsurface occurs. DNA homology studies revealed that relatedness was based on depositional origin rather than depth or site location. These findings suggest that deep subsurface bacteria have had limited contact with surface organisms over time and may have been isolated for millennia. The results in this study demonstrated that subsurface bacteria are as competent as surface bacteria in tolerating DNA damage induced by UV light and peroxide. This suggested that these subsurface bacteria had conserved DNA repair mechanisms despite the lack of exposure to solar radiation. In addition to the conservation of enzymatic DNA repair, there are several physiological characteristics that may contribute to the overall DNA damage resistance, including pigmentation and cell wall thickness.

The sensitivity to UV radiation of several microorganisms of different habitats was also investigated by Gascón et al. (1995). Organisms used were *Rhizobium meliloti*, *Rhodobacter sphaeroides*, *Escherichia coli* and *Deinococcus radiodurans*. Two mutants with a non-functional SOS DNA repair system were included, as well as a mutant in the synthesis of pigment (carotenoids). The results revealed that *Deinococcus radiodurans* is an extremely resistant bacterium, *Rhizobium meliloti* was more resistant than *Rhodobacter sphaeroides* and *Escherichia coli* was the most sensitive bacterium tested. The high sensitivity of the mutants with defunct SOS DNA repair systems was verified.

C.1.3 HIGH-ENERGY NEUTRONS

High-energy, densely ionizing neutrons are well known to be more efficient per unit absorbed dose at killing cells than is sparsely ionizing, low linear energy transfer (LET) radiation such as gamma- or X-rays. Although not expected to occur in the Yucca Mountain repository, studies on the high energy neutron effects on microbes nevertheless can provide more insight into the molecular damage inflicted by radiation. There is strong evidence that an important critical molecular target for ionizing radiation causing cellular lethality is DNA. In neutron interactions with target molecules, direct effects in which the target is itself excited and/or ionized by the particle or its secondaries are predominant, rather than the indirect effects that involve the products of water radiolysis. This was shown by the use of quenchers. The study by Peak et al. (1995) compared DNA damage caused by low LET gamma-ray photons and high-LET neutrons in order to gain more insight into the mechanisms of action of these two types of radiation. Phage plasmid DNA was exposed to both types of radiation in the presence of known OH radical quenchers. Of four quenchers tested (acetate, formate, azide and mannitol), all were able to reduce the yields of both single and double strand breaks. This study provided evidence that neutrons, which are much more lethal and mutagenic than gamma-rays, caused less measured damage to DNA in these model systems. It has been suggested that at least some of the lesions inflicted by neutrons must be different in nature than those caused by gamma-rays. The breakage of DNA in cells by neutrons has long been considered to be largely direct, caused by the interaction of the dense column of ionizations set in motion by neutrons with DNA itself in contrast with the indirect type of damage caused by gamma-radiation in which OH radicals are thought to play a major role. This work has shown that a component of both single and double DNA strand breaks, caused by neutrons in this system, is also indirect, i.e., mediated by scavengeable OH radicals.

Knudson et al. (1995) determined the sensitivity of *Bacillus* spores to neutron and gamma radiation. They concluded that spores of all four species tested (*B. subtilis, B. pumilus, B. anthracis, B. thuringiensis*) were more sensitive to neutron than to gamma radiation. Dry spores of all four species tested were more sensitive than hydrated spores to gamma radiation and hydration/desiccation cycles did not affect the sensitivity of *B. subtilis* and *B. pumilus* spores to neutron radiation. A concentrated *B. anthracis* spore suspension was the most resistant to gamma radiation.

C.1.4 MICROWAVES

Little is known about the basic and general relationships between microbial destruction and microwave exposure, especially with regard to the kinetics of destruction of irradiated bacterial cells. Fujikawa and Ohta (1994) looked for patterns of bacterial destruction in solutions by microwave irradiation. Their results showed that the relationship between bacterial destruction and microwave irradiation was affected by factors such as a high salt concentration in the medium. At 60 g/L, recovery of irradiated cells was significantly suppressed and at 75 g/L, no viable colonies were observed. Growth phase and temperature also affected the results. It is well-known that bacterial cells in logarithmic phase are more sensitive to heating than those in stationary phase. A similar phenomenon was observed for the effects of microwave irradiation. In addition, *E. coli* cells grown at 44°C were more resistant to microwave radiation than those grown at 22-36°C. The acquired resistance at 44°C may be related to a specific set of heat shock proteins synthesized in the cells when exposed to temperatures some degrees above their normal growth temperature.

C.1.5 ELECTRON BEAM

The electron beam is also most often used in the food industry (e.g., Singh 1992, Shamsuzzaman et al. 1989). Radiobiological studies were carried out with three strains of *Listeria monocytogenes* by Farag et al. (1990). Resistance characteristics were determined under a variety of conditions, using both gamma rays and high-energy electrons. Results showed that *Listeria monocytogenes* in various suspending materials, differing widely in chemical composition, could readily be killed by irradiation. Gamma rays and high-energy electrons were equally effective. The measured D_{10} values were consistent with literature values and indicated that a relatively modest pasteurizing dose of 5 kGy would reduce the population level of *Listeria monocytogenes* by 5 to 10 orders of magnitude in a low moisture environment. Reduction of viable numbers in high-moisture materials would be even greater.

C.2 RADIATION RESISTANCE OF *DEINOCOCCUS RADIODURANS* AND *ESCHERICHIA COLI*

C.2.1 *DEINOCOCCUS RADIADURONS*

The study of Mattimore and Battista (1996) on the radiation resistance of *Deinococcus radiodurans* is of considerable importance to the specific environment in a Yucca Mountain repository, because they concluded that the functions necessary to survive ionizing radiation in *D. radiodurans* are also needed to survive prolonged desiccation. The *Deinococcaceae* are a small family of non-spore-forming bacteria which exhibit a remarkable capacity to resist the lethal effects of ionizing radiation. Doses survived are 5.2 kGy with no loss of viability, and survivors have been recovered after 20 kGy. It has been determined that the radioresistance of this species is a direct result of its ability to efficiently repair the DNA damage generated during irradiation, an evolutionary process selected for organisms that could tolerate massive DNA

damage. The radioresistance of *D. radiodurans* cannot be an adaptation to ionizing radiation because there is no selective advantage to being ionizing-radiation resistant in the natural world. There are no terrestrial environments that generate such high fluxes of ionizing radiation and it must, therefore, be assumed that the radioresistance of *D. radiodurans* is an incidental use of the cell's DNA repair capability. Ionizing radiation-sensitive mutants of D. *radiodurans* are also sensitive to desiccation. Radiation resistance and desiccation resistance are functionally interrelated phenomena; by losing the ability to repair ionizing radiation-induced cellular damage, the organism is sensitized to the lethal effects of desiccation.

Udupa et al. (1994) concluded that a dose of 6 kGy is on average required to inactivate a single CFU of *D. radiodurans* R1. In terms of DNA damage, this dose of radiation will induce approximately 200 double strand breaks, over 3000 single strand breaks and >1000 sites of base damage per *D. radiodurans* genome. All available evidence indicates that the ability of *D. radiodurans* to tolerate ionizing radiation is a function of the ability of this species to repair the DNA damage generated by ionizing radiation, but relatively little is known about the actual DNA repair mechanisms involved other than that to date two deinococcal proteins have been directly associated with ionizing radiation resistance: the *rec* gene product (a homolog of RecA protein), and the *pol* gene product (a homolog of *E. coli* DNA polymerase).

Dehydration induces DNA double strand breaks, either during dehydration or upon re-hydration of these cultures. Similar DNA double strand break gel patterns between radiation damage and dehydration damage suggest that radical chemistry is involved in the formation of double strand breaks. The damage inflicted during desiccation accumulates slowly and DNA double strand breaks are not obvious in *D. radiodurans* cultures until 8 days after desiccation. Beyond 28 days it is difficult to detect intact chromosomal DNA and by 42 days only low molecular weight DNA is apparent. But no loss in viability occurs over the first 14 days. In *E. coli*, the same kinetics of double strand break accumulation occurred. The rapid loss of viability in dried *E. coli* cultures (10^3 fold by the second day of desiccation) was not accompanied by overt evidence of DNA double strand breaks indicating that multiple DNA double strand breaks were not responsible for the observed lethality (Mattimore and Battista 1996).

In these desiccation studies with *D. radiodurans*, the periodic opening of the desiccator for sampling increased the relative humidity temporarily from 5% to 60% which lowered the survival of this organism by almost an order of magnitude compared to keeping the humidity low. It is, therefore, possible to lower the survival of desiccated cultures of *D. radiodurans* by subjecting them to cycles of desiccation and partial re-hydration. This could potentially be a very important factor in microbial survival in the Yucca Mountain environment, where intermittent precipitation would be the norm.

The role of DNA repair in the desiccation resistance of *D. radiodurans* is not unlike that ascribed to DNA repair in spores of *Bacillus* sp. During their dormancy, spores accumulate DNA damage that cannot be repaired until the spore germinates. The lack of water within the spore prevents enzymatic activity and therefore DNA repair. Similarly, no evidence has been observed of DNA repair in *D. radiodurans* cultures while they are desiccated, and it was assumed that desiccation-induced DNA damage cannot be repaired until the organism is re-hydrated. In *D. radiodurans* it

is not so much resisting damage as the capacity to repair damage which appears the most developed. Mattimore and Battista (1996) concluded that *D. radiodurans* is an organism that has adapted to dehydration and that its DNA repair ability is a manifestation of that evolutionary process. They believe that *D. radiodurans* is ionizing radiation resistant because it is resistant to desiccation and because desiccation resistance appears to require extensive DNA repair.

Double strand break induction and rejoining after ionizing radiation was analysed in *D. radiodurans* and a radiosensitive mutant by pulsed-field gel electrophoresis by Grimsley et al. (1991). Following 2 kGy, migration of genomic DNA from the plug into the gel was extensive but was not observed after 90 min. post-irradiation recovery. By this time, *D. radiodurans* chromosomes were intact as demonstrated by restoration of restriction clevage patterns characteristic of unirradiated cells. Following exposure to 4 kGy, double strand break rejoining took approximately twice as long, 180 min. Restoration of double strand breaks in the radiosensitive mutant strain (which appeared defective in recombination) was markedly retarded both at 2 and 4 kGy.

Harada et al. (1988) did an Arrhenius plot analysis of the mechanism of thermotolerance induction in the radioresistant *D. radiodurans.* Arrhenius plots of eucaryotic cells showed inflection points at about 43°C. For *D. radiodurans* cells such an inflection point was not observed and the data from this organism fell on a straight line from 42 to 60°C. These results show that *D. radiodurans* cells may be adaptive because of their ability to induce thermo-tolerance in the various changes under high temperature environments.

Minton (1994) presented a microreview of DNA repair in *D. radiodurans* because, while it is known that the extreme radioresistance in this organism possesses exceedingly efficient DNA repair, the molecular mechanisms responsible remain poorly understood. Following high exposures to UV (500 J/m^2) this organism carries out extremely efficient excision repair accomplished by two separate nucleotide excision repair pathways acting simultaneously. One pathway requires the *uvr*A gene and appears similar to the UvrABC excinuclease pathway defined in *E. coli.* The other excision repair pathway is specific for UV dimeric photoproducts but is not mediated by a pyrimidine dimer DNA glycosylase. Instead, it is initiated by a second bona fide endonuclease that may recognize both pyrimidine dimers and pyrimidine-(6-4) pyrimidones. After high doses of ionizing radiation (15 kGy) *D. radiodurans* can mend >100 double strand breaks per chromosome without lethality or mutagenesis. Both double strand break mending and survival are *recA*-dependent, indicating that efficient double strand break mending proceeds via homologous recombination. *D. radiodurans* contains multiple chromosomes per cell, and it is proposed that double strand break mending requires extensive recombination amongst these chromosomes, a novel phenomenon in bacteria. Thus, *D. radiodurans* may serve as an easily accessible model system for the double strand break initiated interchromosomal recombination that occurs in eukaryotic cells during mitosis and meiosis.

Wang and Schellhorn (1995) studied the induction of resistance to hydrogen peroxide and radiation in *D. radiodurans* Though bacteria of the radiation-resistant genus *Deinococcus* have a high resistance to the lethal and mutagenic effects of many DNA-damaging agents, the

mechanisms involved in the response of these bacteria to oxidative stress are poorly understood. To investigate antioxidant enzyme responses in *Deinococcus* sp., the catalase activity produced by these bacteria was measured and the sensitivity of these bacteria to hydrogen peroxide was tested. *Deinococcus* sp. had higher levels of catalase and was more resistant to hydrogen peroxide than *E. coli* K12. The high levels of catalase produced by *D. radiodurans* were, in part, regulated by growth phase. Cultures of *D. radiodurans* when pretreated with sublethal levels of hydrogen peroxide, became relatively resistant to the lethal effects of hydrogen peroxide and exhibited higher levels of catalase than untreated control cultures. These pretreated cells were also resistant to lethality mediated by UV light and gamma rays. These results suggest that *Deinococcus* sp. possess inducible defense mechanisms against the deleterious effects of oxidants and ionizing and UV radiation.

C.2.2 *E. COLI*

The study by Bresler et al. (1980) on mutants of *Escherichia coli* K12 with enhanced resistance to radiation is also of importance to the Yucca Mountain environment, because it deals specifically with the isolation and study of cross-resistance to various agents. After 44 cycles of radiation with increasing doses of gamma rays and subsequent cultivation of the irradiated cultures of *E. coli* K12 strain AB1157 to the stationary phase, nine independently induced Gam^r mutants with enhanced resistance to the lethal effect of radiation were isolated. The growth modification factor characterizing the relative radioresistance of the mutants as compared to the wild type was 5.5 for the strain Gam^r 444, having maximum resistance (at the survival level of 10^{-1}). Most of the mutants were very resistant to the lethal effect of H_2O_2. The Gam^r mutants were also hyperresistant to UV light, methylmethane sulphonate, and mitomycin C, although to a lesser extent than to gamma radiation. The Gam^r mutants were not always superior to the wild type in their resistance to nitrous acid. An increase in the cell size in the radioresistant mutants compared to the wild type was observed.

Bresler et al. (1980) also discussed how a significant increase in the resistance of *E. coli* (and *Salmonella typhimurium*) cells to the lethal actions of UV and gamma rays was caused by several plasmids, including the R factors of drug resistance. In all cases there was reason to believe that the enhanced radioresistance was a consequence of improved working of the DNA repair system. Other mechanisms of increasing radioresistance are also possible: increase in the DNA content (polyploidy), modification of the spectrum of primary damage to DNA due to endogenous protectors and activation of the enzymes eliminating the toxic products of irradiation, and changes in the cell membrane composition.

In a further study, Bresler et al. (1982) studied the formation and repair of single- and double strand breaks in the DNA of gamma-irradiated cells of three *E. coli* strains (parental strain AB1157 and mutants Gam^r 444 and Gam^r 445). The mutants did not differ from the wild type either in the initial yield of single strand breaks or in the rate or volume of repair of single strand breaks upon post irradiation incubation in the growth medium. However, the rate of double strand breaks in mutants was considerably lower than in the wild type both immediately after irradiation and after 3 h post-irradiation incubation in the growth medium. The decrease in the double strand break yield in Gam^r mutants correlated with their relative radiation resistance. It

was also found that in Gam^r mutants the degradation of DNA after gamma or UV irradiation decreased compared to the wild type. Bresler et al. (1982) proposed that the increased radiation resistance of Gam^r mutants is associated with a decreased formation of enzymatically induced double strand breaks in DNA, due to the decrease in the exonuclease degradation of DNA, and also to the increased efficiency of double strand break repair.

Francia et al. (1985) studied the ability of the R46 R factor and its derivative pKM101 (two drug-resistant plasmids) to modify the sensitivity of *E. coli* K12 to ^{60}Co gamma-radiation. Both plasmids enhanced bacterial survival after ^{60}Co gamma-radiation. The R46 R factor gave the studied wild-type (with respect to the excision and recombination repair system) strain a significant level of protection against both UV and ^{60}Co gamma-radiation. This effect was dependent on *recA*$^+$ genotype but not on *recB*$^+$, *recB*$^+$*recC*$^+$, and *recF*$^+$ genotypes. 5-Fluorouracil eliminated the R46 R factor from the parent and its *rec*$^-$ mutant strains. These strains lost not only the antibiotic resistance coded for R46 R factor but their radioresistance as well. The *recA*$^+$ and the *lexA*$^+$ genes control the inducible DNA repair and the events are collectively termed SOS responses. It is most probable that the plasmids R46 and pKM101 specially enhance the ability of the cell to carry out SOS processes. It is also possible that among other gene products of R46 or pKM101 there is one (or more), which is homologous with one of the DNA repair enzymes and thus can directly take part in DNA repair.

Munson (1963) studied radiation induced mutations in growing cultures of *E. coli*. Cultures of a tryptophan-requiring strain of *E. coli* (in a medium containing tryptophan) were irradiated continuously (with ^{137}Cs source) over many generation times at constant dose-rates between 50 and 1400 rad/h (0.5 and 1.4 Gy/h). The viability under these conditions was at least 90%. The mutant-frequency increased linearly with time, as would be expected for a population mutating at a constant rate. The rate of increase, which is equal to the mutation-rate per mutable unit (gene), was proportional to the dose-rate. Supplementary experiments with X-rays gave the same proportionality constant at dose rates of 500 rontgen/min. Over the temperature range 37°C to 16°C the change in mutant-frequency per rontgen fell by a factor of two and the growth rate by a factor of 7. It was concluded that radiation damage to DNA which is expressed as a mutation is subject to a spontaneous repair process, which is more effective at 16°C than at 37°C.

Gram-negative bacteria possess several transport systems for the uptake of branched chain amino acids. Amino acid uptake has been shown to be inhibited by visible and near UV light. Robb et al. (1978) demonstrated a decreases in the rate of apparent uptake of radioactive leucine by *E. coli* as a result of UV and visible light. Two possible explanations for this decrease in amino acid uptake were proposed. Firstly the radiation may cause a generalized non-specific membrane breakdown such that the rate of leu leakage approaches the leu uptake rate. Secondly there may be an inactivation of the uptake systems or associated energy coupling systems. This work confirms the latter possibility for leu uptake systems: radiation inactivates an essential component or components of the uptake mechanism per se and is not the result of generalized leakage of the cell membranes.

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APPENDIX D:

EFFECTS OF PRESSURE ON MICROBIAL SURVIVAL

D.1 ATMOSPHERIC PRESSURE

In general, microorganisms are not affected by changes in atmospheric pressure, except at very low atmospheric pressure such as in an artificial vacuum or the upper atmosphere where water may evaporate and oxygen becomes scarce, which may inactivate or kill microbes.

D.2 DEEP SEA PRESSURE

Bacteria in the deep sea and in geological formations are subject to large pressures (hydrostatic or lithostatic, respectively). It has been shown that steady hydrostatic pressure in the range of 1 to 400 atmospheres (0.1 - 40 MPa) has little or no effect on the growth and metabolism of most microorganism. However, sudden decompression may rupture cell membranes due to the release of gas bubbles. Hydrostatic pressures in excess of 400 atmospheres (>40 MPa) tend to inhibit or stop growth of terrestrial and shallow-water organisms, but not of so-called barotolerant deep-sea organisms which may be exposed to pressures as high as 1000 atmosphere (100 MPa).

Most research into the pressure sensitivity of microorganisms has been carried out with deep-sea organisms. Although barophilic (i.e., optimal growth pressure >40 MPa or >400 atmospheres) organisms have been isolated, it seems that mostly the deep-sea community is only moderately barotolerant rather than barophilic, and its activity is severely constrained, though not halted, by the prevailing high hydrostatic pressures. Tests have shown that low temperatures prevailing in the deep sea (2-4°C) only limit deep-sea microbial activity moderately but that the combination of low temperatures and high pressure imposes severe limitations on microbial activity, unless the organisms are barophilic. The generally observed trend for specific growth rate profiles of barophilic deep-sea bacteria indicate optimal high-pressure growth near their upper temperature limit for growth. It is noteworthy that thus far all strictly barophilic microbes studied seem to group within a particular branch of a single genus (i.e., *Shewanella*) (Kato and Bartlett 1997 and references therein).

At least some of the barophilic bacteria studied so far have survived decompression during recovery from the deep sea, so controlled temporary decompression may have no lethal or irreversibly damaging effects. High hydrostatic pressure appears to inhibit microbial synthesis of RNA, DNA and proteins, and affects membrane transport functions and rates of activity of various enzymes, probably by distorting the configuration of those enzymes. Many of the basic properties of barophiles that enable their survival at extremes of pressure remain to be elucidated but several genes whose expression is regulated by pressure, or which appear to be critical to baroadaptation, have been uncovered (Kato and Bartlett 1997) in DNA samples recovered from sediments from the deepest ocean trench.

D.2.1 HOT SMOKER VENTS

Hot smoker vents are submarine geothermal vents where mineral-laden hydrothermal fluids with temperatures of 200 to 350°C build up huge rock chimneys. The water in these vents is sterile,

with no evidence of life at 250°C, in line with the dramatic destruction of the building blocks of life at those temperatures. The temperature along the walls of the chimneys is much lower, and these locations are teeming with life (10^8 cells of one particular hyperthermophile per gram of rock, Stetter (1995)). Almost all the hyperthermophiles isolated from these environments are obligate anaerobic, reduce S and are heterotrophic. The source of organics is still unknown and may be derived from sediments. In contrast to the marine sites, continental hot springs are often oxic and highly acidic and from them many autotrophic thermoacidophiles have been isolated. Marine hyperthermophiles require quite high salt concentrations and one would expect quite different species in freshwater (continental) environments, yet several species have been isolated from both types of ecosystems. (Several hyperthermophiles have been isolated from freshwater springs on three different continents. All of the hyperthermophiles were discovered in geothermally-heated ecosystems and, therefore, viable hyperthermophiles may be ubiquitous in geothermal systems. They appear to readily survive adverse conditions in the dormant state and even fastidious anaerobes appear to be relatively insensitive to oxygen.) Deep sea hyperthermophiles are not dramatically affected by growth under the high pressures of their natural environments. An obligately barophilic or extremely halophilic hyperthermophile has yet to be isolated (Stetter 1995).

Enzymes from organisms living in deep sea vents where boiling water and crushing pressure combine become more stable and active when the pressure is increased. The same appears to be true for enzymes from thermophiles that are not adapted to high pressure. These enzymes gained stability when pressure was increased. High pressure appears to be an extrinsic stabilizer of some but not all proteins of hyperthermophiles (Reeve 1994). The maximum growth temperatures of hyperthermophiles (88 to 103°C) increased by <1°C under very high pressures (200 to 300 atm), although growth rates for some isolates did increase by about 15% and higher temperatures were tolerated but no species survived longer than 5 minutes at 150°C (Reeve 1994).

Salt can enable both special halophilic enzymes and ordinary enzymes to survive in solvents (Flam (1994)). Some unusual salts dramatically increase the heat resistance in vitro of enzymes purified from some hyperthermophiles. High internal salt concentrations should also help to prevent the denaturation of double-stranded regions of nucleaic acids (Reeve 1994).

D.3 OIL RESERVOIRS

Deep geothermally-heated oil stratifications are a novel non-volcanic high temperature environment for hyperthermophilic Archaea. H_2S formation (reservoir souring) and 10^6 viable hyperthermophiles per liter of extracted fluids yield evidence for hyperthermophilic communities within oil-bearing Jurassic sandstone and limestone found about two miles below the bed of the North Sea and below the Alaskan North Slope permafrost soil (Stetter et al. 1993, Stetter 1995). The in situ temperature is about 100°C. The minimum growth temperature of the oil field hyperthermophiles suggest that they are unlikely to be active in surface soils, oil and groundwater flows at ambient temperatures. The predominant form of nutrition in these wells is unknown at present. The novel hyperthermophiles that grow anaerobically on a component of

crude oil may be a critical part of a complex reservoir food net. Stetter et al. (1993) pose the question of where these organisms come from. They suggest that survival since the formation of the oil reservoirs is unlikely because all reservoirs had been flooded with seawater. Hyperthermophiles are known to survive in cold seawater, hence they could have been introduced when the oil field wells were flooded during the oil recovery process. There is only a low population of hyperthermophiles in seawater but the reservoirs are flooded by as much as 127 000 m^3 of seawater per day, and even though the water is treated with biocidal chemicals, this may not be effective in killing all microorganisms. Some hyperthermophiles isolated from oil wells are similar to those found in marine hot vents. All of the hyperthermophiles from oil reservoirs appear to reduce sulphur compounds which may be provided by reservoir minerals, the crude oil and seawater. The production of H_2S contributes to biological souring of deep hot wells (Stetter et al. 1993).

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