HEAVY METAL TOXICITY IN BIOREMEDIATION: MICROBIAL CULTURES AND MICROSCOPY

THESIS

Jason B. Goodbody, 1st Lieutenant, USAF

AFIT/GEE/ENV/97D-06

DEPARTMENT OF THE AIR FORCE
AIR UNIVERSITY
AIR FORCE INSTITUTE OF TECHNOLOGY

Wright-Patterson Air Force Base, Ohio

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Jason B. Goodbody, B.S.
1st Lieutenant, USAF

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Dr. Larry Burggraf; Member

Major Jeffrey Martin; Member

Dr. Charles Bleckmann; Chairman

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Jason Goodbody

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Abstract

This research employed a variety of microscopy and spread plating techniques to observe the effects of heavy metal treatments on a toluene-selected bacterial population.

Microbial colonies were cultured on spread plates and the resulting numbers were compared to respiration data. The mechanisms of reproduction were demonstrated to be more sensitive to metal treatments than were the mechanisms of respiration.

Phase contrast, Gram stain, fluorescent microscopy, were used to compare and document a wide variety of bacteria resulting from different metal treatments as well as from environmental changes within the source bioreactor.

The removal of sensitive bacteria and the selection of metal tolerant species resulting from metal treatments was observed. Species that were initially unobserved within the bioreactor appeared to dominate when competing types of bacteria were removed and more agreeable environmental conditions were present.

The use of fluorescent stains to differentiate between live and dead bacteria when treated with heavy metals proved to be impractical as the bacteria exhibited auto-fluorescence. Such new findings, however, did aid in the characterization of different types of bacteria and offered new techniques for potential heavy metal toxicity measurements as well as differentiation methods.

HEAVY METAL TOXICITY IN BIOREMEDIATION: MICROBIAL CULTURES AND MICROSCOPY

I. Introduction

Overview

This thesis effort explores the mechanisms of heavy metal toxicity to toluene selected bacteria through the use of microscopy and microbial cultures. An indicator of bacterial robustness and health, metabolic activity is often characterized by the bacteria's consumption of dissolved oxygen (DO). A reduction in DO consumption indicates that a significant inhibition to the microbial activity has occurred. This inhibition can be attributed to cellular death, in which the number of viable cells participating in oxygen consumption is reduced, or non-lethal debilitation of metabolic activity. Respirometry, or the measurement of biological oxygen demand (BOD), gives little insight into the extent to which either mechanism is operating.

Through the use of microbial cultures, bright field, phase contrast, and fluorescent microscopy, as well as Gram staining procedures, the impacts of heavy metal toxicity on bacteria can be better defined.

Past Research

While there has been extensive study on the effect of heavy metals upon bacteria, typically, such studies have been conducted in sterilized environmental samples and have used only single species. The use of heterotrophic bacteria in terrestrial and aquatic environments, while difficult to culture and maintain, are essential for the understanding of the effects heavy metals have upon their activities.

The presence of heavy metals have been shown to inhibit primary productivity, photosynthesis, nitrogen fixation, detritus and litter decomposition, and bioremediation in soils, sediments, and surface waters.

The Problem

Bioremediation. In accordance to the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), often times referred to as Superfund, over 1200 hazardous waste sites are listed on the National Priorities List (NPL). As of 1991, an additional 30,000 sites were under consideration under this program, and the Environmental Protection Agency (EPA) estimates that an additional 4000 sites are in need of review. (Giamporcaro, 1993: 138) The escalation of the number of such sights requires the implementation of continuously improving remediation technologies. There are several effective physical and chemical methods for the removal of hazardous wastes. These include the use of landfills, incinerators, air stripping, and long-term storage.

The environmental fate of organic chemicals in soils is dependent upon the physical and chemical characteristics of both the compound and the contaminated soils.

Abiotic mechanisms may transport the compounds from the area of interest or slightly alter their structure; however, complete mineralization of organic compounds to carbon dioxide and water is strictly the result of microbial activities. Bioremediation is dependent upon these microbial activities and is becoming an increasingly popular technique for removing substances hazardous to the environment and to human health. (Atlas and Bartha, 1993:422)

Physical methods for remediating the environment are often costly, time consuming, and not completely effective. Interest in bioremediation increased after the Exxon Valdez incident in 1989, where more than $1 million a day was spent to scrub the oil contaminated rocky beaches of Prince William Sound, Alaska. (Atlas and Bartha, 1993:422) Bioremediation can be performed on-site, with limited equipment, and at a fraction of the cost incurred through the use of more conventional methods. (Atlas and Bartha, 1993:422)

Biodegradation rates are known to be influenced by such factors as the substrate concentration, the presence of inhibiting or enhancing substrates, temperature, pH, water availability, the redox environment, microbial activity, and nutrient availability. Not only are bioremediation processes directed toward organics, microorganisms are also commonly used in the removal of metals from preferred materials.

Metal Toxicity. High concentrations of metals in remediation sites are toxic to microbial growth and function. There are many routes of toxicity and, as a result, there are differences in opinion as to the primary mechanisms involved. The presence of heavy

metals, regardless of the primary causes of toxicity, impairs the full use of microorganisms in normal detritus degradation, bioremediation of hydrocarbons harmful to human activity and the environment, or in nitrogen fixation and oxygen production.

Objective

The objective of this thesis effort is to explore the mechanisms of metal toxicity and the various methods for determining cellular viability.

Agar spread plates for colony growth will be used to determine the number of viable cells remaining after treatment with heavy metals. The number of bacterial colonies grown from surviving and perhaps even damaged bacterial cells can be correlated with the metabolic activity recorded in parallel experimental efforts executed by Captain Pat Marbas. Such correlation, or lack there of, will provide insight as to the method of metal toxicity.

Light microscopy, to include Gram and fluorescent staining techniques, will also be employed to better characterize the populations being evaluated and to quantify the live/dead ratio in a given sample.

II. Literature Review

Materials

Toluene. Toluene was chosen because of its common use in industry, its familiar presence as a contaminant in groundwater, and its ability to be degraded aerobically by soil microorganisms. Toluene is often used in microbial studies because many of the environmental and microbial factors which affect its extent and duration in soils, is representative of similar fates of a wide variety of other environmentally offensive aromatic compounds.

Properties of Toluene. Toluene, C_7H_8, is a naturally occurring aromatic compound and, along with Benzene, Ethylbenzene, and Xylene (BTEX compounds) is a constituent of crude oil. Often produced in petroleum refineries and coal tar distillation, it is used as a component of fuels, paints, coatings, dyes, gums, oils and resins. (De Zuane, 1990: 256)

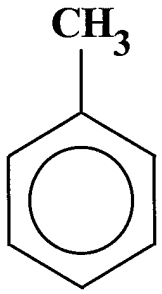


Figure 2-1 Chemical Structure of Toluene

Table 2-1 Properties of Toluene

Molecular Formula	Mol Weight gm/mol	Specific Density	Solubility g / 10^6 g H_2O	Boiling Point	Vapor pressure mm Hg/atm
C_7H_8	92.15	0.866	515 ± 17 @ 20 C	111 C	20.1 @ 20 C

(Testa and Winegardner, 1991: 28)

Ecological Aspects of Toluene. Toluene is moderately toxic through inhalation or ingestion and can be tolerated without noticeable effects up to 200 ppm in ambient air. (Manahan, 1993: 753) Exposure to 500 ppm may cause nausea, headaches, impaired motor coordination, and possible kidney and liver damage. Massive exposure can lead to cardiac sensitivity and narcotic effects that could lead to a comatose reaction. (De Zuane, 1990: 256)

Toluene can be oxidized enzymatically to form easily excreted by-products and, as a result, is much less toxic than benzene. Because of its decreased toxicity, toluene is often used as a substitute for benzene in industry.

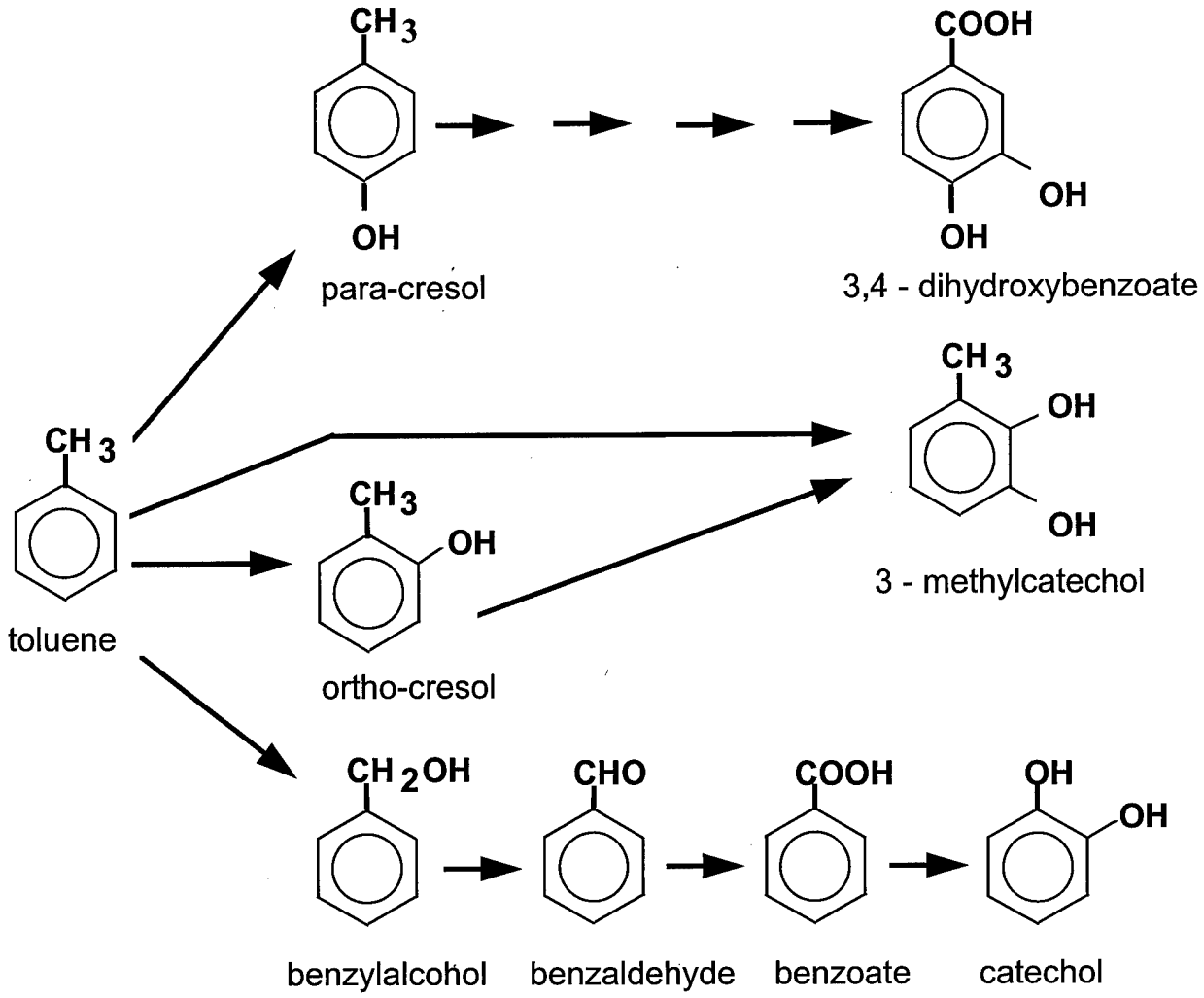
Over the past 20 years, the most likely cause of BTEX aquifer contamination is due to accidental release from underground storage tanks. (National Research Council, 1993: 104) The United States Environmental Protection Agency (USEPA) has regulated industry to restore ground water contamination of BTEX compounds to safe health levels. For toluene, the health standards for maximum contaminant level (MCL) and secondary levels (SMCL) are 2 mg/L and 0.04 mg/L respectively. (De Zuane, 1990: 257) As a result of its moderate toxicity and its common affiliation with more toxic substances, toluene becomes of notable interest for those involved in efforts to improve upon remediation technology.

Biodegradation of Toluene. Provided there is adequate dissolved oxygen in the ground water, low levels of toluene, in the ppb or low ppm range, can be readily

biodegraded by indigenous soil microbes. (National Research Council, 1993: 105) In situ biodegradation activities have been considered a practical low cost alternative for contaminated ground water management. Under proper management, in situ bioremediation can effectively reduce the plume size as well as control the migration of toluene and its related hydrocarbons (BTEX). (National Research Council, 1993: 104)

Aromatic hydrocarbon degrading bacteria are remarkably diverse in their catabolic nature, employing a wide variety of pathways, dependent upon both suitable environmental conditions and the type of organisms present. (Leddy and others, 1995; Lee and others, 1995; Landa and others, 1994) Enzymes excreted by microorganisms are solely responsible for this degradation and are known as oxygenases. These enzymes, usually mono - and dioxygenases, are surprisingly non-specific, allowing for the catabolism of BTEX, trichloroethylene (TCE), 1,1 dichloroethane, 1,1,1 - trichloroethane, chloroform, as well as a variety of aromatics. Provided that the compounds are not saturated with halogens, having at least one carbon-hydrogen bond, they are usually biodegradable. (Landa, 1994)

The degradation of toluene is a complicated process that requires many discrete steps. The entire process may require the action of several species of organisms within the microbial population; these organisms most likely act on the toluene at different stages of the process. Examples of such pathways are noted in Figure 2-2.



(Duetz and others, 1994; Leddy and others, 1995)

Figure 2-2 Example Pathways for Bacterial Degradation of Toluene

It is this non-specificity of enzymatic activity which makes toluene a suitable primary substrate, facilitating the degradation of the more toxic substances mentioned above. Andrew Landa investigated the simultaneous catabolism of toluene and TCE by a strain of bacteria known as *Pseudomonas cepacia* G4, a common "toluene-oxidizing, TCE-degrading bacteria" (Landa and others, 1994).

Those enzymes that contribute to the first steps in the breakdown of toluene also effectively catalyze the catabolism of TCE (Fan and Scow, 1993). This supports field observations that indicate that the most effective biodegradation occurs when BTX and/or halogenated aromatics are found in mixtures as opposed to their individual components (Lee and others, 1995). Researchers have had difficulty in extrapolating laboratory investigations of a single compound under controlled conditions (pH, nutrient availability, temperature, etc.) to real field situations where a variety of conditions complicate remediation efforts (Landa and others, 1994; Lee and others, 1995; Atlas and Bartha, 1993: 430; Fan and Scow, 1993).

Metals

Metals are typically defined as those compounds which form positive ions when in solution and form oxides, rather than acids, when introduced to water.(Collins and Stotzky, 1989: 31) The term "heavy metal" is typically used as these metals are recognized as environmental pollutants. They have also been regarded as "heavy" because their specific gravities are greater than five.

Nieboer and Richardson (Nieboer and Richardson, 1980: 3) proposed a classification system that took into account the metal's chemistry. In this manner, metals such as aluminum, which have relatively low specific gravity of 1.5, can be regarded for their role as an environmental pollutant. (Walker and others, 1996: 5)

Nieboer and Richardson separated the essential and non-essential metals into three classes based upon their impacts as pollutants. Class A ions are known as oxygen seeking and have a more ionic character to the bonds in which they form. Class B metals are known as sulfur/nitrogen-seeking and form covalent bonds. Borderline metals have the characteristic of both.

Table 2-2 Class A / Class B Metal Classification

Class A	Borderline	Class B
Calcium	Zinc	Cadmium
Magnesium	Lead	Copper
Manganese	Iron	Mercury
Potassium	Chromium	Silver
Strontium	Cobalt	
Sodium	Nickel	
	Arsenic	
	Vanadium	

(Walker and others, 1996: 5)

Class B metals can displace essential borderline metal ions in biomolecules such as metalloenzymes. Their ability to form stable organometallic cations in aqueous solution makes them highly lipid soluble. As a result, these compounds can accumulate in cellular material and exert toxic effects. (Collins and Stotzky, 1989, 34)

Growth Media (Agar)

Agar is the primary solidifying agent in bacteriology. Its propensity to gel from a molten state when cooled to 40^0C yet remain solid up to 65^0C makes it highly desirable medium with which to work. Its transparency allows for easy analysis of colony growths. (Krieg and Gerhardt, 1994: 217)

Sterilization of Media. Autoclaving is a process by which most media used in microbiology are sterilized, and typically, requires high pressures and temperatures. The heat and high pressure utilized with this process, for all practical purposes, kill all microorganisms and bacterial spores. While *thermophiles,* or heat loving (and often pressure tolerant) microorganisms can persist in such conditions, they are unlikely to be encountered in the laboratory. (Cote and Gherna, 1994:169)

Agar (Spread) Plates. Developed by Robert Koch, spread plates were instrumental in the advancement of bacteriology as a science. The agar plate, as it is also known, allowed for the isolation and enumeration of bacterial colonies originating from viable cells. Spread plates are created by pipetting a sample onto the surface of a solidified nutrient medium, or agar, and the cells are dispersed (spread) with a wire or glass spreader.

Microorganisms

For 3.5 billion years, or over 75% of the earth's existence, prokaryotic organisms are believed to have been thriving and adapting within a changing environment. Throughout this period, all life and its ubiquitousness has been a function of its ability to

interact with its surroundings. Bacteria, of all organisms, have demonstrated their abilities to thrive in the widest range of habitats and in what other organisms would consider the most toxic of environments.

Because microorganisms are so vital to natural systems and becoming more so in human industrial activities, it is important to understand the conditions in which these microorganisms operate. It is therefore essential to understand what substances and conditions have detrimental impacts upon microbial existence in order to protect natural cycles as well as increase efficiency in anthropogenic processes.

Activated Sludge. Activated sludge is a microbiologically mixed culture of uncontrolled micro- and macro-organisms. Typically, this assemblage is comprised of 95% prokaryotic organisms (bacteria) and 5% eukaryotic organisms (protozoa, rotifers, higher invertebrates) (Richard, 1989:1). The wastewater stream entering the activated sludge process is composed of organics and inorganics that may be undesirable for release into the natural water system. It is the microbial biomass that is responsible for metabolizing or breaking down these chemicals or transforming them into environmentally acceptable forms. The primary organisms responsible for much of the activated sludge process are the floc-forming bacteria, which grow upon wastewater organics and settle out under gravity leaving a clarified effluent.

Sewage sludge is often used as fertilizer in agriculture, supplying essential nutrients such as nitrogen, phosphorous, calcium, and magnesium to farmlands. Heavy metals in sewage sludge more often originate from industrial activities such as smelting,

mining, manufacturing, and chemical and pesticide production. (McGrath and others, 1994) It is through the application of sewage sludge on farming lands which results in elevated metal concentrations in soil systems.

The metals tend to be tightly bound to soil particles and tend not to be removed from an area without physical means such as purposeful remediation or, simply, soil erosion. In sludge, or soils with high organic content, heavy metals are less mobile as they are often chelated to these organic particulates. In acidic conditions, however, the metals are made more available and are taken in by microorganisms and plants and are entered into the food chain and can undergo biomagnification. (McGrath and others, 1994)

Much of our knowledge on the ecological effect of metals on plants and animals does not carry over to the microbial world. In the last 20 years, and more recently since Eastern Europe began its efforts in environmental clean-up, increased concern was raised that relatively small metal concentrations had such large effects on soil microbial activity. (McGrath and others, 1994; Laskowski and others, 1992)

Toluene Degraders. Numerous studies in the biodegradation of toluene have shown that the most common degraders of this substance are of the family genus *Pseudomonas* (Landa and others, 1994; Lee and others, 1995; Duetz and others, 1994; Fan and Scow, 1993). These aerobic Gram-negative bacilli (or rods) generally feed upon multi-carbon substrates (Holt and others, 1994: 94) and are frequently found in soil,

growing around neutral pH and at mesophilic temperatures (Microbionet, 1997; Holt and others, 1994; 94).

The following species have been shown to contribute to the degradation of toluene as well as degrade its catabolites such as catechol and its substituted forms (Figure 2-2) via two approaches: the direct attack to the aromatic ring, dioxygenated at the 2,3 position, for example, resulting in 3-methylcatechol (Duetz and others, 1994); or the methyl group can by oxidized by monooxygenase resulting in an alcohol group. The following species of *Pseudomonas* that are commonly attributed to the degradation of toluene are *P. cepacia, P. mendocina,* and *P. putida* (Duetz and others, 1994; Fan and Scow, 1993).

Cell Physiology.

Internal Physiology. Bacteria, like the more complex eukaryotic organisms, have all the inner workings for cell function, cellular division and growth, genetic transcription and translation, and energy production. All of this activity within a single bacterium typically takes place within a volume of 1.5 - 2.0 μm^3 (Beveridge, 1989: 9). Bacteria differ from more complex organisms (eukaryotes) in their lack of segregated organelles. (Brock and others, 1994: 15).

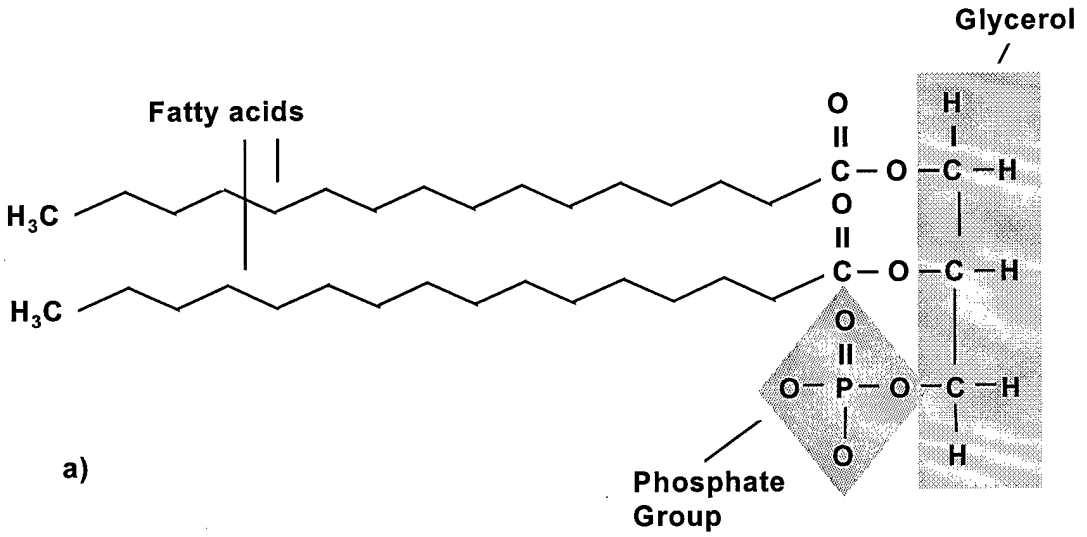
External Physiology. What often times differentiates various genera of bacteria is the manner in which they operate within their environment. And as such, their structure and chemical characteristics are often effective indicators of their abilities and function. How the bacteria alter their environment is equally important as how they are

impacted by their surroundings. Much of this interaction, or at least what can be observed, takes place throughout and external to the outer structures.

The enveloping layers that make up the outer structures of the bacterial cell consist of a cytoplasmic membrane, a capsule, sheaths, an S-layer, and flagella. (Brock and others, 1994: 12)

Cytoplasmic Membrane. All cells have a highly selective permeable barrier known as the *cytoplasmic membrane*, separating the life sustaining metabolic activity of the cell from its environment and allowing all food materials, nutrients, and waste products to pass. The cytoplasmic membrane is normally only 8 nm thick and is comprised of a phospholipid bilayer, which is structured such that the hydrophobic components of the phospholipids (fatty acids) are internal to the bilayer and the hydrophilic components (glycerol) are external and exposed to the external aquatic environment. (Brock and others, 1994:51) (Figure 2-3)

It is through this cytoplasmic membrane, the cell can isolate itself osmotically from its surroundings, concentrating necessary nutrients within the cell. If this membrane is damaged, the cell lyses, its contents leak out into its surroundings, and the cell becomes in equilibrium with its environment. In effect, the cell dies.



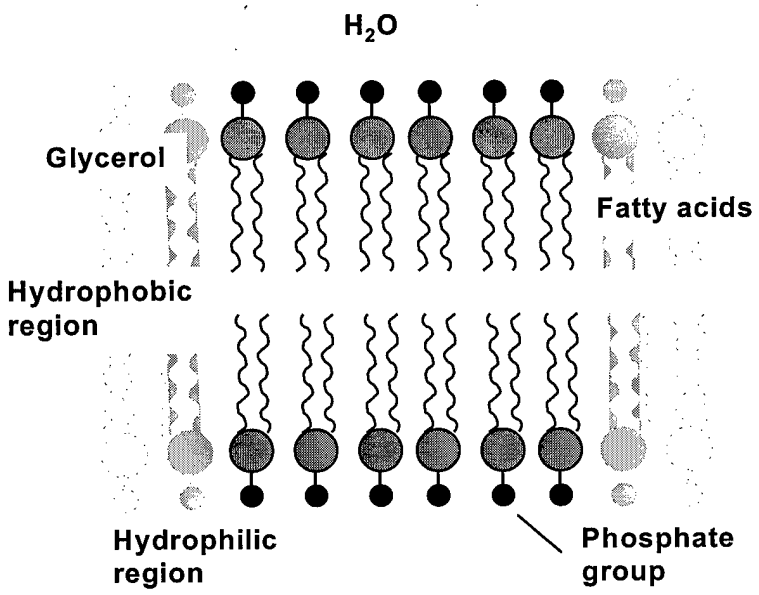


Figure 2-3 Phospholipid Structures

a) Typical structure of phospholipid b) Fundamental structure of phospholipid bilayer (Brock and others, 1994)

S-Layers. S-layers are paracrystalline arrays consisting of proteinaceous material, which is sometimes found on the exterior of the cell wall. The S-layer requires the presence of calcium or magnesium as an essential ingredient, acting as cement for these planar arrays. (Beveridge, 1989: 15). The S-layer is typically not expressed unless the cell is under environmental pressure as it is a costly structure to produce and maintain. (Beveridge, 1989: 16)

Capsules and Sheaths. Typical of bacteria in aquatic environments is the possession of an envelope structure known as the capsule. The capsule provides a formidable barrier to antibodies and contributes to biofilm formation. The capsule layer extends 0.5 to 1.0 micrometers beyond the cell surface and is constructed of repetitive sequences of sugar derivatives or proteinaceous materials depending upon the family of bacteria. (Beveridge, 1989: 16)

Regardless of the structural component, capsules are mostly water and thus alternate between gel and liquid states, providing an opportunistic environment in which metal ions can freely interact. (Beveridge, 1989: 16) As the capsule is primarily anionic, and contains carboxylate groups, it further acts as a chelator of bivalent cations. Along with the cell wall and outer membrane, the capsule plays a key role in metal accumulation.

Sheaths are fibrous cellular growths that encapsulate the growing chains of cells making up the capsule surrounding the cell walls of some Gram-negative bacteria.

Gram Types. Bacteria are difficult to characterize as pathways for degradation are often common across widely varying groups of bacteria. More so, visual observation in terms of shape and motility says nothing of species or even genus characterization. Gram staining is often employed to divide the bacteria into two major groups referred to as Gram-positive and Gram-negative bacteria. The variances in cell structure are the foundation for this technique of categorization.

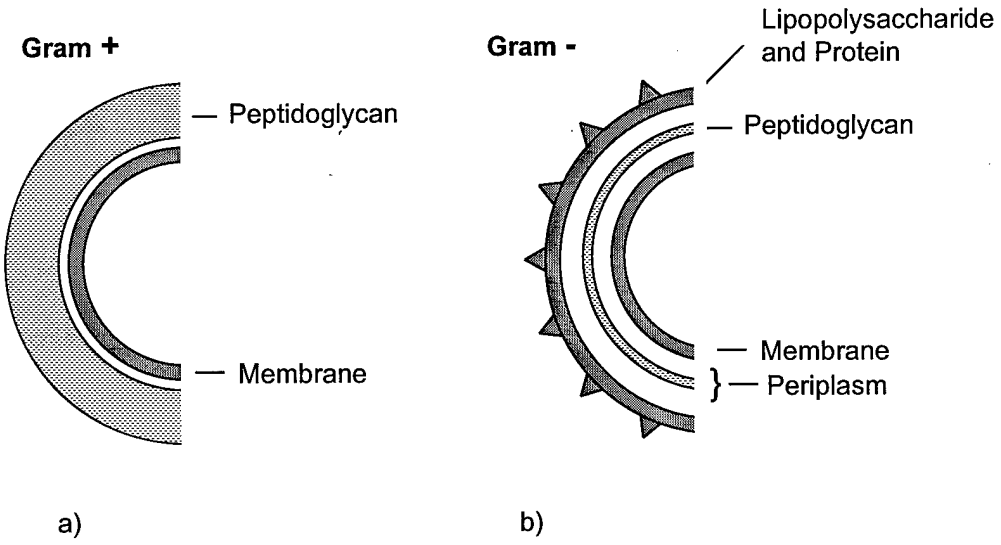


Figure 2-4 Cell Walls of Bacteria

a) Schematic diagram of Gram-positive Bacteria b) Schematic diagram of Gram-negative Bacteria

(Brock and others, 1994)

Eubacterial Gram-Positive Walls. Gram-positive bacteria cellular structure is rather simple compared to its counterpart, Gram-negative bacteria. The cell wall consists primarily of a cytoplasmic membrane and a thick layer of peptidoglycan (Gram-negative bacteria have a much thinner layer). This peptidoglycan layer consists of repeating glycan chains, composed of two sugar derivatives, N-acetylglucosamine and N-acetylmuramic acid, and various amino acids which form peptide cross-links. The combination of the glycan chains and the amino acid cross-links can create a very rigid matrix, which maintains the structure of the cell wall. This peptidoglycan layer can constitute as much as 90% of the cell wall in Gram-positive bacteria and can be 20 layers thick. (Atlas, 1993: 62)

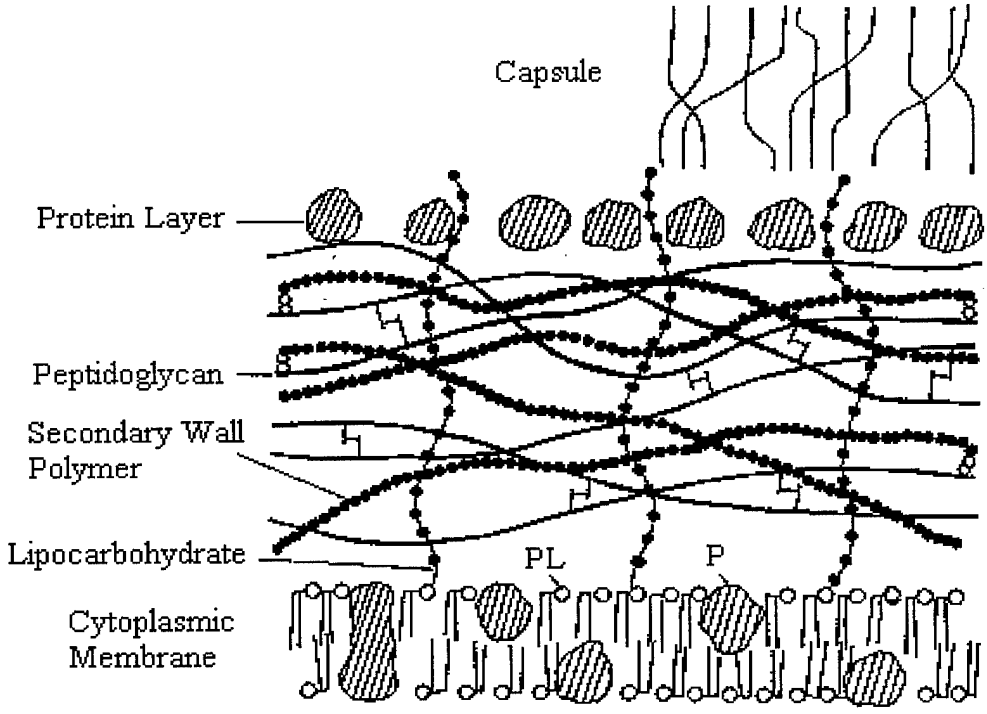


Figure 2-5 Gram Positive Cell Envelope

(Hancock and Poxton, 1988: 7)

The carboxylate and phosphate groups within the cell wall are attractive metal binding sites. By removing or neutralizing these groups from the cell walls, studies indicated that it was these groups that were major contributors as metal binding sites. (Doyle, 1989: 284)

Secondary polymers attached to the peptidoglycan layers known as teichoic or teichuronic acids are intensely strong chelators of heavy metals. (Beveridge, 1989: 14) It is the presence of these teichoic acid groups, the relative preponderance of carboxylate groups and other binding sites, and the relative thickness of the cell wall which give Gram-positive bacteria a high affinity for heavy metals, resulting in a higher sensitivity to metals in the environment. (Doyle, 1989:289)

Eubacterial Gram-Negative Walls. The Gram-negative peptidoglycan layer, conversely constitutes only about 10 % of the cell wall and may be present in only a single layer. (Beveridge, 1989: 14) The remainder of the membrane structure of Gram-negative bacteria takes the form of an outer membrane (OM) layer. This outer membrane is a bilayer membrane as well, consisting of phospholipids lining the inner leaf of the membrane and lipopolysaccharides (LPS) forming the outer leaf. (Doyle, 1989: 299) Proteins are scattered throughout the OM acting as specific and non-specific membrane channels. The OM is anchored to the peptidoglycan layer by lipoproteins and acts as a secondary barrier providing additional protection from harsh chemicals, antibiotics, enzymes, and detergents. (Brock and others, 1994: 794)

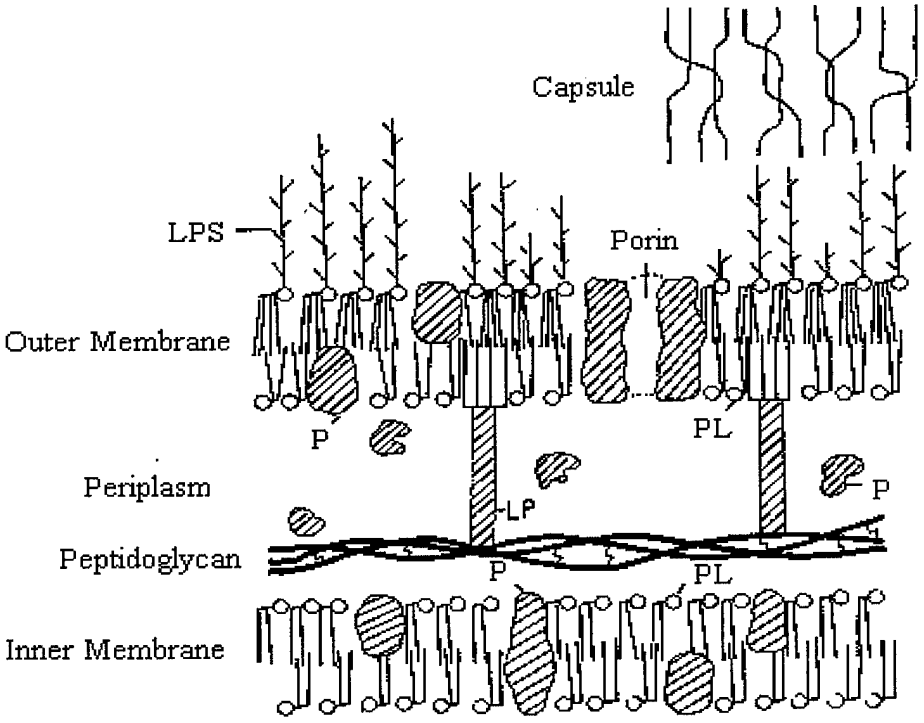


Figure 2-6 Gram-Negative Cell Envelope

(Hancock and Poxton, 1988: 17)

Motility. Many prokaryotic organisms are motile, allowing them to move from region to region within their microenvironment in search of more hospitable, nutritive surroundings. Flagellar movement is most common among bacteria, resulting from the energetic rotation of long, thin protein strings (flagella). (Brock and others, 1994: 66) Such structures are difficult to see under the light microscope and are observed more readily after staining or under an electron microscope.

The arrangement of flagella on the bacterial cell varies between species. Generally, flagellar structures are arranged singly at the pole (monotrichous), in tufts at the pole (lopotrichous), at both poles (amphitrichous), or over the entire surface of the

cell (peritrochous). The motion of such cell types results from the rotation of the wavelike structure around the point of attachment to the cell. (Brock and others, 1994: 67) Generally, peritrichously flagellated bacteria move slowly and smoothly in a strait line while polar flagellated bacteria move more erratically in a spinning motion. (Brock and others, 1994: 68)

Intimate Relationship With Metal Ions. Metal salts have been ever present throughout the evolution of aqueous living systems. As a result, the effective management and utilization of such metals in solutions has been well developed in the microbial realm.

Much of the metal salts within the oceans, lakes, streams, and estuaries are in the form of magnesium, calcium, potassium, and sodium salts. (Silver and others, 1989) While many other constituents are clearly available in solution, no element having a concentration less than 2 nmol in the ocean is generally considered essential for life. (Beveridge, 1989: 7) Any such rare element found in high concentrations and not ordinarily encountered in a natural system would be considered toxic to an organism. Even elements that are essential in trace amounts (copper, iron, and zinc), when found in high concentrations, can also be toxic.

Essential and trace metals can be captured in the outer portions of the cell such that the organism can be considered to be continuously enveloped in a "brine" of various ions. (Beveridge, 1989: 16) This phenomenon of capturing metals can act as the first step in the bacteria's active accumulation and assimilation of otherwise trace metals at

concentrations several orders of magnitude above background levels. (Atlas, 1993: 290) This concentration of metal ions may have developed as a method for accumulating essential cations if the bacteria finds itself in a more dilute environment. (Ferris, 1989: 303) The outer membrane may also immobilize heavy metals, thus preventing their penetration into the cell.

Microorganisms' ability to oxidize metals, thus bringing them out of solution, plays an important role in the biological cycling of metals on a global scale. Microorganisms are also considered for their ability to accumulate metal ions within and on the membrane surface of the cell at concentrations several orders of magnitude above background levels. Such interactions are so predominant that they can often be utilized for economic or ecological gain.

Often microorganisms are used effectively in mining operations. Extracting, or leaching, of valuable metal from low-grade ore is often difficult and expensive to pursue. In sulfide-containing ores, however, it is possible to utilize sulfur-oxidizing bacteria (*Thiobacillus ferrooxidans*, for example) in the recovery of copper and uranium (Atlas, 1993: 441). Microorganisms' ability to solubilize metal ions through the oxidation process raises the potential for the extraction of rare metals or the removal of heavy metals from industrial run-off (Atlas, 1993: 439).

Mechanisms of Metal Toxicity and Tolerance. High concentration of metals are toxic to microbial growth and function. As a result, remediation of hydrocarbons harmful

to human activity or the environment can be significantly reduced in the presence of heavy metals.

There are many mechanisms of metal toxicity. While anions in solution have been shown to reduce the toxicity of metals, this reaction can work in the opposite manner. Metals can, conversely bind with nutritive anions as well as essential enzymes resulting in their decreased level of metabolic efficiency. More documented toxicity mechanisms to be discussed further include cationic cell wall displacement, transport into cell, and interaction with biomolecules.

Cell Wall Displacement. Beveridge, Collins, Stotzky, and others assert that metal toxicity is largely the result of cell wall displacement. The substitution of a metallic ion by another can cause severe disruption of the membrane wall structure of bacteria, thus causing cell lyses and death or severely reducing cellular metabolism, nutrient uptake, and discharge of waste materials.

Bacterial membrane surfaces are generally hydrophilic and possess an electronegative charge. As a result, metal and non-metal cations are often found saturating the membrane surface. (Ferris, 1989:) The cellular wall structures, in fact, often require Mg^{2+} and Ca^{2+} to act as electropositive cement, lessening the repulsive forces of the electronegative LPSs of Gram-negative membranes and teichoic acids of Gram-positive membranes. These metals also contribute to the anchoring of the outer membrane to the underlying peptidoglycan layers. (Doyle, 1989: 302)

The teichoic acids and carboxyl groups found in the peptidoglycan of Gram-positive bacteria are active metal chelators. Above normal absorption of heavy metals and their propensity to replace the essential Mg^{2+} and Ca^{2+} (which maintain membrane structural integrity) is believed to be the primary cause of metal toxicity. (Doyle, 1989) Because the peptidoglycan layer in Gram-negative cell walls is much thinner than that of Gram-positive cell walls, the amounts of metal chelated, and thus the overall toxicity, is much less in Gram-negative bacteria. The lack of teichoic acid (a strong metal chelator) in Gram-negative cell walls is also presumed to contribute to reduce metal toxicity. A baseline of toxicity may be observed because of the predominance of available phosphate groups attached to the external portion of the lipids in the cytoplasmic membrane (phospholipid bilayer). These phosphate groups provide adequate chelate sites for metals and are characteristic of both Gram types.

In 1992, Collins and Stotzky presented evidence that the presence of heavy metals can alter the net charge of the cell, thereby impacting various physiological functions of the cell. This effect also reduces the ability of the cell to effectively interact with its surroundings by reducing its ability to bind with other cells and soil particulates. (Collins and Stotzky, 1992). Further research by this pair, however, raised questions as to the survival affects metals have upon bacteria via this mechanism. (Collins and Stotzky, 1996)

While, accumulation of metals at the surface can be morphologically damaging, this activity can ultimately reduce the metal's entry into the cytoplasm and more sensitive

cellular functions. In this manner, toxicity can actually be reduced when considering other mechanisms of toxicity.

During exponential growth of most bacteria, much of the wall is sloughed off during each cell division. This is a result of inside-to-outside wall growth, ensuring that the wall materials are continually being pushed out from the plasma membrane. (Doyle, 1989: 287) This binding of metals to the cellular surface is noted as a key mechanism reduced metal toxicity in Diaz-Ravina and Baath's work in bacteria metal tolerance. The metals become increasingly unavailable as a result of binding to this organic matter.

Transport of Metals Into Cell. The transport of metal ions into microorganisms can occur through two mechanisms: metal specific carriers may sequester essential nutrients such as Mg^{2+} and Ca^{2+} and bring them inside the cell; and the non-specific transport of metals complexed to substrates having their own specific transport system.

Heavy metals can be taken in to the cell by existing transport systems designed to accumulate Na^{+}, K^{+}, and other biologically necessary cations. Once inside the cell, enzymes, oxidases, and reductases can neutralize these metals, which would normally prove toxic.

Interaction With Biomolecules. Exoenzymes used in the sequestering of essential nutrients, detoxification of materials including metals, and in the breaking down of macro-organics often employ a metallic core element as its structural component. Metal ion replacement in these proteins and enzymes, such as zinc, which is an essential

component of 150 enzymes, can severely reduce metabolic function. (Ochiai, 1991; Walker and others, 1996; 6) This effect can be prevalent if the binding energy between the toxic ion to the ligand site is greater than that of the characteristic metal ion. (Bertini and others, 1985:925)

Metals can even complex with the acetic or phosphoric acid groups on the macro-protein, thereby modifying its conformation and rendering it useless. Such toxicity can be considered reversible if the toxicants are quickly removed from the system. The microorganisms can recover by producing more enzymes.

Metal Tolerance. Diaz-Ravina and Baath offered evidence that increased metal tolerance was found in bacterial populations when exposed to increasing concentrations of Zn, Cd, Cu, and Ni over incubation periods between 4 to 12 months. This tolerance was a function of both concentration and length of time of incubation. At low concentrations, an increased level of tolerance was detected after only 2 days for Zn exposure. At higher concentrations, metal tolerance was observed after a much longer period of time (4 months to a year). (Diaz-Ravina and Baath, 1996)

Their research shed light on the mechanisms of metal tolerance in terms of population dynamics, showing that initial observed metal tolerance is a result of immediate death of sensitive species and long-term tolerance is a result of competitive abilities and adaptation of surviving bacteria.

The competition of inorganic cations also contributes to the conditions that determine the amount of metal toxicity. The presence of class A metals such as

magnesium and calcium have been shown to compete for binding sites and thus reducing the toxicity of the heavy metals. (Collins and Stotzky, 1989: 56)

Organic matter within the ecosystem, reduces the bioavailability of heavy metals, thus reducing their toxicity. Extensive studies have been done in the use of the synthetic chelator ethylenediaminetetraacetic acid (EDTA) as a method for removing metals from the environment and thus reducing toxicity in a variety of organisms (Collins and Stotzky, 1989: 58). Natural chelators such as amino and humic acids, peptones and other complex soluble organic substances also reduce metal toxicity by strongly binding to these cations. (Collins and Stotzky, 1989: 58)

Characterization and Enumeration

Diluents. Bacterial cells, if found in microbially dense, soupy conditions, may require dilution to allow for microscopy, enumeration, analysis for genetic or metabolic properties, and general growth. Cells in dense suspensions are often times more hearty as the bacteria metabolize with the help of materials leached from neighboring cells. Diluted bacterial samples are thus more susceptible to environmental change.

Distilled or tap water is not recommended for use as dilution water. Tap water may contain trace amounts of heavy metals and detergents that can significantly reduce or cease cellular metabolic function. Distilled or de-ionized water is not appropriate as it is osmotically hypotonic to bacteria and is not buffered against pH change (Koch, 1994: 255). A protein-laced phosphate buffered saline is generally used as a dilutant. The presence of proteins, such as gelatin or peptone, chelates metals and detergents and

phosphate salts buffer to near neutrality while also chelating metals and detergents typically found as contaminants. (Koch, 1994: 255)

When working towards understanding heavy metal toxicity, it is essential to limit key chelating agents in nutrient and dilution solutions. Without such precautions, the effects of heavy metals in solution is difficult to determine as such metals will precipitate out of solution.

Colony Growth and Viability. Bacterial viability is often defined as the ability to multiply within a nutrient solution or form colonies on solid growth medium. As discussed above, bacterial reproduction and metabolism can be disrupted or inhibited by a variety of toxicants, both biological and chemical. Antibiotic or disinfectant susceptibility is measured by the ability to limit bacterial growth. How bacteria react to such agents can be determined by a variety of methods.

The determination of the number of live healthy cells within a sample is driven by health concerns and as such is often used in medical, toxicological, and water sampling. The determination of the presence of bacteria, as in environmental assays, is often accomplished through the use of plate counts. This method essentially determines the number of viable cells capable of multiplying when introduced to an appropriate growth medium. This ability to multiply, as first noted by Robert Koch and detailed by Gerhardt in *Methods for General and Molecular Bacteriology* (Gerhardt, 1994: 248) is based simply on the notion that they must be able to "initiate and complete cell division". While some cells may be alive, they may be damaged such that further cell division is

impossible. This distinction is critical in plate growths and is characterized by the ability of cells to undergo indefinite growth over an extended period of time. These viable cells are referred to as colony forming units (CFU). The use of this terminology under the assumption that one viable cell will produce one colony of its daughter cells after sustained reproduction. (Koch, 1994: 249) (Bitton, 1994: 47)

There are two common approaches for the determination of CFU. The *pour plate method* involves the mixing of 0.1 - 1 mL of microbial suspension with the molten agar medium during or prior to pouring into petri dishes. The *spread plate method* involves the pipetting of 0.1 mL of sample onto the surface of a hardened agar medium. (Bitton, 1994: 47) It is the *spread plate method* that is employed in this laboratory effort.

Light Microscopy. Microscopic evaluation of microorganisms is routinely accomplished by light microscopy. The direct observation of microorganisms was made possible around 1673 when Dutch inventor Anton van Leeuwenhoek (1632-1723) developed the first compound light microscope. (Brock and others, 1994: 16). The light microscope has had a substantial influence in the development of microbiology as a general science and in the evolution of medical and environmental research.

Three types of light microscopy are routinely used in microbiology; *phase-contrast*, *bright-field*, and *fluorescence*. In this effort, phase-contrast and bright-field microscopy are transmitted light techniques. That is, light originates from a lamp below, and is "transmitted" through, the specimen.

Bright-field Microscopy. Bright-field microscopy is the most elementary as well as oldest bacteriological technique. It is most commonly used in experimental microbiology with the aide of staining techniques as bacteria are difficult to observe and characterize under bright-field.

Phase-contrast Microscopy. Phase-contrast microscopy was developed as an alternative to bright-field techniques in order to improve upon the distinction or contrast of cells from the surrounding medium. Small differences in refractive indexes between the cells and their surrounding can be translated into observable differences in visible intensity by specially made optical devices (Kapitza, 94: 17). Materials of higher refraction index slow the light wave and create a "phase shift" from its original condition, in which the waves are in phase.

Fluorescence Microscopy. Fluorescence is the luminescence of substances brought about by the absorbence of light (photons) by the material, excitation of its electrons, and immediate emission of electromagnetic radiation as these electrons make transitions to lower energy states.

One of the attractive features of fluorescence is its sensitivity, with some methods maintaining limits of detection as low as the parts-per-billion range. (Scoog, 1992: 174) The number of chemical systems that luminesce, however, limits such methods.

Fluorescent materials can only absorb light at specific wavelengths. The emitted radiation, conversely, exhibits a spectrum that is characteristic of the species of interest and can be used for both qualitative and quantitative analysis. (Scoog, 1992: 174) Some

fluorescent materials occur naturally and will fluoresce readily under UV light. Chlorophyll is such a pigment. Other materials can be treated with a reagent or dye which binds to the material of interest and fluoresces.

Fundamentals of Fluorescence. White light radiation includes all spectral colors, which can be observed when passed through a prism and the light is dispersed. These spectral colors are classified by their wavelengths, λ, which are measured in nanometers (nm) (10^{-9} m). The visible spectrum starts at the upper end at 750 nm and is observed as red light. The spectrum ranges from red to orange, yellow, green, blue, purple, and through to violet, which registers at 400 nm. Below 400 nm is the invisible ultraviolet (UV) and above 750 nm is the invisible infrared. (Table 2-3).

Table 2-3 Spectral Colors

Wavelength	Color
340-400 nm	Near ultraviolet (UV)
400-430 nm	violet
430-500 nm	blue
500-560 nm	green
560-620 nm	yellow to orange
620-700 nm	orange to red
over 700 nm	near infrared (IR)

The fluorescent reemission of electromagnetic radiation by the absorbing molecules normally occurs within billionths of a second (Kapitza, 1994: 20) and expresses a wavelength which is slightly "red-shifted". That is, the emitted light is less energetic, with wavelengths about 20 to 50 nanometers longer than the radiation that excited the fluorescence.

Absorbed UV can be reemitted as visible light, blue light can be emitted as green or any less energetic emission, down the spectrum to red. (Kapitza, 1994: 20) This shift in wavelength to lower frequencies is known as the *Stokes shift.*

Whether a substance fluoresces or not is dependent upon the structure of the molecule and its immediate environment. Many organic molecules, especially unsubstituted aromatic hydrocarbons, exhibit strong fluorescence at ultraviolet or visible wavelengths. (Rost, 1992: 28)

One of the more influential environmental factors in biological fluorescence microscopy is the binding of fluorescence dyes, or *fluorochromes*, to organic and inorganic compounds. The binding of a fluorescent molecule to a macromolecule can alter its fluorescent properties. Because the fluorescent characteristics of fluorochromes can vary according to their fate within a biological system, these dyes can be used as probes in microscopy. (Rost, 1992: 31)

Fluorescent Dyes. Fluorescent dyes have an affinity for a specified cellular component and can attach to specific areas of the biological specimen or tissue and leave other areas unstained. These characteristics make this technique valuable for qualitative analysis. As an example, a certain fluorescence emission may result if a fluorochrome binds to an intracellular protein. If fluorescence is observed, depending on the known mechanisms, one might assert that a metabolic activity is present, or perhaps cell lyses has occurred indicating severe cellular damage or death.

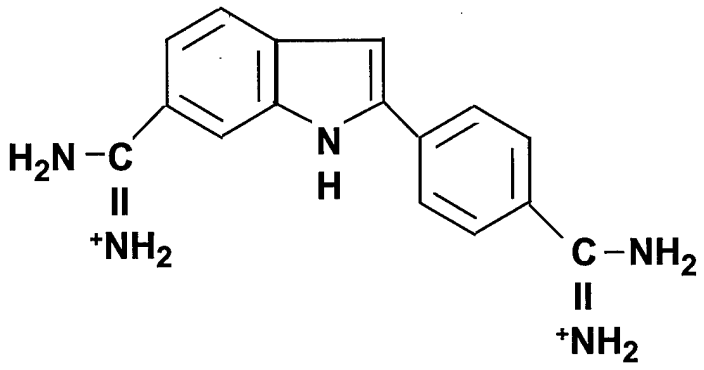
In the differentiation between live and dead cells, it is useful to consider one of the most telling of mechanisms for cellular death, that of cell lyses due to the compromise of the plasmic membrane. Cyanine dyes are a family of nucleic acid stains that commonly have a high affinity for nucleic acids, with little or no bonding to other molecules and low intrinsic fluorescence when not bound to these nucleic acids. (Haugland, 96: 144)

The stains to be used in this effort consist of two nucleic acid reagents and a fluorescently labeled wheat germ agglutinin (WGA) as a Gram-stain determinant. Sytox® Green nucleic acid stain is a cyanine dye and is a useful identifier of dead cells because it is impermeant to cell membranes and therefore will only stain those cells whose cytoplasmic membrane has been compromised. In addition, it is non-selective toward nucleic bases allowing for universal determination for virtually all cells. The dye-bound nucleic acid of these dead cells, whether they be Gram-positive or Gram-negative fluoresce bright green when exposed to a 450-500 nm source. (Haugland, 1996: 149). Sytox® Green nucleic acid stain is often used as a DNA counterstain for chromosome labeling, for bacteria enumeration, and, in combination with other stains, for viability determination. (Haugland, 1996: 144).

A stain used in coordination with Sytox® Green, for bacterial viability determination, is DAPI (4',6-diamidino-2-phenylindole) (Haugland, 1996: 151). This reagent is an indole, which refers to a group of water soluble cell-permeant, DNA binding stains that fluoresce bright blue. DAPI binds to adenine and thymine sequences of DNA, presumably owing to the fluorochrome's increased fluorescence by its displacement of

water molecules within the minor grooves of the DNA sequence (Haugland, 1996: 151). The chemical structure of DAPI is shown below in Figure 2-7.

Figure 2-7 DAPI (4', 6-diamidino-2-phenylindole) Stain



Fluorescent Filters. The appropriate selection of fluorescent filters are crucial for successful fluorescence microscopy. An exciter filter transmits only the desired radiation required for the method being used and suppresses all other unneeded wavelengths of radiation. (Holz, 1977: 7) A barrier filter suppresses excessive excitation light, not absorbed by the specimen, which would otherwise interfere with fluorescence observation. (Figure 2-8)

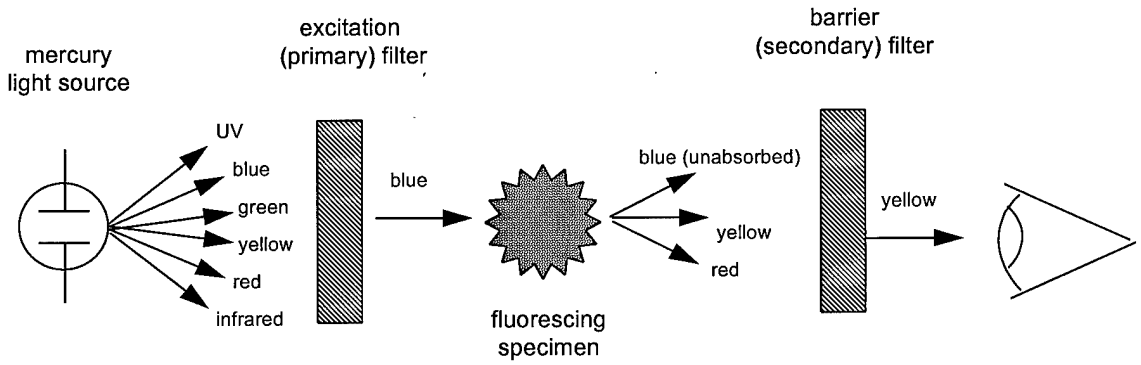
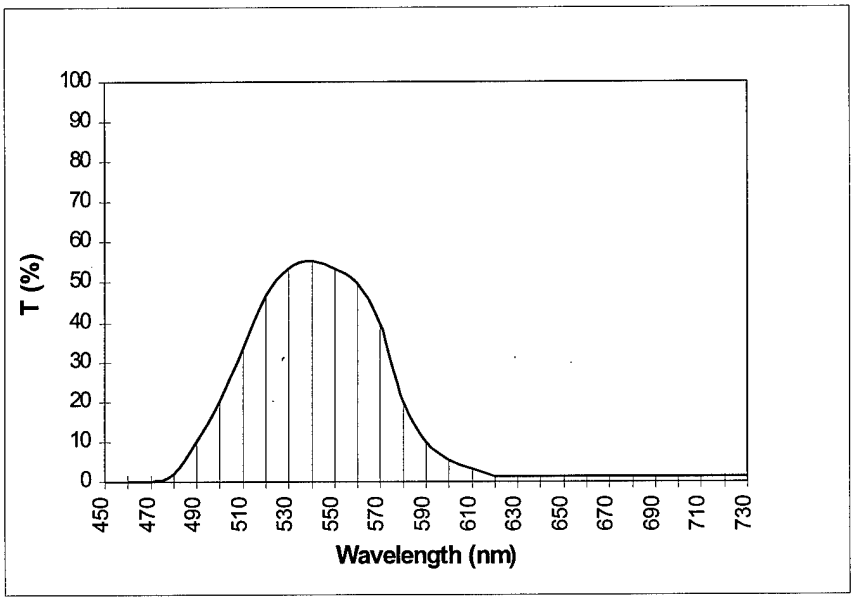


Figure 2-8 Diagram of Fluorescent Elements for Blue Excitation

(Holz, 1977: 7)

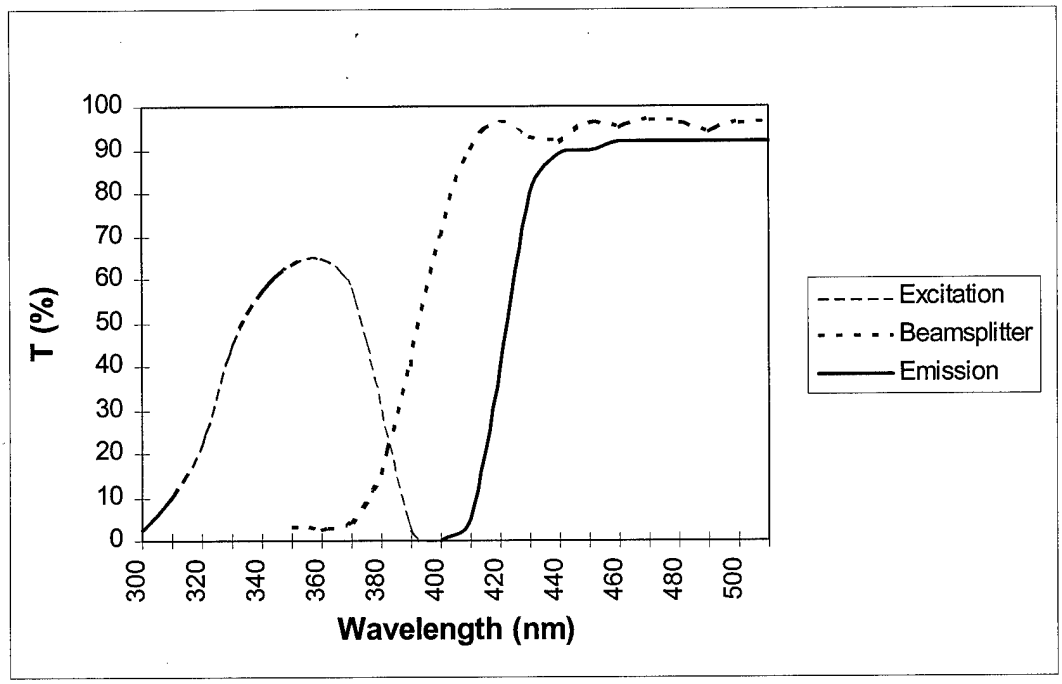
A filter essentially cuts off undesired parts of the spectrum. The amount of light at various wavelengths that is not lost due to reflection or absorption is measured in terms of a percentage of its transmittance (T %). The shape of transmittance curve characterizes the filter designation. A wideband filter would transmit over a wide wavelength band as shown as an example in Figure 2-9. This transmittance is typical of glass filters and would be designated in this instance as G 540, the number indicating the peak transmitted wavelength. Examples of a band pass filter (BP 530), shortwave pass filter (KP 520) and longwave pass filter (LP 570) are shown in Appendix A.

Figure 2-9 Wide-band Filter



The filters provided by Zeiss were designed for observation of various fluorochromes in industry. Filter set 02, as shown in Figure 2-10, is appropriate for observing the DAPI fluorochrome, requiring excitation at 345 nm and emission at 425 nm. Note it is a combination of a wideband filter for excitation (G 345), and shortwave pass filter for emission (KP 425). Filter sets 09 and 15 can be seen in Appendix B. Filter set 09, required for the Sytox® Green nucleic acid stain, excites at 490 nm and emits at 525 nm. Filter set 15, required for the Texas red stain, excites similarly to rhodamine detecting filters at 540 - 560 nm and emits at 580 nm.

Figure 2-10 Filter Set 02



Gram Staining. Staining techniques are imperative in bright-field microscopy as they increase their contrast as well as reveal various external physiological structures that would otherwise go unnoticed. The Gram stain is considered a differential stain because ensures that chemically and physiologically different cells are stained. The Gram stain was developed by Christian Gram in 1884 and was thus used long before the composition of the external cellular walls and membrane could be effectively observed and understood. Since that time, this reaction has become a powerful tool in medical and environmental bacteriology and is often the first step in bacterial characterization. (Brock and others, 1994: 46).

The Gram reaction is dependent upon the differences in permeability of cell walls resulting from structural differences. In the staining process, the cells are flooded with crystal violet, which enters the cellular structures and turns all cells purple. The cells are then treated with an iodine solution, which acts as a mordant, complexes with the crystal violet stain, and fixes the color within the cell wall. An organic solvent, typically an ethanol/acetone mixture, is used to decolorize the cells and wash out the crystal violet. The thick cell walls of the Gram-positive cells and the abundance of peptidoglycan layers, do not allow the crystal violet-iodine complex to be washed out whereas the thinner peptidoglycan layer of the Gram-negative cells do not fix the stain. The smear is then counterstained with a red dye so that the unstained Gram-negative cells can be easily observed under bright-field microscopy. The resulting observation is blue-purple Gram-positive cells and red-pink Gram-negative cells.

III. Methodology

This chapter reviews the methodology used in the laboratory effort supporting this thesis. This laboratory effort was conducted between 16 May 1997 and 7 Oct. 1997 in parallel with the efforts of Capt. Pat Marbas and I will refer to his thesis, *Metabolic Inhibition of a Toluene Enriched Microbial Population Due to Lead: Verification of a Free Metal Ion Toxicity Model* (Marbas, 1997) for much of the preparation of the microorganisms, their manipulation, introduction to metal treatments, and any adjustments to growth solution and feeding/cleaning schedule required for his laboratory work.

Microorganisms

The initial population of microorganisms used in this experiment was retrieved as activated sludge from Tank 4 of the Fairborn water reclamation plant. Two liters of this activated sludge was transported to the laboratory in a plastic container. In the laboratory, a toluene-selected microbial population was cultured and stored within a bioreactor constructed from a 2 gallon opaque, plastic gasoline container. Keeping the population free from direct or incident light ensures that no phototrophic organisms propagate.

Oxygen Consumption

In Marbas' effort, the measurement of the consumption of molecular oxygen was the method chosen for the determination of respiration rate and thus an indirect measure

of metabolic activity of the bacteria. As a simple aromatic hydrocarbon, toluene is degradable primarily under aerobic conditions. Because molecular oxygen is necessary for the initial steps of biodegradation, the dissolved oxygen (DO) techniques employed in Marbas' lab are appropriate indicators of metabolic activities.

Light Microscopy

Initial observations of the bacteria were accomplished in order to establish a baseline from which the population underwent succession over the course of the laboratory effort. Microscopy was utilized in concert with the spread plates in these experiments. These microscopic observations were conducted through the use of bright-field and phase-contrast, staining, and fluorescence microscopy.

Microscope. For these efforts, a Zeiss Axioskop microscope was used. This light microscope is designed for use in the medical and environmental fields, for the examination of cell and tissue specimens. The microscope is equipped with a series of objectives at increasing magnifications of 5x, 10x, 20x, 40x and 100x. The eyepiece also provides a 10x magnification. The overall magnification of the microscope can be determined as such:

$$\mathbf{M}_{\text{microscope}} = \mathbf{M}_{\text{objective}} \times \mathbf{M}_{\text{eyepiece}} \qquad (3.1)$$

M = magnification

(Kapitza, 1994:32)

-- such that a 20x objective and the 10x eyepiece micrometer would provide an overall magnification of 200-fold.

At high magnification (100 x objective), Zeiss immersion oil is required for better clarification and resolution of bacteriological slide preparation. The refraction index for the oil is n = 1.51, which is equivalent to the refractive index of the glass slides and coverslips. This technique also ensures that light is not reflected away from its path to the objective resulting from the phase changes between the glass and the surrounding air. (Kapitza, 1994:4)

Transmitted Light Microscopy. In transmitted light microscopy, the specimen is illuminated by light originating from a halogen lamp housed below. The light is projected through the specimen by means of a condenser. The light is then transmitted up through the objective and after a series of prisms and focusing lenses, the image can then be seen through the eyepiece. (Kapitza, 1994:8)

Fluorescent Microscopy. In contrast to transmitted light microscopy, the type of fluorescent microscopy used in this effort is epi-fluorescence, or incident light microscopy. In this instance, ultra-violet light is transmitted from an additional light source fixed to the rear of the microscope. The light is transmitted through a heat-protection filter and through a selection filter. The light then reaches a dichroic beam splitter, which reflects the short wave exciting light down through objective and on to the specimen. The resulting emission is then gathered by the objective and transmitted up through the dichroic beam splitter allowing the longer wavelengths of the re-emitted light to pass and travel on to the eyepiece for observation. (Kapitza, 1994:21)

The protocol for the introduction of fluorescent dyes to the suspended bacteria is detailed in Appendix C. Initial gram-staining observations indicated that Gram-positive bacteria were rare and the application of the WGA gram stain diluted the sample to the point that it was difficult to isolate enough bacteria for evaluation. As a result, this part of the procedure was discarded and bacterial viability using the DAPI and Sytox® Green stains was implemented as an isolated effort.

The proper filter sets were received late in this effort and the use of this technique could not be fully integrated into the evaluation of the treated and untreated microbial populations. The microorganisms were observed under UV-light without the dyes and were confirmed as exhibiting auto-fluorescence. This caused difficulty in differentiating live and dead cells resulting from the fluorochrome emissions, as their own natural fluorescence washed out and distorted the results.

Wet Mounts. Phase-contrast microscopy is most effective for examining wet mounts. In the preparation of wet mounts, a 0.2 - 0.3 µl sample of bacterial microcosm is applied, with a pipette or syringe, to a clean glass microscope slide and covered with a glass coverslip. To avoid drifting of bacteria due to convection currents and evaporation, petroleum jelly is applied to the edges of the coverslip with a flat toothpick, creating a seal and isolating the specimen. For aerobic bacteria, motility can only be observed for a short time as oxygen is soon depleted. As the samples were fairly dilute, motility was observed for 15 to 20 minutes without loss in activity.

Gram Staining and Morphology. Initial Gram stains were conducted in order to get comfortable with the techniques involved. While this method is an effective differential technique, experience in this technique is the only adequate assurance that observations will be fruitful.

The protocol for the preparation and staining of the sample smears is detailed in Appendix D.

Throughout this laboratory effort, Gram stains were conducted in order to track the succession of the microbial population. In each observation, various morphologies, and their relative ratios were recorded. This identification was accomplished by adjusting the specimen stage such that the cell of interest fell directly under the measuring scale (reticle) in the eyepiece. For 100x magnification, the minor "ticks" represent 0.000999 mm (a result of calibration) or roughly 1 μm. Enumeration was accomplished by rotating the reticule in the eyepiece and counting all relevant cellular units within the sweep. This enumeration was not absolute, that is, *# per mL*, as is common in bacteriology, but instead was intended to observe the emergence of otherwise rare morphologies, the dissipation of otherwise common forms, and the relative ratios of these morphologies as the laboratory effort proceeded.

Spread Plates

Spread plates were used as the primary determinants of microbial health in this laboratory effort.

Nutrient Agar. Nutrient agar plates were prepared for each colony growth activity. For each preparation, 23 grams of Bacto ® nutrient agar was added to 1 liter of de-ionized water. In order to ensure that the agar is dissolved completely and that the medium is completely sterile, the mixture must be run through an autoclave at 121° C and 15 psi above ambient pressures.

After swirling the mixture thoroughly, but not so as to agitate the solution and cause air bubbles to form, the medium was allowed to sit and cool down until it could be handled. For extended use, the bottle was placed in a water bath of about 55-60 ° C to maintain the agar in a liquid state. Each preparation created roughly 35-40 9-cm petri plates.

Preparation of plates. For the preparation of each plate, the liquid agar was poured into each petri dish such that half the volume was filled (15 - 20 mL was sufficient, giving a thickness of 0.24 to 0.32 cm). Adequate thickness avoids limiting nutrients or localized drying. The plates were allowed to sit out overnight to dry and were then inverted and placed in an Ambi-Hi-Low chamber at 10 ° C for indefinite storage. Inversion ensures that the water vapor emitting from the warm agar and condensed on the lid of the petri dish does not drip back onto the agar surface. Prior to use, the plates were allowed to warm to room temperature.

Dilutions. The preparation of appropriate dilutions of cells suspended in aqueous media is based upon intuition, trial-and-error, and careful calculation. Typically, a 1 % dilution is used (Koch, 1994: 255), but in this instance, the sample is microbially

concentrated and further dilutions were required. Optimally, the number of colonies that form as a result of proper dilution should range between 30 and 300. Results falling outside this range are difficult to statistically justify as they may not represent actual conditions. The difficulty in creating the suitable dilution comes not from the variability in the methodology, but from the varying conditions of the bioreactor and the toxic effects of the metal treatments.

Preparing Dilutions. It is essential that adequate dilutions are created to ensure an accurate colony count. If the sample from the microcosm is too dilute, too few colonies will grow and confidence in the count will be limited. (Atlas and Bartha, 1993: 186) If the sample is not dilute enough, too many colonies will appear, counting will be cumbersome, colonies will grow into each other reducing colony distinction, and inhibitory effects due to localized nutrient limiting effects may occur.

Initial runs were accomplished to determine an appropriate dilution to use for the spread plates. Given that a variety of conditions might impact bacterial population robustness, a series of dilutions provides a rough range in which to get good data in future plate growths. For each of these initial runs, three replicates were created for various dilution percentages. An initial dilution stock was prepared. Roughly 20 mL of innoculum from the bioreactor was added to dilution water for a total of 200 mL in a 200 mL sterilized glass flask. This simulates the ratio of innoculum to dilution water normally found in the BOD bottles during the metal runs in Capt. Marbas' laboratory effort, that of a 1:10 dilution of microorganisms.

The dilutions are described as percentages of the original microcosm, that which can be found in the bioreactor. A 40 mL extraction, added to 60 mL of dilution water (100 mL final volume), for example, is described in this effort as a 40 % microcosm dilution. With the initial stock described above, further dilutions were made on an exponential scale (0.001 %, 0.01 %, and 0.1 % for example) to ensure that a dilution range could be established with confidence and viable counts would be "bracketed" by at least one of the three dilutions (Table 3-1). The initial dilution stock was kept homogenous with a magnetic stirrer during extractions. This ensures that extractions from this stock are representative of the microcosm. Disposable plastic pipettes were used to prevent contamination between cultures during these serial dilutions.

Table 3-1 Serial Dilutions

Dilutions (stock	Drawn from 1st Dilution (mL)	Dilution water volume (mL)
2nd Dilution -- 0.01%	2.00	20
3rd Dilution -- 0.001 %	0.20	20

Dilution Determination Runs. This section will describe successive dilution determination runs for the purpose of establishing reliable range in which to expect good plate counts. These runs were also intended to gain confidence in the procedures, as poor methods and frequent contamination can be easily observed within dilution runs. Optimally, confidence is gained if, for example, reducing the microbial dilution by a factor of ten should reduce resulting colony counts by the same factor. This signifies that

one should continually expect a one-to-one relationship between dilution and colony count.

The following methodology describes the initial run. Subsequent runs were essentially identical, with only the value of the dilutions varying.

With an initial dilution (stock) of 10%, the next dilution (0.1%) was created by using a 1 mL pipette to extract 0.2 mL from the initial stock and adding dilution water for a final volume of 20 mL in a sterilized 50 mL capped tube. The tubes and caps were sterilized in an autoclave prior to their use. The second and third dilutions (0.01 % and 0.001 %) required the introduction of 2.0 and 0.2 mL, extracted from the *first* dilution, to dilution water for total volumes of 20 mL. This dilution setup was used as an example in Table 3-1.

This resulted in, three capped tubes containing 1:100000, 1:10000, and 1:1000 ratios (or 0.001, 0.01, and 0.1 % dilutions) of bioreactor microbial extractions in total dilution water solution were prepared. These samples were then ready for enumeration through the use of spread plates

Spread Plate Technique. This technique was employed throughout the laboratory effort and for all dilution and metal runs.

Spread plates were prepared to establish appropriate dilutions for future population determination. Prior to application of the microbial solution, the capped tubes were immersed in a sonification chamber to separate any floc that may have formed. This ensures that the CFU are optimally single bacterium and not colonies of tens or

hundreds. This is also very effective in resuspending any bacteria that may have congregated at the bottom of the bottle, allowing for a good distribution of specimen within the solution. Phase-contrast microscopy was periodically employed to confirm the distribution and relative separation of bacteria.

A disposable plastic pipette was used to apply 0.1 mL of the final dilution onto the agar surface of the petri plate. Because the cells may have a tendency to aggregate in situ, the drops were immediately spread over the surface. The spreader used in this technique was constructed out of a glass bar, bent into an "L" shape using the heat from an alcohol flame. The spreader was sterilized by immersing in alcohol and then ignited over an alcohol flame prior to each application. The spreader was immersed in the small pool of sample on the agar surface. Hydrophilic attraction of the glass spreader quickly draws the liquid to the outside of the plate. The plate was then rotated under the spreader so that the sample is distributed over the entire plate.

In order to gain experience in this technique, and achieve a uniform coverage as close to the edges as possible, this procedure was attempted several times prior to experimental runs. The resulting colonies were observed to improve spreading techniques. The drying of the agar overnight, prior to spreading, was important because the agar should be dry enough such that the 0.1 mL inoculation is absorbed in the agar within 15 to 20 seconds. This allowed for the plates to be immediately inverted so that any condensation would not drip on to the plate, thus blending the growths.

The protocol for the determination of appropriate dilutions was conducted as follows:

Three capped bottles and their varying dilutions were set out on the table from lowest microbial dilution to highest. For each dilution level, three agar plates were placed in front of each bottle. Approximately 0.5 mL of sample was extracted with a 1 mL pipette from the first bottle and 0.1 mL of sample is placed upon each of the three plates, one after the other. Because these samples are serially diluted, the same pipette could be used to extract samples from the remaining two bottles and applied to the plates in the same manner without concern for contamination. For this run, as well as runs to follow, a blank was performed by applying 0.1 mL of dilution water so as to ensure a sound protocol, free of method-generated contamination. This application can be visualized in Table 3-2.

Table 3-2 Dilution

Run # 1

Plate #	Dilutn %
1A	0.001
1B	0.001
1C	0.001
2A	0.01
2B	0.01
2C	0.01
3A	0.1
3B	0.1
3C	0.1
Blank	-

After the samples were applied to the plate surface, the sterilized spreader was used to distribute the sample as described above. Before each application the spreader was sterilized so as to avoid cross-contamination.

The spread plates were then inverted and incubated for 24 hours in a room with relatively constant temperature, around 30 $^{\circ}$C. Under an illuminating 4 power magnifying glass apparatus, the colonies were then enumerated with a hand counter.

Two additional runs were conducted and the counts for all three runs are presented in Table 3-3, Table 3-4, and Table 3-5.

Table 3-3 Dilution Run # 1 and Counts

Dilutn %	Count	cfu/mL (x 10^3)
0.001	9	900
0.001	7	700
0.001	6	600
0.01	46	460
0.01	55	550
0.01	46	460
0.1	450	450
0.1	459	459
0.1	482	482
Blank	2	

Table 3-4 Dilution Run # 2 and Counts

Dilutn %	Count	cfu/mL (x 10^3)
0.001	6	600
0.001	8	800
0.001	6	600
0.005	36	720
0.005	45	900
0.005	35	700
0.01	75	750
0.01	60	600
0.01	80	800
0.05	330	660
0.05	295	590
0.05	320	640
Blank	1	

Table 3-5 Dilution Run # 3

and Counts

Dilutn %	Count	cfu/mL ($x 10^3$)
0.001	10	500
0.001	12	600
0.001	7	350
0.005	52	520
0.005	48	480
0.005	66	660
0.025	257	514
0.025	260	520
0.025	238	476
Blank	0	

A total of 6 dilution runs were accomplished but only the last three produced useful information. Single digit counts can be ignored as they are not necessarily representative of the robustness of the population. We can see here that there is wide variation *between* runs, but little variation *within* the runs. An analysis of variance was conducted to confirm this. A Tukey comparison of means indicated that runs 1 and 3, as identified above, were found to have no statistical differences between them and thus can be categorized into one homogenous group. Run 2, however was determined to have a mean that was statistically different than the other groups. Because of expected variability in the microbial population, caused by time of sampling, availability of nutrients, and other environmental effects, one should expect variability between the readings. The lack of variability *within* the runs gives credence to the procedures implemented. The runs also indicated that when the concentration of bacteria is

increased, the number of counts similarly increase. Once rough dilution ranges were established, and confidence was gained in the procedures, spread plates were then prepared for the metal treatment runs conducted by Capt. Marbas.

Metal Runs

For each metal run, spread plates were prepared within two hours after metal treatments. This allowed for dissolved oxygen measurements to be completed. The protocol for these plate counts was similar to that of the dilution determination runs. Capt. Marbas predetermined the volume of the microcosm within the dilution water as a necessity for establishing appropriate BOD readings. This usually varied around a 1:10 microcosm to total volume ratio (or 10% microcosm dilution). These variations in dilution, while necessary for the respiration measurements were inconsequential because the subsequent serial dilutions could be adjusted to ensure adequate resulting counts.

For each metal run, BOD bottles were retrieved after the completion of the dissolved oxygen measurements. These bottles represent each of the stages of metal treatment, as can be seen in Table 3-6

Table 3-6 Lead Treatment 1 (Aug 30)

Microcosm (mL)	Treated Dilutn water (mL)	Total Volume	Sample Dilution
30	275	305	0.0984

Metal Conc (ppm)	10	5.00	2.50	1.00
Actual conc	9.02	4.51	2.25	0.9

These four metal-treated bottles, along with a microcosm blank (noted as M-blanks hereafter) were set out on the table from lowest metal concentration to highest. The dilutions were established similarly to that described above in Dilution Determination Runs section. The first dilution was created by adding a 0.5 mL sample (in the case of the lead treatment # 1), of each of the five bottles, to dilution water for a total volume of 50 mL in a sterilized 100 mL flask. These dilutions were placed in a sonification chamber for 3 minutes and then the bacteria were kept suspended and dispersed throughout the solution with a magnetic stirring rod. The next two dilutions used extractions from the first dilution as shown below.

Table 3-7 Lead Treatment 1 Serial Dilutions (Aug 30)

	innoc (mL)	dil water (mL)	dilution %	innoc (mL)	2nd Dil (mL)	2nd Dil %	innoc (mL)	3rd Dil (mL)	3rd Dil %
metals	0.50	50.00	0.0984	10.00	20.00	0.0492	2.00	20.00	0.0098
blanks	0.25	50.00	0.0492	4.00	20.00	0.0098			

Note the difference in dilutions between the metal treatments and the blanks. This is a result of known reduction of CFU upon metal treatment and necessary adjustments to ensure adequate counts. Dilutions were made for each BOD bottle, yielding a total of 14 separate containers. Spread plates were then created with two plates per sample, yielding a total of 29 plates, including a blank for quality control. The remaining metal runs executed throughout the laboratory effort were conducted similarly but with varying concentrations of metal treatments as well as varying dilutions, adjusted according to that which was required to perform an adequate plate count.

Colony Growths

As a result of initial spread plate growths, the resulting colonies were observed to have varying textures and colors. The color of the colony is typically characteristic of the types of proteins being synthesized by the bacteria. Because protein synthesis is a direct result of transcription of different genetic codes, colony color can be used to effectively differentiate between varieties of bacteria species.

After 48 hours of colony growth, the varying types of colony color were noted for different treatments of metals, including an untreated sample. Additionally, swipes from these colonies were taken, resuspended in 10 mL dilution water, and observed via wetmount and Gram stain microscopy. In this manner, the morphologies of bacteria associated with each colony were observed.

Measuring Toxicity

Metabolic Inhibition. In past research (Hansen, 1995), and research running concurrently (Marbas, 1997) the toxic effect of metal treatments was determined by the ratio of the rate of oxygen consumption of a treated sample divided by the rate of oxygen consumption of an untreated sample. This ratio is known as the normalized metabolic activity (NMA) (Hansen, 1996: 77).

$$NMA = \frac{\text{Rate } O_2 \text{ Consumption}_{\text{Metal Treated Sample}}}{\text{Rate } O_2 \text{ Consumption}_{\text{Untreated Sample}}} \qquad (3\text{-}2)$$

(Hansen, 1996: 77)

Where:

$$\text{Rate } O_2 \text{ Consumption} = \frac{DO_{\text{initial}} - DO_{\text{final}}}{\Delta \text{ Time}} \qquad (3\text{-}3)$$

An NMA of 0 would indicate no metabolic activity in the treated microcosm caused by complete metal inhibition or toxicity. An NMA of 1 would indicate no metabolic inhibition.

Colony Forming Units (CFU). The numbers of colonies observed in plate counts is a result of both the dilution of the applications and the number of CFU in relation to total number of bacteria in solution. The dilution effects can be accounted for and normalized to the number of CFU present in a 1 mL sample out of the BOD bottle. The reduction in number of CFU resulting from toxic effects, however, can be directly observed from colony counts when compared with those resulting from M-blank counts.

This toxicity effect is *now* represented by the death of bacteria or the result of substantial cellular damage such that the bacterial cells are no longer reproductively viable. In either case, this toxicity effect is described as the ratio of the number of CFU per mL of the treated culture divided by the number of CFU per mL of the untreated culture. This ratio will be termed as *normalized colony forming units per mL* (NCFU/mL) and can be compared to NMA for insight into the mechanisms of toxicity.

$$NCFU = \frac{CFU / ml_{\text{Metal Treated Sample}}}{CFU / ml_{\text{Untreated Sample}}} \qquad (3\text{-}4)$$

An NCFU of 0 would indicate no reproductive activity and thus no colony growth resulting from the treated microcosm caused by complete metal toxicity. An NCFU of 1, however, would indicate no metabolic toxicity.

Effect of Dilution on Plate Counts. In coordination with a respiration test designed to determine the effects of population size on DO readings (performed on 2 Sept.), plate counts were conducted so as to better understand such dependencies. These tests were similar to the dilution runs performed at the beginning of this laboratory effort and similar results are expected; that there is a one for one relationship between the size of the population (as regulated by dilutions) and the number of CFU observed. The dilutions were prepared such that the size of the microcosm varied as shown in Table 3-8. Each sample was further diluted by extracting a 0.1 mL sample and adding dilution water for a total volume of 100 mL, creating another 1000-fold NCFU dilution. This ensured that the number of colonies would again fall within the optimal 30 - 300 range.

Table 3-8 Dilution Test (2 Sep)

Microcosm (mL)	Treated Dilutn water (mL)	Total Volume	Sample Dilution	Total dilution %
20	280	300	0.06667	0.00667
30	270	300	0.1	0.01
40	260	300	0.13333	0.01333
50	250	300	0.16667	0.01667
60	240	300	0.2	0.02

IV. Data Analysis and Discussion

This chapter reviews and analyzes the data collected in accordance with the methodology described in Chapter 3.

Morphological Identification of the Bacterial Population.

Light microscopic investigations were conducted periodically throughout this effort in order gain an understanding as to the type of the bacteria being sustained as well as to record the succession of the population over the duration of the laboratory effort. In initial observations, dated 15 June 1997, the bacterial population of the bioreactor was quite diverse, as could be expected when originating from an activated sludge microbial population. With the toluene saturated nutrient water, however, a more homogenous population might have been expected, as a toluene-selected population would have been established. Only a toluene selected bacterial population would have been thriving. Even toluene-tolerant species would be starved for a more agreeable food source and would be scarce, perhaps only surviving off of dead cellular material.

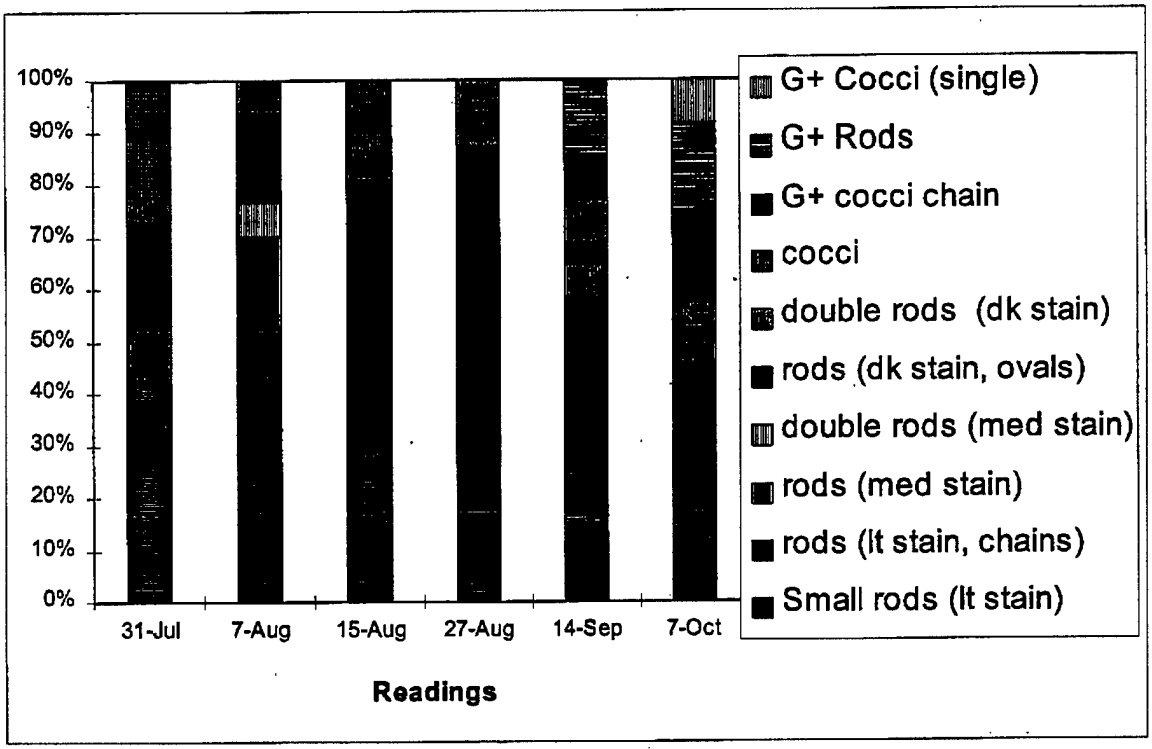
Gram Stain and Morphology. The nuances of Gram staining in determinative bacteriology became evident in the first few trials. If air drying of the sample/smear was done too slowly, or the heat fixation was accomplished at too high temperatures, the cells appeared as if they had burst, expanded, or just been warped by these techniques, which, if done correctly, are generally not so physically destructive. Staining was performed satisfactorily after a few trials.

As shown in Table 4-1, the morphology of the bacterial population was evaluated periodically throughout the duration of the experimentation. The bacteria were categorized in terms of shape, size, and stain. For Gram-negative bacteria, some forms had greater affinity for the counter stain and thus had a more intense or darker stain. The bacteria were labeled as having a *lt*-light, *med*-medium, or *dk*-dark counterstain. The size of the rods was described as the length times the circumference (or width) and the cocci were assigned a rough estimation of their diameter. The ratios of these morphologies can be seen in Table 4-1 and visualized in Figure 4-1.

Table 4-1 Morphology Ratios

Morphology	Dimensions (um)	31-Jul	7-Aug	15-Aug	27-Aug	14-Sep	7-Oct
Small rods (lt stain)	1.2-1.5 x .5	42%	52%	29%	18%	30%	29%
rods (lt stain, chains)	2 - 4 x 0.5 - 0.8	0%	0%	19%	35%	28%	17%
rods (med stain)	2 - 3 x 0.5 - 1	11%	18%	0%	0%	6%	4%
double rods (med stain)	2 - 3 x .8 - 1	0%	6%	0%	0%	0%	0%
rods (dk stain, ovals)	1.5 - 2 x .8 - 1.2	21%	17%	33%	35%	4%	3%
double rods (dk stain)	1.5 - 2 x .8 - 1.2	4%	6%	6%	13%	8%	4%
cocci	1 - 1.5	21%	0%	13%	0%	0%	1%
G+ cocci chain	d = .3 - .5	2%	0%	0%	0%	9%	17%
G+ Rods	2 x 1	0%	0%	0%	0%	14%	17%
G+ Cocci (single)	d = .5	0%	0%	0%	0%	0%	8%

Figure 4-1 Bacterial Morphologies



The criteria for determination of morphology was consistent throughout this period except for the first two readings. In this case, the light stained rods of both unicells and chains (typically two cells) were classified together on the premise that the chained rods were simply dividing cells. While this may be a valid assumption, it may not be the case and there may be, in fact, two separate types of bacteria. In Figure 4-1, an imaginary line could be drawn, cutting the "small rods (lt stain)" columns for 31 Jul and 7 Aug down the middle, and incorporating an unknown percentage of "rods (lt stain, chains)".

The possibility that these 10 different morphologies are representative of any "type" or species of bacteria is quite small. In fact, more meticulous inspection with a

more powerful microscope would probably present a greater variety of different morphologies, showing distinct differences where none were previously observed, or creating sub categories from a more general morphology. This effort was intended to show the ratios of sub-populations and note their trends as the whole population experiences a succession resulting from a changing environment, altered by either exogenous or self-initiated factors.

Note the relative consistency of the small light stained rods as well as the dark stained rods and their doubles (in this case I believe the double rods are just rods undergoing division but I felt it was necessary to separate them if this isn't the case). These morphologies are fairly consistent until the last two readings. It was here I noticed some severe changes in the population. Gram-positive cocci and rods were beginning to proliferate in an environment not previously suited for their sustainment. The presence of Gram-positive bacteria raised concern that subsequent BOD and spread plate readings would result in abnormal results. Later, I will show this to be the case.

Wet Mount Microscopy and Motility. Wet mount observation was not the method of choice for enumeration or even rough classification. The specimen were highly mobile, coming in and out of the field of view as they moved normal to the microscope slide, making cell counts and identification difficult. When used in tandem with Gram-staining, however, there was a higher confidence in the observations as certain morphologies could be recognized between methods. Also, the effects of evaporation/air

drying and heat fixing upon cellular integrity and shape during Gram staining could be noted and taken into account when morphological differences were being determined.

The bacteria were highly mobile. Stationary cells were present but appeared to be dead cells or those that became fixed to either the glass coverslip or the glass microscope slide. Three distinct forms of motility were observed;

Polar Flagellar Movement I. This group consisted primarily of single or double rods of all recorded types. The rods would move quickly throughout the field of vision in a unidirectional manner, indicating, perhaps, a single polar flagella. The typical rotary motion was clearly evident as the rods would move in a wagging fashion. This was further supported when the some rods would appear to find themselves normal to the slide, such that the observer would be viewing them head-on. The bacteria would rotate as a corkscrew, spinning rather quickly as if its flagella were being held firm. This spinning resembled a corkscrew motion indicating a singular, helically shaped flagellum, as opposed to a flagellar tuft, which would have produced more of a wagging motion.

Those rods in doublets or triplets underwent the same sort of motion with the "rear" cell doing a majority of the work. The tandem seemed to move much slower and with less vigor, indicating that the work of a single cell encumbered by the mass of the other conjoined cell(s). This would also support the possibility that a majority of these joined cells may, in fact be a result of observation during cell division rather than the bacteria operating as a chain of cells encased in a capsule.

Polar Flagellar Movement II. This group of rods had unidirectional motion but in a wagging manner. This may indicate polar flagellar tufts but without meticulous dark-field microscopic analysis, this can not be confirmed. On rare occasions, long (5-8 μm) thin rods would move in a similar manner. This wagging, however was more snakelike and the motion appeared to be a result of the main cell's (or group of cells') contortions and less than that of the action of a flagella.

Random Motion. These bacteria appeared to be short stout rods or even cocci. Typically cocci are not noted for their motility (Brock and others, 1994:48, 67-68). The motion was erratic, somewhat vibrational, and in no particular direction. This motion could not be a result of Brownian motion as the movement was more intense than that of nearby inert material. The movement could be the result of peritrochous flagella, in which the flagella is attached to points around (*peri*) the cell (Brock and others, 1994: 68). This morphology, however usually results in unidirectional motility as well, thus another source of locomotion may be involved.

Fluorescence Microscopy

The results of the fluorescence microscopy were inconclusive in terms of differentiation of live and dead cells. Roughly 80 percent of the cells were shown to exhibit auto-fluorescence, which washed out any indication of live/dead determination. In coordination with Gram staining and phase-contrast microscopy, the breakout of observed auto-fluorescence of the bioreactor population, dated 7 Oct, is shown in Table 4-2. Morphologies that were previously classified into their major groups on the basis of

appearance, can now be broken down into subgroups depending upon the extent and type of auto-fluorescence they exhibit. Auto-fluorescence may result from certain proteins and enzymes specific to the fluorescent species.

Table 4-2 Cellular Auto-Fluorescence (7 Oct)

Morphology	Dimensions, μm	Fluorescence
Small rods (lt stain)	1.2-1.5 x .5	Yellow/green under filters 02 and 09
rods (lt stain, chains)	2 - 4 x 0.5 - 0.8	Yellow/green under filters 02 and 09
rods (med stain)	2 - 3 x 0.5 - 1	Yellow/green under filter 02, none under 09 Yellow/green under filter 02 and blue under 09
rods (dk stain, ovals)	1.5 - 2 x .8 - 1.2	None
double rods (dk stain)	1.5 - 2 x .8 - 1.2	None
cocci	1 - 1.5	Blue under 02
cocci chain (assume G+)	d = .3 - .5	Bright yellow/green under 09

These results support the idea that many of the bacteria are of the Genus *Pseudomonas*. This is supported by the arguments that while some members are targeted as degraders of toluene and other aromatics, some soil species are known to exhibit fluorescence as well. The species, which is commonly understood to both degrade toluene as well as fluoresce, is *P. putida*. (Duetz and others, 1994; Brock and others, 1994: 778)

Dilutions

The initial set of dilutions was accomplished early on in the laboratory effort to establish an appropriate range in which to expect good spread plate results. The results

from these tests were promising and allowed for the subsequent metal runs to be implemented with confidence. These results were presented in the methodology section.

Effect of Dilution on Plate Counts

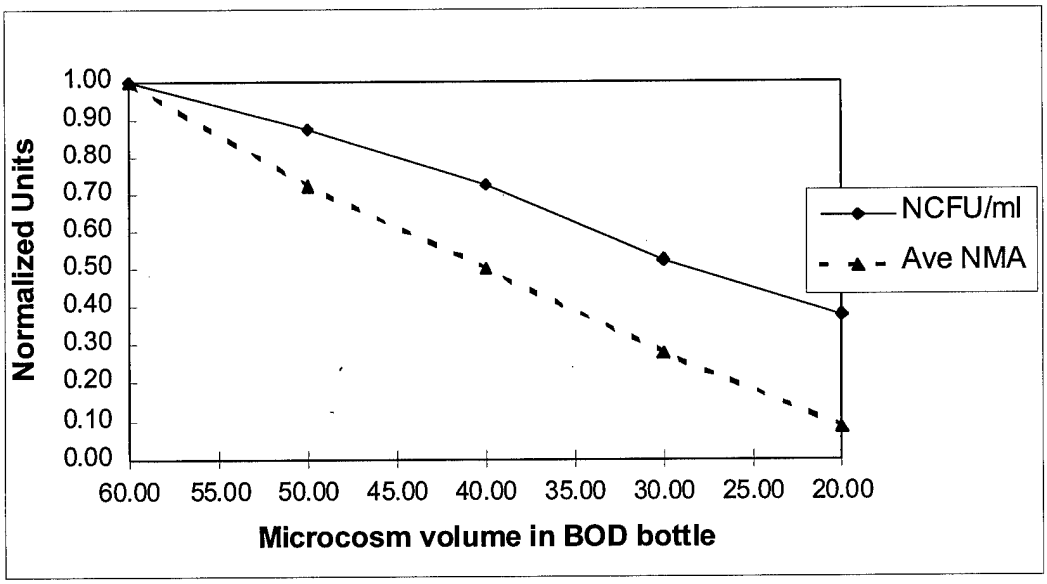
This test was run in conjunction with Capt. Marbas' dilution test conducted on 2 September by which he measured oxygen consumption as a function of varying microbial population size. His concern was that there might have been synergistic or inhibitive effects resulting from microbial concentration during BOD tests. If the microcosm population was too dilute, the interaction between the bacteria, which are necessary for the degradation of organic material, would be impacted. If the population was too concentrated, excreted toxins may inhibit the metabolism of neighboring bacteria.

The spread plate count was conducted to support any data produced from these metabolic measurements and to ensure that the procedures and microcosm extractions were accurate. Table 4-3 shows how well these plate counts correlate with the increase in microcosm inoculum volume as well as the contrast with respiration data (respiration data is depicted as Ave NMA (average normalized metabolic activity). These relationships are shown graphically in Figure 4-2 as well.

Table 4-3 Dilution Test Counts (2 Sep)

Microcosm (mL)	cfu/mL 1 x 10^3	cfu/mL 2 x 10^3	ave cfu/mL x 10^3		NCFU/mL	Ave NMA
20.00	970	900	935		0.38	0.08
30.00	1280	1300	1290		0.52	0.28
40.00	1750	1840	1795		0.73	0.50
50.00	2020	2300	2160		0.87	0.72
60.00	2540	2400	2470		1.00	1.00

Figure 4-2 Dilution Test: Comparing Colony Growths and Respiration (2 Sep)



When considering colony counts, the increase in microcosm concentration results in the number of CFU increasing in nearly a 1 for 1 relationship. The respiration data, however, exhibits a far steeper decline as concentration is reduced. This may be in part to the reduction of neighboring enzymatic activities due to dilution of microcosm. Such dilution may reduce respiration, thus slowing metabolism without inducing a chronic effect upon the population, which would show up in colony formation.

The Effect of Metals on Spread Plate Counts

The manner in which colonies are formed as a result of spread plating is important to the ability to link NCFU/mL to NMA. There are two possible assumptions under which this link is appropriate: 1) The nutrient agar is as general a food source as anticipated and virtually all bacteria metabolizing toluene subsequently are represented in colony formation; or 2) the small population represented in the colony counts is as

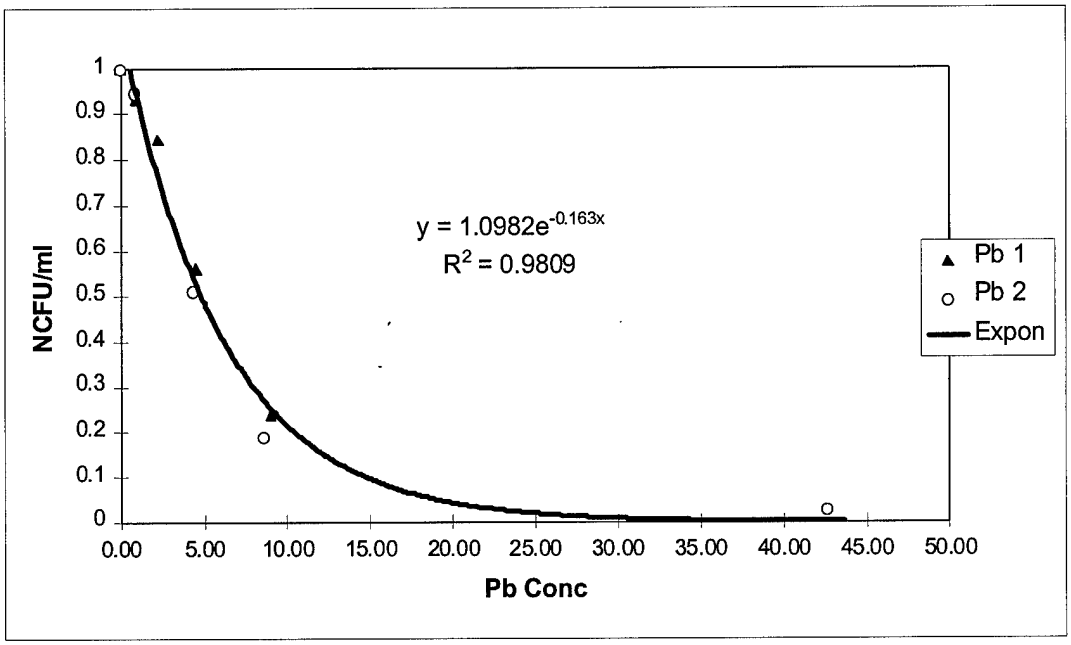
equally effected by metal treatments as is the larger population, whose response is documented by respiration measurements.

Toxicity Function. The normalized colony forming units per mL (NCFU/mL) can be represented in a manner consistent to that described in recent research; that of a toxicity function ($C_N^{-1} - 1$). This metal toxicity represents the impact the free metal ion concentration has upon the reproductive viability of the bacterial cells. This colony-based toxicity function can be correlated to a toxicity function ($A_N^{-1} - 1$) indicating the metal toxicity as it impacts metabolic activity, or more specifically, respiration rate.

The Effect of Lead on Spread Plate Counts. The data collected for this effect is a result of two separate runs conducted on 30 Aug (lead treatment 1) and 13 Sep 1997 (lead treatment 2). The preparation for these two runs followed the same methodology. (Marbas, 1997) Although the tests were conducted roughly two weeks apart, establishing a significant difference between the two populations, as was evident in the morphology observations. A third run was conducted later (7 Oct 97) but the population had undergone considerable changes and the results were not representative of the earlier tests. The tabularized counts for all three lead runs are presented in Appendix E.

Lead treatment 1, which ranged in concentrations from 0.87 to 8.69 ppm, and lead treatment 2 which ranged from 0.87 to 43.3 ppm, when presented together, is shown below in Figure 4-3. The effect lead has on NCFU/mL, indicates an exponential reduction in colonies as the concentration of lead is increased.

Figure 4-3 Combined Spread Plates for Pb Treatments 1 and 2 (30 Aug & 13 Sep)



Even at high concentrations of lead (43 ppm), there were still a considerable number of CFU. This survivability can be attributed to the manner in which the bacterial samples were handled. The methodology requires the bacteria to undergo acute heavy metal toxicity resulting from their relatively brief introduction to a noxious environment, typically two hours. The samples were then removed and placed in a more nourishing environment during preparation for spread plating. This allowed for the surviving population to recover and those that were viable were able to form colonies.

The lower concentrations of both treatments overlay quite well as if the readings came from the same population and same metal treatment run. Because of the ever-changing population, however, such apparent correlation is weak. This becomes evident

when these colony counts are presented in terms of lead activity measurements determined by Capt. Pat Marbas.

Figure 4-4 and Figure 4-5 represent the colony count (NCFU/mL) and respiration (NMA) data from lead treatments 1 and 2 as a function of lead activity in solution. (Activity measurements and respiration data provided by Capt. Pat Marbas)

Figure 4-4 Lead Treatment 1: NMA vs. NCFU/mL (30 Aug)

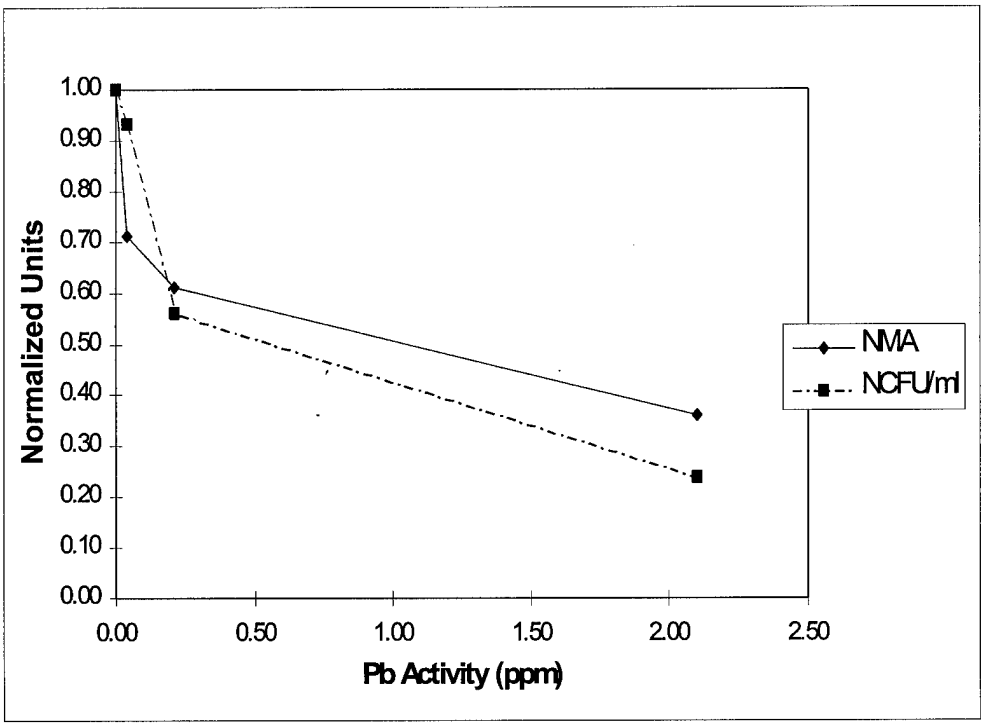
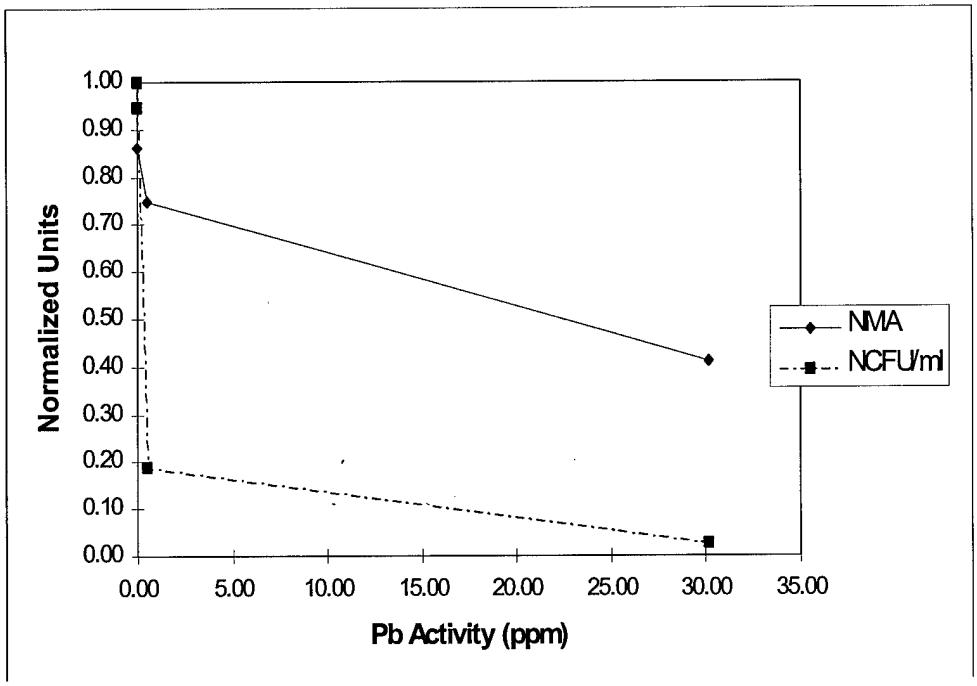
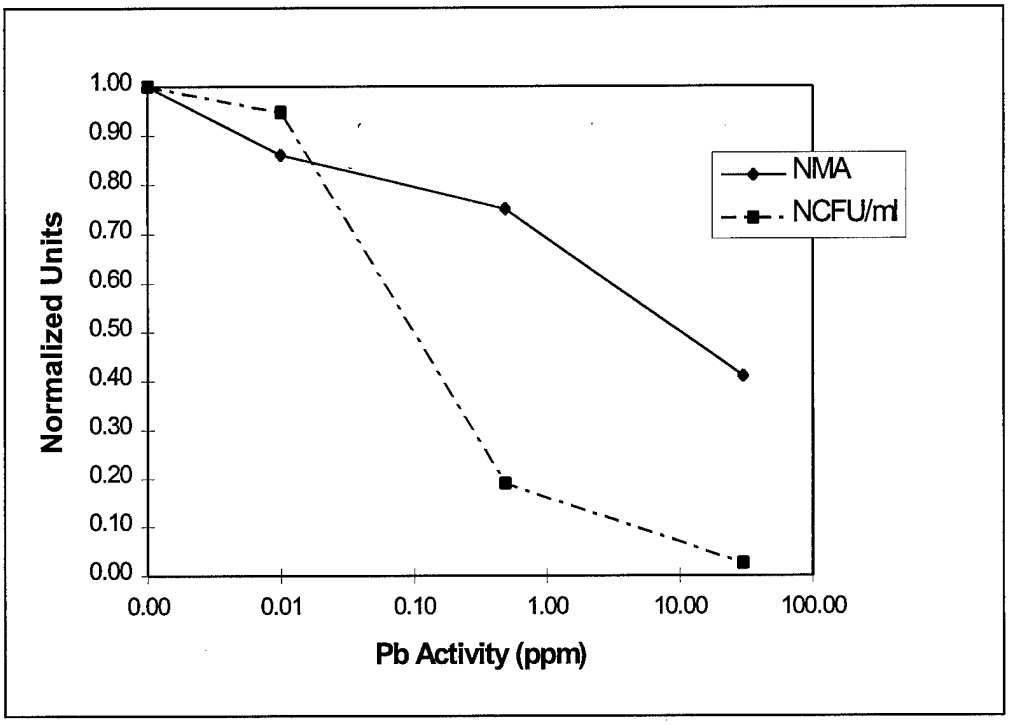


Figure 4-5 Lead Treatment 2: NMA vs. NCFU/mL (13 Sep)



The differences between respiration and colony counts are significant at high concentrations such that it would be useful to observe these differences on a logarithmic scale to better discern the two.(Figure 4-6)

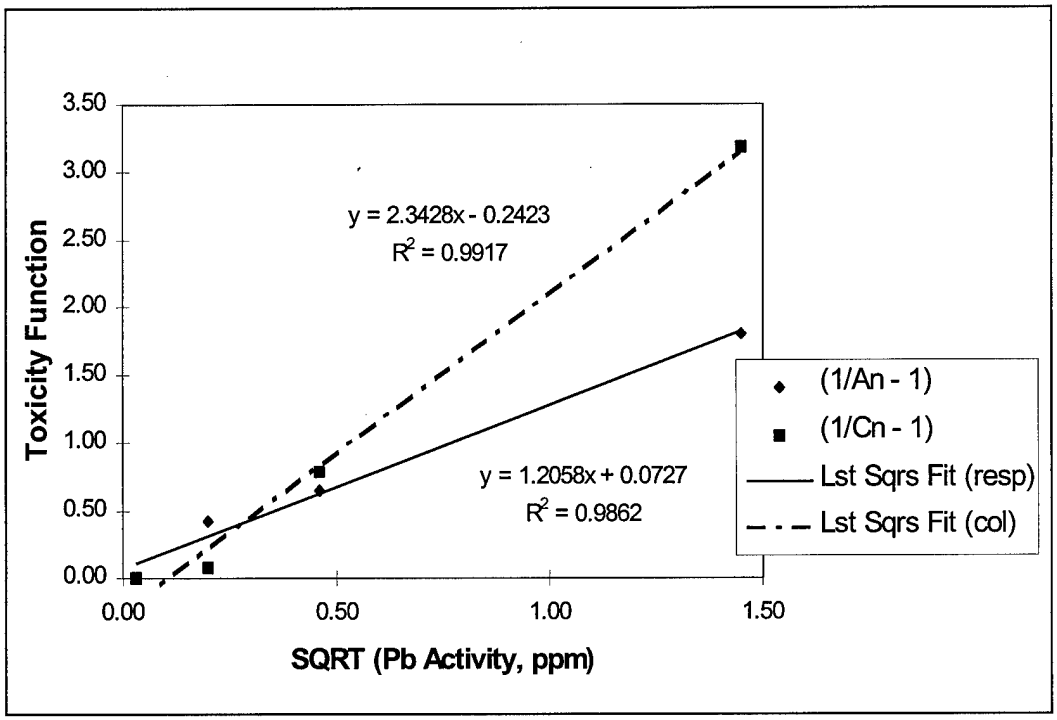
Figure 4-6 Lead Treatment 2: NMA vs. NCFU/mL On Log Scale (13 Sep)



Note that at low concentrations, the toxicity of lead upon colony formation, and thus reproduction, is slightly less than that of respiration. This might suggest that trace amounts of lead, while not necessarily toxic, are inhibitive to metabolic activity. Whether this is a result of inhibition of enzymatic function, ionic transport, or of bacterial self-inhibition as a survival technique, is speculative. At higher concentrations, however, the effects of lead treatment have severely reduced colony formation while having less of an impact upon respiration. This suggests that the mechanisms for reproduction are highly sensitive to metal treatments.

The differences between NMA and NCFU/mL become more evident when they are plotted in terms of toxicity function with the square root of lead activity as the independent variable. A least squares fit can be approximated (Figure 4-7).

Figure 4-7 Lead Treatment 2: Toxicity Function for Respiration and Colony Counts (13 Sep)



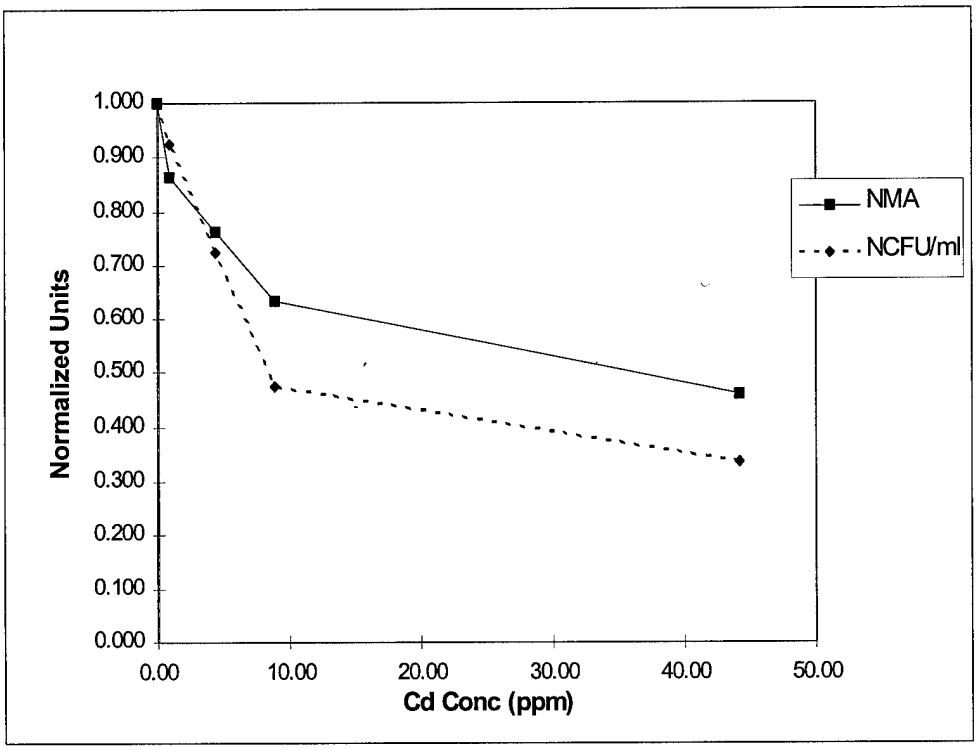
These results show a significant increase in toxicity to colony growths as compared to respiration activities as well. Similar charts can be seen for lead treatment 1 (Aug 30) in Appendix F.

The Effect of Cadmium on Plate Counts. The first cadmium treatment (10 Sep) was inconclusive, showing no significant trends regarding the concentration of cadmium and plate counts. A concurrent BOD measurement also lacked reliable results (Marbas,

1997) and the run was attempted again. (Note Appendix E for both runs and their counts).

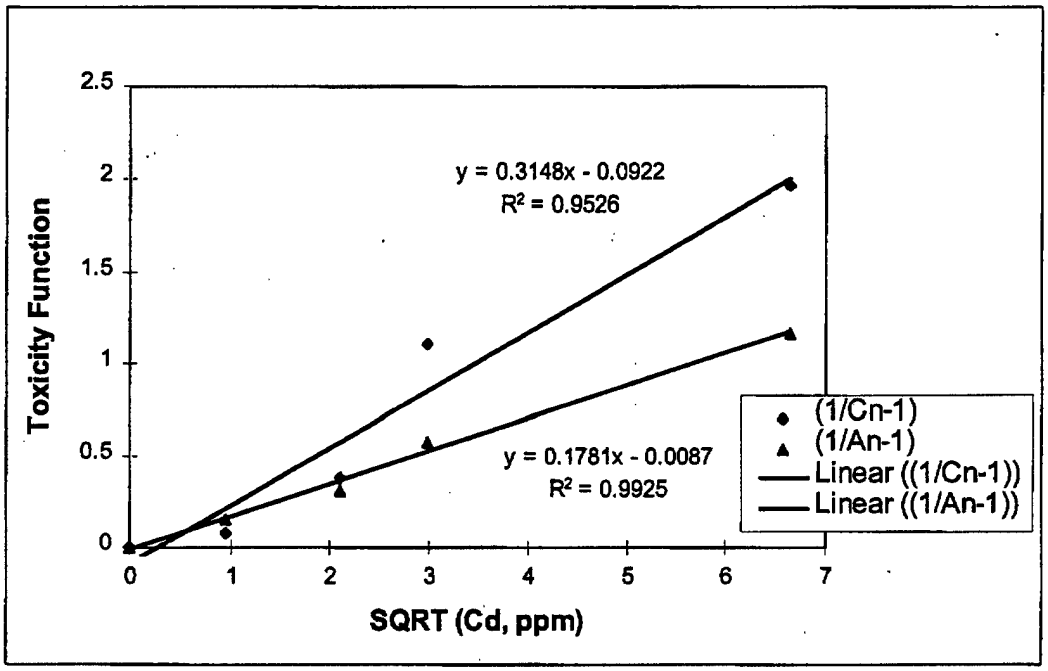
The second treatment (12 Sep) provided more reasonable results indicating a good reduction of colony counts, as well as reduced metabolic activity, as cadmium concentration is increased. In this effort, cadmium activity measurements were not recorded as the scope of such investigations anchored around lead measurements. Figure 4-8 depicts the contrast between respiration measurements and colony counts.

Figure 4-8 Cadmium Treatment: NMA vs. NCFU/mL (12 Sep)



These results are also depicted in terms of a toxicity function in Figure 4-9.

Figure 4-9 Cadmium Treatment: Toxicity Function vs. SQRT [Cd] (12 Sep)



Note once again that the toxicity as measured by colony counts is greater than that of respiration measurements. This suggests, as in the lead treatments, that the mechanisms of reproduction are more sensitive to heavy metals than the mechanisms of respiration.

Evaluating the Effects of Metal toxicity. The effects of lead and cadmium on the growth of bacterial colonies were noteworthy. At concentrations near 40 - 45 ppm of both cadmium and lead, the NCFU/mL registered at 0.34 and 0.026 respectively indicating the toxicity of lead greater than that of cadmium by a factor of roughly ten.

The free ion activity for these metals was not compared, which raises questions as to the availability of these metals in solution. However, when approaching this comparison from a practical point of view, understanding that cadmium is less toxic whether this results from differences in ligand-metal (chemical) or cellular (biological) interactions is notable. It is precisely these differences which result in the distinction of toxicity.

Colonies, Morphology, and Metal Toxicity

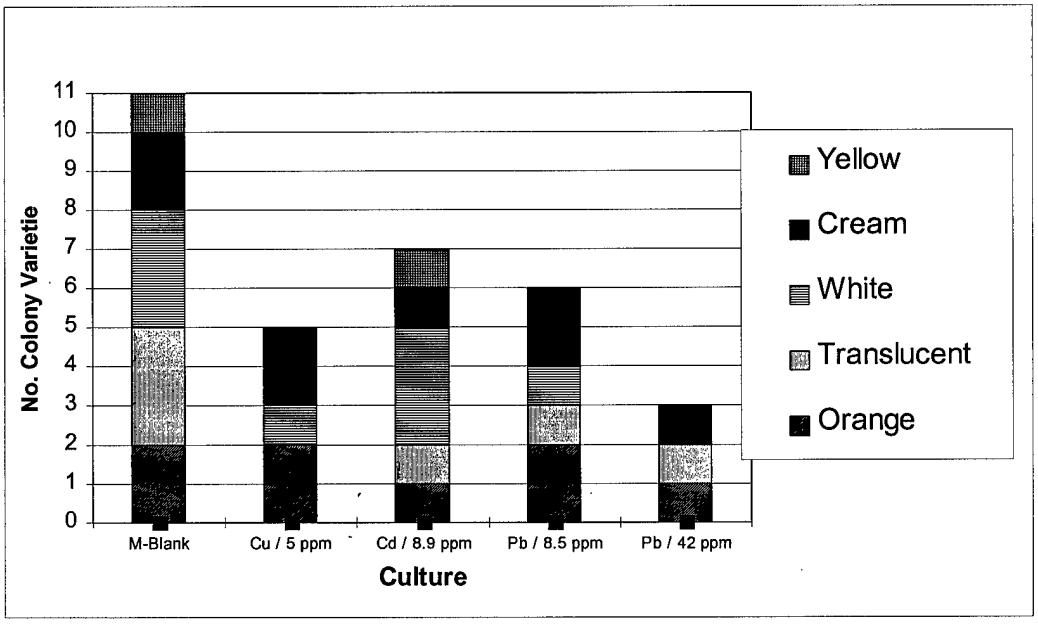
The number of different morphologies within each colony, after a 48 hours culture, was greater than expected. The number ranged from one to three distinct morphologies. Ideal culturing conditions would allow a single type of bacteria to act as the CFU. Methods such as sonification and dilution should promote this. A CFU, however, is widely accepted in microbiology as just that, a colony forming unit. It may be from cells of unrelated bacteria or a single organism.

Colonies benefit from the synergy of different bacterial species and a colony resulting from a single species may not be as resilient to nutritive changes. It should not be surprising then to find a colony made up of two or three distinct species. It may be likely, however, that the varying morphologies merely result from specialization within the colony to such a degree that slight changes in phenotype (environmentally induced changes in form and function from an unchanged genotype, or genetic makeup). Some morphological observations, such as in the white colonies for example, consisted of cocci (round) bacteria of diameter 0.5 μm and two types of rods of diameter 0.8 μm and lengths of 6 – 10 μm and 2 – 3 μm. While these two bacilli may represent the same type of

bacteria, it would be speculation to note anything other than their morphological differences without more intense characterization tests.

Upon observation of the morphologies, and their associated colonies, it became evident that the metal treatments affected the bacterial population in two ways. First, as both respiration and spread plate measurements indicate, the entire population is inhibited and reduced in numbers by the metal treatments. The second influence of metal treatments takes part in observable selection of "types" of bacteria. This is evident in two ways. As seen in Figure 4-10, the number of different colony growths is reduced after metal treatments. The M-Blank (untreated sample) and the lead treatments were from lead run # 2 conducted on 13 Sep. The copper run was conducted on 28 Aug and the Cadmium run on 12 Sep.

Figure 4-10 Bacterial Morphologies After Metal Treatment



Those colonies found to be present after culturing an untreated sample are not completely represented in the metal treated samples. Some "types" did not survive to form colonies. The copper treated sample was absent of the translucent colonies, while the lead and cadmium treated samples lacked yellow colonies. Increased lead treatment retained only orange, translucent, and cream colonies indicating that the resident bacteria were perhaps less sensitive to the treatment.

In addition to colony changes, the total number of observably distinct morphologies were significantly reduced as a result of metal treatments. For example, the white colony held three morphologies resulting from an untreated sample. An 8.9-ppm cadmium treatment did little to reduce this number, yet a 5-ppm copper treatment reduced the number of morphologies within the white colony to one. Increased concentration (42 ppm) of lead reduced the numbers more dramatically. These results indicated that different metal treatments had different effects on bacteria.

The different morphologies, their description, and their associated colonies can be found in the colony section of Appendix G. It is notable that those forms which are represented in the M-Blank colonies are not necessarily found in the metal treated colonies. It would simplistic to find a set of five morphologies in an M-Blank (A, B, C, D, and E) and in colonies resulting from metal treatments, we might find subsets of (A, B, E) or (B, D), for example. Instead, we find that morphologies found in cadmium treated cultures, for example, are observed as such: (G, P, Z), completely different and previously unobserved. In fact, relatively few morphologies observed in certain M-Blank

colonies are still found after metal treatments. These include, light stained rods (dimensions 2 - 4 x 0.5 - 0.8 μm) found in white colonies after copper treatments, rods (2 - 4 x 1 μm) in orange colonies after both copper and lead treatments, and dark stained rods (1.5 - 2 x 1 μm) in cream colonies after lead treatments. Most morphologies found in colonies resulting from metal treatments are previously unobserved, suggesting a selection of certain types of bacteria, killing off those that typically dominate and allowing rare types to proliferate.

Aggregation of Identification Techniques

Appendix G: Bacterial Identification Matrix, brings together three methods of identification utilized in this effort. These observations include Gram-stain microscopy, observed auto-fluorescence under, and morphological identification within colonies. Because Gram staining was used as a supporting technique, it was not surprising to find that much of the bacteria from the bioreactor identified in the fluorescence observations were also found in Table 4-1 as those found to dominate throughout the laboratory effort.

The M-Blank results indicate that those bacteria which proliferate in the bioreactor do not appear to dominate the colony growths. This suggests that a portion of the bacteria contributing to respiration measurements are not likely to form colonies on the nutrient agar. For a particular metal treatment, only a few types are contributing to colony formation.

V. Conclusions

This chapter reviews the results of this thesis effort, addresses its implications, and suggests efforts for further research.

Summary of Effort

In this thesis effort, a wide range of different microbial techniques was applied in order to gain a better understanding of the mechanisms of metal toxicity. Throughout the laboratory effort, the correlation of results between NMA measurements and colony counts was promising. Even the anomalies in the cadmium and lead runs produced similar "non" results in both experimental efforts. While this provided confidence in the methods and instruments, it indicated that there may have been some contamination or overall population effect early on in the methodology for that given experiment. Typically, the metal and dilution runs that provided good results for the measurement of respiration activity, also provided satisfactory results for colony counts.

Morphology

Throughout the laboratory effort, the bacterial population in the bioreactor, from which the microcosms were extracted, underwent succession. Bacterial morphologies seemed to disappear and reappear as their environment changed. These changes were only roughly documented and further research in this arena would do well to monitor the population as meticulously as possible.

More importantly, the presence of Gram-positive bacteria in the bioreactor towards the end of the laboratory effort raised questions as to the repeatability of later BOD and colony counts.

Observances of morphological changes resulting in metal treatments were important for two reasons: First, there was confirmation that some types of bacteria were more sensitive than others. Also, metal treatments were shown to reduce the overall numbers of bacteria regardless of "type", as expected.

Fluorescence

Differentiation of live/dead bacteria via fluorescence was confounded by auto-fluorescent species. Thus the observances of the bacterial samples under fluorescence did not provide any data concerning cell viability. In coordination with Gram staining and phase-contrast techniques, however, morphologies that were identified previously, could be broken down into subgroups depending upon the extent and type of auto-fluorescence they exhibited. Because some members of the genus *Pseudomonas* are known as degraders of aromatic hydrocarbons (including toluene) and species of *Pseudomonas* from soils are also known to auto-fluoresce, an argument can be made that some of the bacteria studied in this effort were of the genus *Pseudomonas.* More specifically, this may also suggest the presence of the species *P. putida,* which is documented to fall into both categories. This cannot be confirmed through this effort, as the absolute characterization of such types generally requires enzymatic and DNA tests.

The results of these tests also implied that when working with heterogeneous soil or sludge bacteria in environmental efforts, the use of general fluorescent methodology may not be appropriate. The presence of fluorescent *Pseudomonas* bacteria may render the viability tests invalid as the natural emission of fluorescence acts as noise to the live/dead results.

Wet Mount Microscopy

This method of observational microscopy was useful in triangulating morphological characteristics in tandem with Gram staining and fluorescent techniques. It is useful to establish an observational baseline, especially when many staining techniques tend to disturb the natural shapes and sizes of the specimens.

Dilutions and Plate Counts

The effect of serial dilutions and the volumetric variation of microcosms introduced to dilution water, both validated the methodology used for this effort. The results showed demonstrably that there was a one for one relationship between the factor by which the sample was diluted and the number of colonies that formed as a result. These initial runs provided dilution ranges in which the metal runs could be evaluated successfully as well as provided confidence in the procedures in use.

The Effect of Metals on Bacterial Viability

The effects of lead and cadmium were most successfully evaluated among the metals, both indicating a strong positive relationship between the concentration of metal salts introduced to a microcosm and the reduction in number of colony forming units.

While lead exhibited a much higher reduction in colony counts than that of cadmium, by nearly a factor of 20, both treatment runs showed a trend in which the toxicity effect leveled off at higher concentrations. Even at a 40 - 45 ppm lead treatment, there were still roughly 20,000 colony forming units per mL. This may suggest that there may be fairly tolerant strains within the population.

Metal Toxicity Vs Inhibition. The plate counts record the effect of metal treatments to the reproductive viability of the bacterial sample. The toxicity effect indicates that the cells are either killed as a result of treatment or that they are damaged in such a way such that they are unable to reproduce past one generation.

The recordings of toxicity function via BOD readings only provide insight into the impacts of metals upon metabolic activity. Depending upon the dominant mechanism of metal toxicity for a selected metal, the cells' metabolism may be inhibited without predominant lethality.

This effort, however, showed that for both lead and cadmium, there is reason to assert that these heavy metals have far more lasting effects on microbial populations than just reduction in metabolic activity. While low concentrations of lead and cadmium had

less of an impact upon reproductive viability than that of respiration, levels past 0.01 ppm seemed to severely inhibit the reproductive capabilities of the bacteria.

Colony observations also suggested that not only is the reproductive viability of the entire bacteria population reduced significantly by metal treatments, but that certain classifications of bacteria are more sensitive than others. While much of the population is reduced in numbers after high concentrations of lead treatment, for example, there was still a small microbial population that was tolerant toward the treatment. Such drastic selection, however, can create a biological bottleneck, making the surviving species vulnerable to inevitable changes in its environment. This raises some concern over the reversibility of heavy metal contamination as well as the bacteria's ability to recover and operate under pre-contaminated conditions.

The colony observations, as well as the morphological matrix, strongly suggested that different species of bacteria are seriously impacted by heavy metal treatment while others seem to be tolerant. This confirms that reduction in metabolic activity is not only caused by the severe reduction in total population, but that entire species and sub-populations are completely eliminated, leaving the observed metabolic activity to the select few species.

Chronic Vs Acute Exposure. Both the plate counts and morphologies resulting from cultured colonies indicate that at even high concentrations of lead, there are still a large number of viable cells. Some appear to represent resistant strains of the dominant species in the bioreactor while others represented rare species able to thrive after the

environmental conditions change and the competition is reduced. The bacteria were removed from the toxic metal salt baths within a couple of hours after treatment and placed on a less noxious growth medium. Even such acute exposure manifested a tolerance to heavy metal treatments due to selection. While the link between acute and chronic metal toxicity and subsequent tolerance is weak, results of an acute exposure survival can provide some indication of the possibility of chronic exposure tolerance. Diaz-Ravina and Baath presented findings that metal tolerance in soil bacterial communities can be developed over a period of months (Diaz-Ravina and Baath, 1996).

Summary

The mechanisms involved in reproduction were shown to be highly sensitive to the toxic effects of heavy metals. This was somewhat surprising considering that when confronted with a noxious environment, many bacteria are known to go dormant until they find themselves in more agreeable surroundings. Thus metabolism could be suspended in order to preserve life. The results of this thesis effort countered such assumptions. While spread plating was shown to be an effective way to support respiration data, its ability to detail the mechanisms of this toxicity is still greatly limited. Finally, the observation of auto-fluorescence, while a detriment to live/dead determination, proved useful in characterizing different types of bacteria, even in the limited capacity in which it was used.

Recommendations

It was demonstrated that the true nature of the microbial population in the bioreactor was not consistent, nor was it well monitored. With this known, there are two directions by which future studies should operate. First, experimentation may require a relatively constant population in order to build sufficient data on population effect of particular metals, requiring tens of experiments per metal over one or two weeks time. For such a requirement, it may be necessary to establish that the experiments are conducted on the same population. Such an assumption would require the reduction in variation in the bioreactor population. The second direction assumes that metal toxicity effects on a changing population is reasonable, better representing field conditions. Environmental and biological conditions of the population (pH, temp, Gram type, dominant morphologies, etc.), at the time of metal treatment must be recorded. Either method requires the continual observation and perhaps control of environmental conditions.

Because fluorescence is a function of chemistry, the differences in intensities and wavelengths of observed emissions can be an effective way of differentiating and characterizing different strains of bacteria. In addition, entire colonies can be observed for fluorescence and compared to emissions from individual cells extracted from the colony. This may help to determine whether different morphologies are of the same strain or are, in fact, communal colonies of several species. In coordination with this

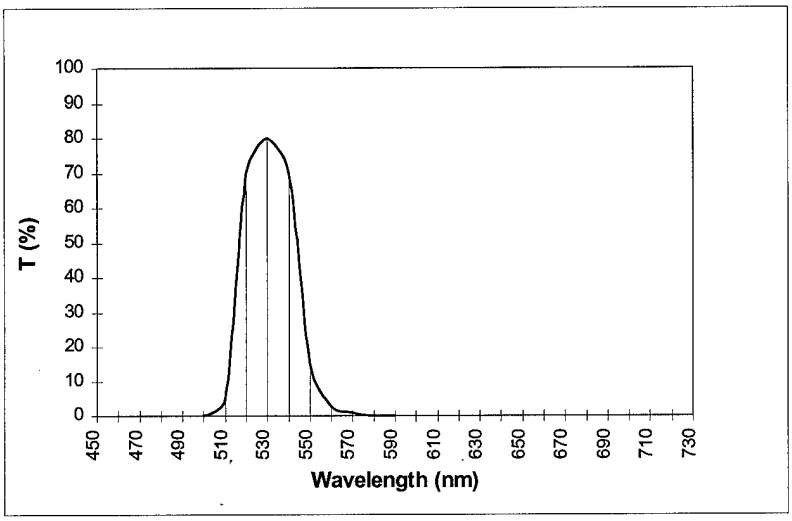
method, colony growths can be resuspended, grown in a nutrient bath, and observed to determine if the different morphologies are specialization resulting from colony growth.

The presence of heavy metals may prove to alter the chemistry and configuration of cellular fluorescent biomolecules. As a result, the reduction of auto-fluorescence and metabolic activity may be correlated. In addition, lead is known to quench fluorescent emissions and thus can be used as a probe to show whether its effect as a poison is a result of external or internal toxicity.

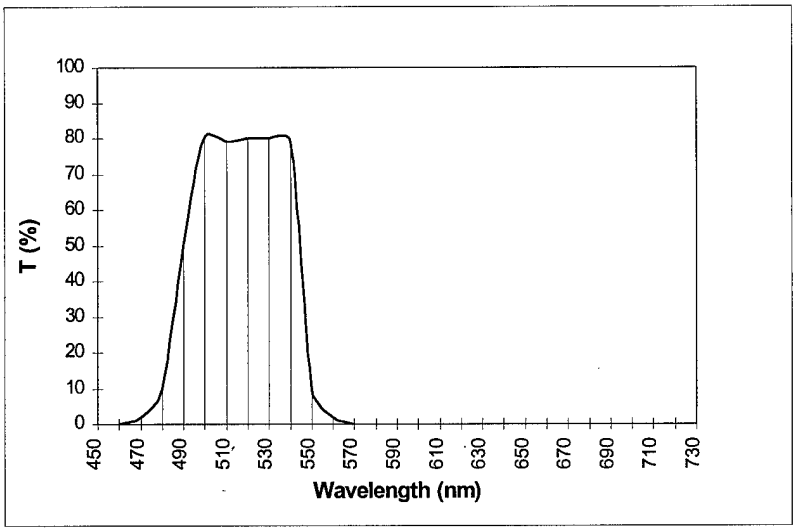
To better understand the difference between chronic and acute toxicity as well as to study the kinetics of heavy metal availability, the time dependence of toxicity on spread plate cultures can be investigated. In this manner, different toxic effects may be observed depending on how long the bacteria are introduced to a noxious environment before removed and cultured. Additionally, spread plating can include toluene laced agar so that those colonies forming bacteria are better representative of the respiring bacteria in the microcosms.

Appendix A: Examples of General Fluorescent Filters

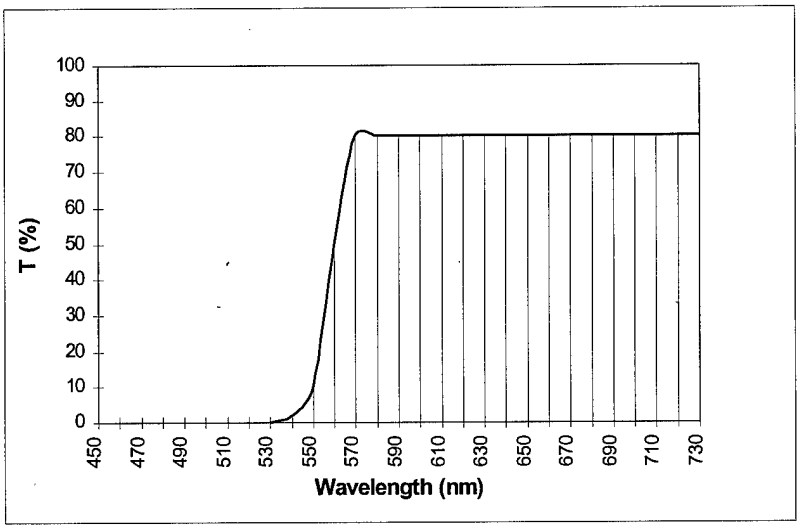
Band Pass Filter: This is also known as a **line filter** and is selective towards a small range of wavelengths



Shortwave Pass Filter: Also known as a **cut-off filter Shortwave pass**, the wavelengths which are shorter than the designated cut-off (here at $\lambda = 540$ nm) are transmitted.



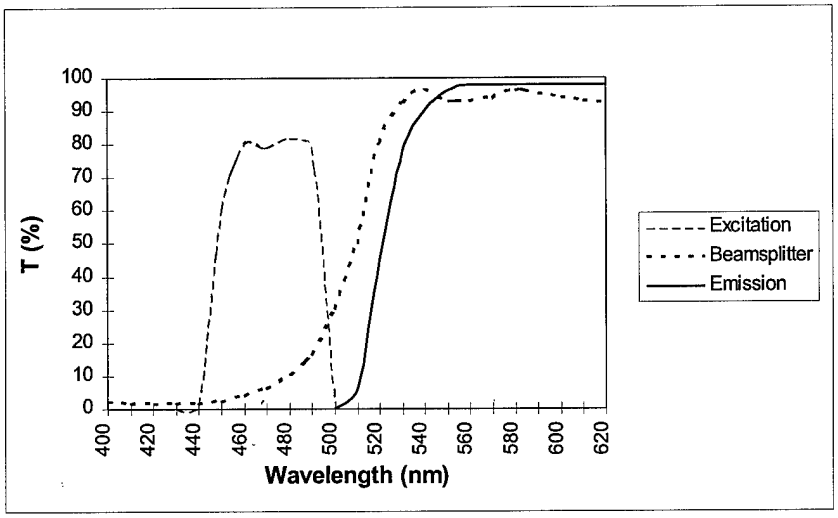
Longwave Pass Filter: Also known as a **cut-off filter Longwave pass**, the wavelengths which are longer than the designated cut-off (here at $\lambda = 570$ nm) are transmitted.



Appendix B: Zeiss Fluorescent Filter Sets

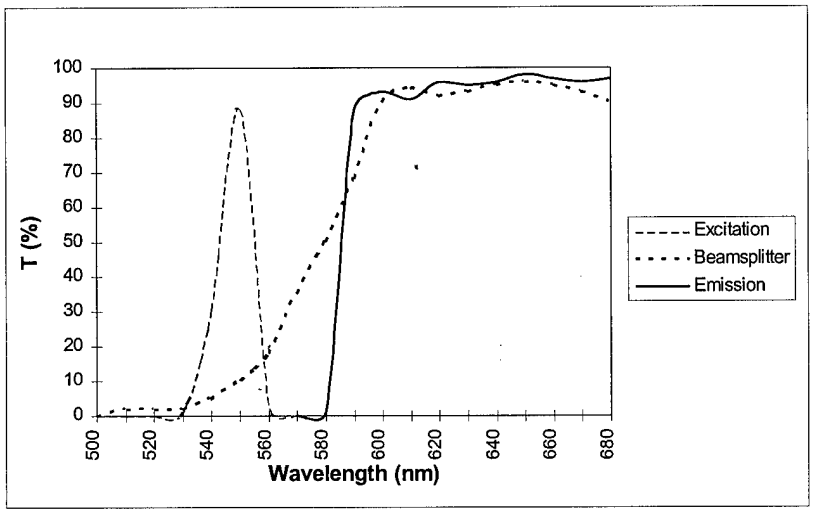
Filter set 09, required for the Sytox Green nucleic acid stain, excites at 490 nm and emits at 525 nm.

Filter Set 09



Filter set 15, required for the Texas red stain, excites similarly to rhodamine detecting filters at 540 - 560 nm and emits at 580 nm.

Filter Set 15



Appendix C: Fluorescence Microscopy Stain Protocol

The stain kit should be stored in the dark at -20^{0}C. Prior to use, the reagents should warm to room temperature and centrifuged briefly before opening the vessels.

Any traces of growth medium must be removed prior to staining the bacteria. Phosphate and carboxylic groups as well as nucleic acids can bind to DAPI and SYTOX Green stains, resulting in wide variation in fluorescence results. Accordingly, phosphate wash buffers must be avoided as they may reduce staining efficiency.

The following protocol is provided by Molecular Probes^{TM}:

1. To prepare a 2 mg/ml stock solution of Texas Red-X conjugate of WGA (*component C*) add 500 µL of 0.1 M sodium bicarbonate (*component D*) to reconstitute the reagent. Use 50 µL for the preparation of 20 aliquots. The remainder can be refrozen for storage as aliquots.
2. Prepare a working solution of the viability indicators by combining 3 µL of the DAPI stain (*Component A*) and 3 µL of the SYTOX Green nucleic acid stain (*Component B*) to 54 µL of water.
3. Centrifuge 50 µL of bacterial suspension (about 5 x 10^7 cells) in a 0.2 µm-pore size spin filter at 2000 rpm for 1 to 2 minutes.
4. Wash the cells in a 50 µL phosphate-free dilution water solution by pipetting up and down several times.
5. Recentrifuge and resuspend in 50 µL phosphate-free dilution water.
6. Add 2.5 µL of the WGA conjugate stock solution (finalizing the concentration to 100 µg/mL) and mix by pipetting up and down thoroughly.
7. Incubate for 5 to 15 minutes at room temperature
8. Centrifuge at 2000 rpm for 1 to 2 minutes to remove the WGA staining solution.
9. Resuspend in 50 µL phosphate-free dilution water.
10. Add 2.5 µL of the Component A/Component B working solution from step 2 and incubate for 10 minutes at room temperature.
11. Add 10 µL to a slide, apply a glass coverslip, seal and observe immediately in the fluorescence microscope.

Modified from Molecular Probes ^{TM} Product Information Sheet
For ViaGram^{TM} Red Bacterial Gram Stain and Viability Kit (V-7023)

Appendix D: Gram Staining Protocol

A sample of 0.2 to 0.5 mL is pippetted onto a glass slide. The smear is dried and heat fixed by placing the slide on a heating apparatus at 55-60 ^{O} C.

Solutions

- Fisher Diagnostics Gram Crystal violet staining reagent
  - Crystal Violet 20 g
  - SD3A Alcohol 200 mL
  - Ammonium Oxalate 8 g
  - Deionized Water 800 mL
- Fisher Diagnostics Gram Iodine mordant
  - Iodine 3.3 g
  - Potassium Iodide 6.6 g
  - Deionized Water 1000 mL
- Fisher Diagnostics Gram Decolorizer
  - SD3A Alcohol 550 mL
  - Acetone 450 mL
- Fisher Diagnostics Gram Safranin counterstain
  - Safranin O 2.5 mL
  - SD3A Alcohol 100 mL
  - Deionized Water 900 mL
- Tap water

Procedure

1. Flood the smear with the crystal violet staining reagent for 1 min.
2. Rinse the smear with a gentle stream of tap water for 2 seconds.
3. Flood the smear with iodine mordant for 1 min.
4. Rinse the smear with a gentle stream of tap water for 2 seconds.
5. Decolorize with flood of ethanol acetone solution until crystal violet stain no longer washes out (20 - 30 seconds).
6. Rinse with gentle stream of tap water for 2 seconds or until no color appears in effluent then blot the film dry with absorbent paper.

Modified from Fisher Diagnostics
Product Information Sheet provided by Fisher Scientific
For Fisher Diagnostics Gram Stain Set (SG 100D)

Appendix E: Metal and Dilution Runs

Micro cosm (ml)	Dilutn water (ml)	Total Volume	Sample Dilution
30	270	305	0.098361

Metal Conc	10	5.00	2.50	1.00	8/30/97
Actual conc	9.02	4.51	2.25	0.90	

	innoc (ml)	dil water (ml)	dilution %
metals	0.50	50.00	0.098361
blanks	0.25	50.00	0.04918

	innoc (ml)	2nd Dil (ml)	2nd Dil %
metals	10.00	20.00	0.0492
blanks	4.00	20.00	0.0098

	innoc (ml)	3rd Dil (ml)	3rd Dil %
metals	2.00	20.00	0.00984

	Culture	Dilutn %	Count	cfu/ml	Comments	8/31/97
1	M-blank	0.04918	>300	#VALUE!		
2	M-blank	0.04918	>300	#VALUE!		
3	M-blank	0.00984	167	1670000		
4	M-blank	0.00984	172	1720000		
5	9.02	0.09836	252	252000		
6		0.09836	275	275000		
7		0.04918	196	392000		
8		0.04918	209	418000		
9		0.00984	55	550000		
10		0.00984	54	540000		
11	4.51	0.09836	>300	#VALUE!		
12		0.09836	>300	#VALUE!		
13		0.04918	>300	#VALUE!		
14		0.04918	>300	#VALUE!		
15		0.00984	86	860000		
16		0.00984	104	1040000		
17	2.25	0.09836	>300	#VALUE!		
18		0.09836	>300	#VALUE!		
19		0.04918	>300	#VALUE!		
20		0.04918	>300	#VALUE!		
21		0.00984	137	1370000		
22		0.00984	149	1490000		
23	0.90	0.09836	>300	#VALUE!		
24		0.09836	>300	#VALUE!		
25		0.04918	>300	#VALUE!		
26		0.04918	>300	#VALUE!		
27		0.00984	-	#VALUE!		
28		0.00984	158	1580000		
29	Blank		3			

Pb Treatment 1

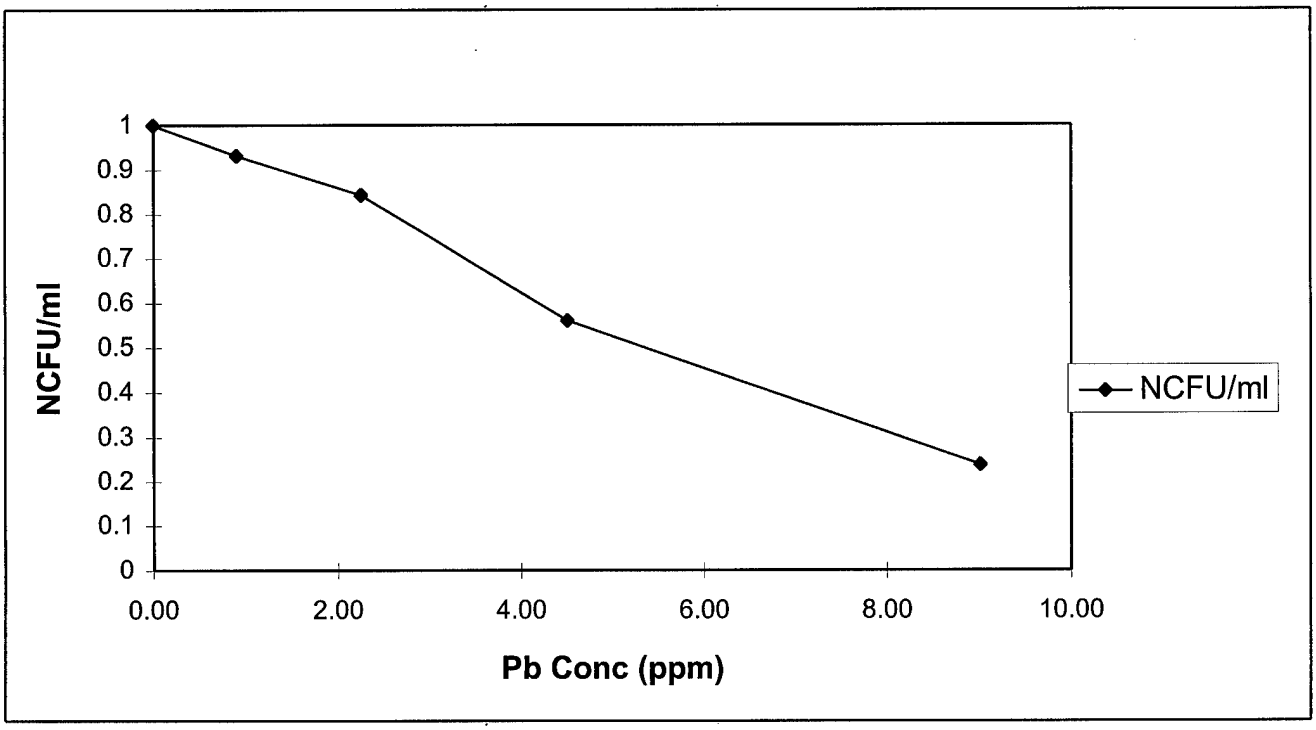
8/31/97

Culture	cfu/ml 1	cfu/ml 2	ave cfu/ml
9.02	392000	418000	405000
4.51	860000	1040000	950000
2.25	1370000	1490000	1430000
0.90	1580000	-	1580000
0.00	1670000	1720000	1695000

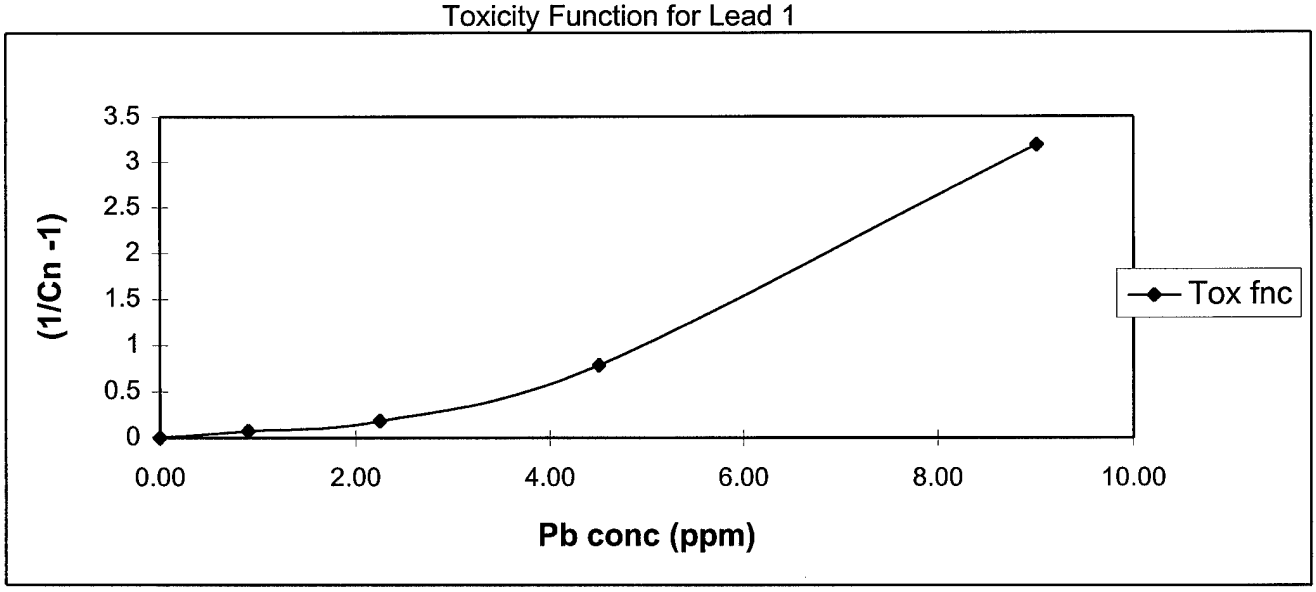
NCFU/ml	Tox fnc
0.239	3.18519
0.56	0.78421
0.844	0.18531
0.932	0.07278
1	0

NCFU/ml	NCFU/ml
0.2313	0.24661
0.5074	0.61357
0.8083	0.87906
0.9322	#VALUE!
1	1

NCFU/ml for Lead Treatment 1



Toxicity Function for Lead 1



Cd Treatment 1

Micro cosm (ml)	Treated Dilutn water (ml)	Total Volume	Sample Dilution
40	260	305	0.13115

Metal Conc	10	5.00	2.50	1.00	9/10/97
Actual conc	8.52	4.26	2.13	0.85	

	innoc (ml)	dil water (ml)	dilution %
metals	0.25	50.00	0.06557
blanks	0.10	50.00	0.02623

innoc (ml)	2nd Dil (ml)	2nd Dil %
4.00	20.00	0.0131
6.00	20.00	0.0079

innoc (ml)	3rd Dil (ml)	3rd Dil %
2.00	20.00	0.0066

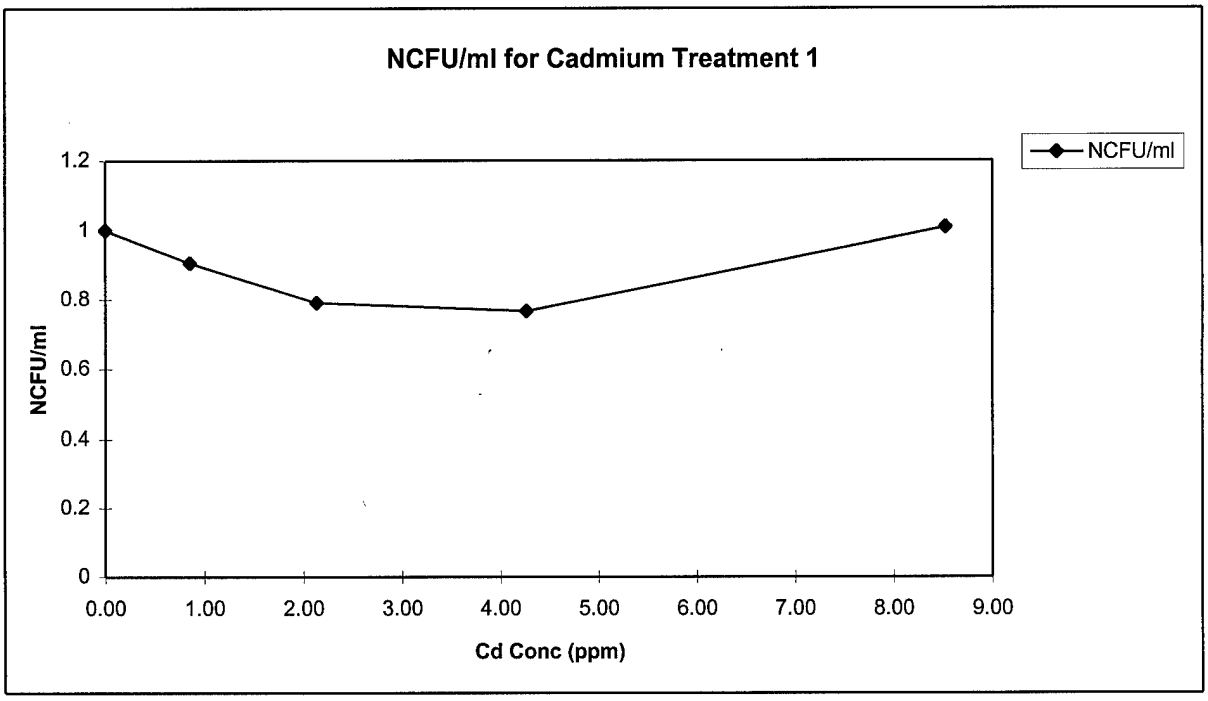
	Culture	Dilutn %	Count	cfu/ml	Comments	9/11/97
1	M-blank	0.02623	>300	#VALUE!		
2	M-blank	0.02623	>300	#VALUE!		
3	M-blank	0.007869	20	333333		
4	M-blank	0.007869	27	450000		
5	8.52	0.065574	>300	#VALUE!		
6		0.065574	>300	#VALUE!		
7		0.013115	36	360000		
8		0.013115	43	430000		
9		0.006557	28	560000		
10		0.006557	22	440000		
11	4.26	0.065574	>300	#VALUE!		
12		0.065574	>300	#VALUE!		
13		0.013115	37	370000		
14		0.013115	23	230000		
15		0.006557	13	260000		
16		0.006557	17	340000		
17	2.13	0.065574	>300	#VALUE!		
18		0.065574	>300	#VALUE!		
19		0.013115	39	390000		
20		0.013115	23	230000		
21		0.006557	15	300000		
22		0.006557	16	320000		
23	0.85	0.065574	>300	#VALUE!		
24		0.065574	>300	#VALUE!		
25		0.013115	41	410000		
26		0.013115	30	300000		
27		0.006557	14	280000		
28		0.006557	19	380000		
29	Blank		2			

Cd Treatment 1

9/11/97

Culture	cfu/ml 1	cfu/ml 2	ave cfu/ml
8.52	360000	430000	395000
4.26	370000	230000	300000
2.13	390000	230000	310000
0.85	410000	300000	355000
0.00	333333	450000	391667

NCFU/ml	Tox fnc
1.0085	-0.0084
0.766	0.30556
0.7915	0.26344
0.9064	0.10329
1	0



Micro cosm (ml)	Treated Dilutn water (ml)	Total Volume	Sample Dilution
30	270	305	0.098361

Metal Conc	50	10.00	5.00	1.00	9/12/97
Actual conc	44.26	8.85	4.43	0.89	

	innoc (ml)	dil water (ml)	dilution %	innoc (ml)	2nd Dil (ml)	2nd Dil %	innoc (ml)	3rd Dil (ml)	3rd Dil %
metals	0.40	50.00	0.078689	4.00	20.00	0.01574	2.00	20.00	0.0079
blanks	0.20	50.00	0.039344	4.00	20.00	0.00787			

	Culture	Dilutn %	Count	cfu/ml	Comments	9/13/97
1	M-blank	0.039344	>300	#VALUE!		
2	M-blank	0.039344	>300	#VALUE!		
3	M-blank	0.007869	22	275000		
4	M-blank	0.007869	18	225000		
5	44.26	0.078689	>300	#VALUE!		
6		0.078689	>300	#VALUE!		
7		0.015738	15	93750		
8		0.015738	12	75000		
9		0.007869	10	125000		
10		0.007869	7	87500		
11	8.85	0.078689	>300	#VALUE!		
12		0.078689	>300	#VALUE!		
13		0.015738	24	150000		
14		0.015738	14	87500		
15		0.007869	10	125000		
16		0.007869	12	150000		
17	4.43	0.078689	>300	#VALUE!		
18		0.078689	>300	#VALUE!		
19		0.015738	33	206250		
20		0.015738	25	156250		
21		0.007869	15	187500		
22		0.007869	10	125000		
23	0.89	0.078689	>300	#VALUE!		
24		0.078689	>300	#VALUE!		
25		0.015738	38	237500		
26		0.015738	36	225000		
27		0.007869	11	137500		
28		0.007869	14	175000		
29	Blank					

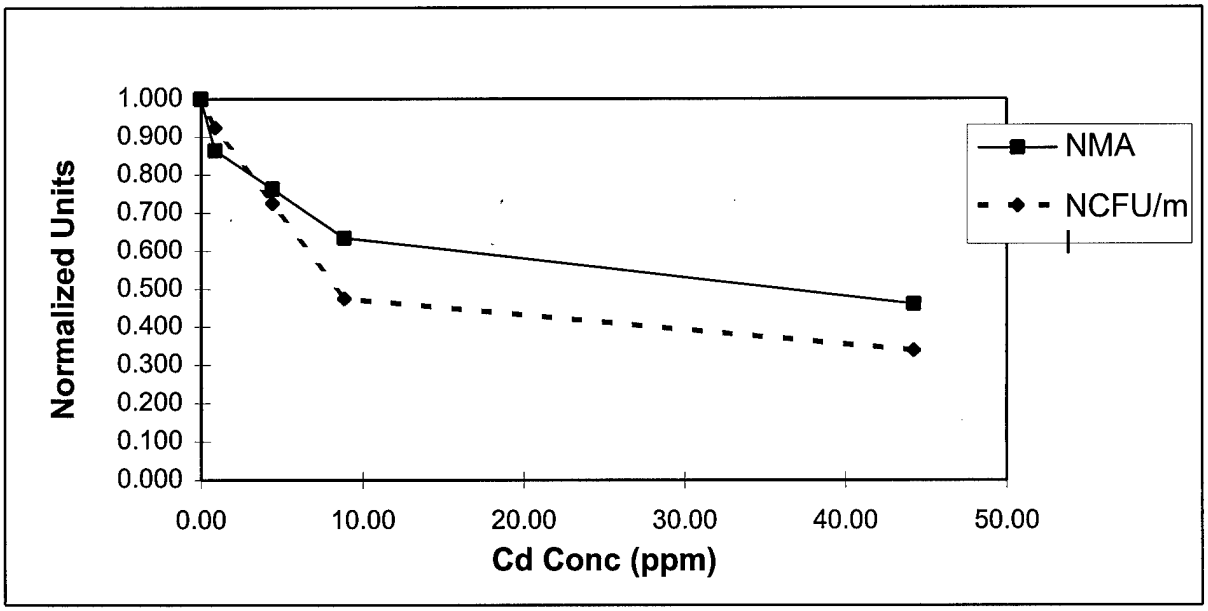
Cd Treatment 2

9/13/97

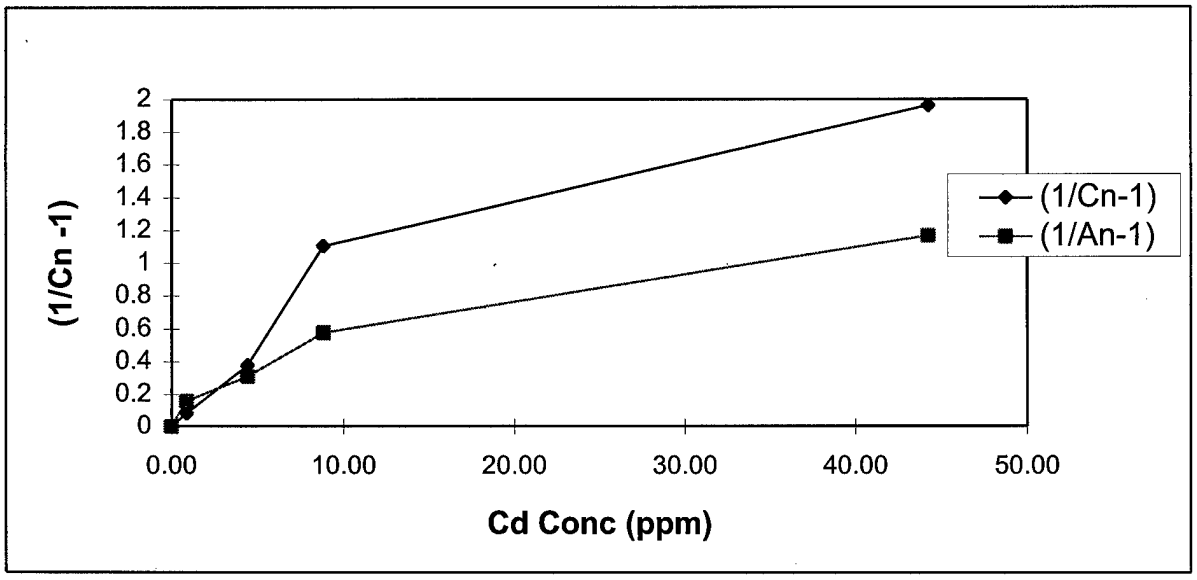
Culture	cfu/ml 1	cfu/ml 2	ave cfu/ml
44.26	93750	75000	84375
8.85	150000	87500	118750
4.43	206250	156250	181250
0.89	237500	225000	231250
0.00	275000	225000	250000

sq root conc	NCFU/ ml	Do meas	NMA	(1/Cn-1)	(1/An-1)
6.653	0.3375	0.00461	0.461	1.963	1.1692
2.975	0.475	0.00634	0.634	1.1053	0.57729
2.104	0.725	0.00763	0.763	0.3793	0.31062
0.943	0.925	0.00864	0.864	0.0811	0.15741
0	1	0.01000	1.000	0	0

NCFPU/ml for Cadmium Treatment 2



Toxicity for Cadmium Treatment



Micro cosm (ml)	Treated Dilutn water (ml)	Total Volume	Sample Dilution
40	260	305	0.131148

Metal Conc	50	10.00	5.00	1.00	9/13/97
Actual conc	42.62	8.52	4.26	0.85	

	innoc (ml)	dil water (ml)	dilution %	innoc (ml)	2nd Dil (ml)	2nd Dil %	innoc (ml)	3rd Dil (ml)	3rd Dil %
metals	0.40	50.00	0.104918	6.00	20.00	0.0315	1.50	20.00	0.0079
blanks	0.10	50.00	0.02623	6.00	20.00	0.0079			

	Culture	Dilutn %	Count	cfu/ml	Comments	9/14/97
1	M-blank	0.02623	157	785000		
2	M-blank	0.02623	144	720000		
3	M-blank	0.007869	50	833333		
4	M-blank	0.007869	55	916667		
5	42.62	0.104918	15	18750		
6		0.104918	16	20000		
7		0.031475	6	25000		
8		0.031475	3	12500		
9		0.007869	1	16667		
10		0.007869	2	33333		
11	8.52	0.104918	115	143750		
12		0.104918	114	142500		
13		0.031475	50	208333		
14		0.031475	62	258333		
15		0.007869	7	116667		
16		0.007869	16	266667		
17	4.26	0.104918	242	302500		
18		0.104918	>300	#VALUE!		
19		0.031475	94	391667		
20		0.031475	91	379167		
21		0.007869	30	500000		
22		0.007869	23	383333		
23	0.85	0.104918	>300	#VALUE!		
24		0.104918	>300	#VALUE!		
25		0.031475	192	800000		
26		0.031475	150	625000		
27		0.007869	60	1000000		
28		0.007869	45	750000		
29	Blank					

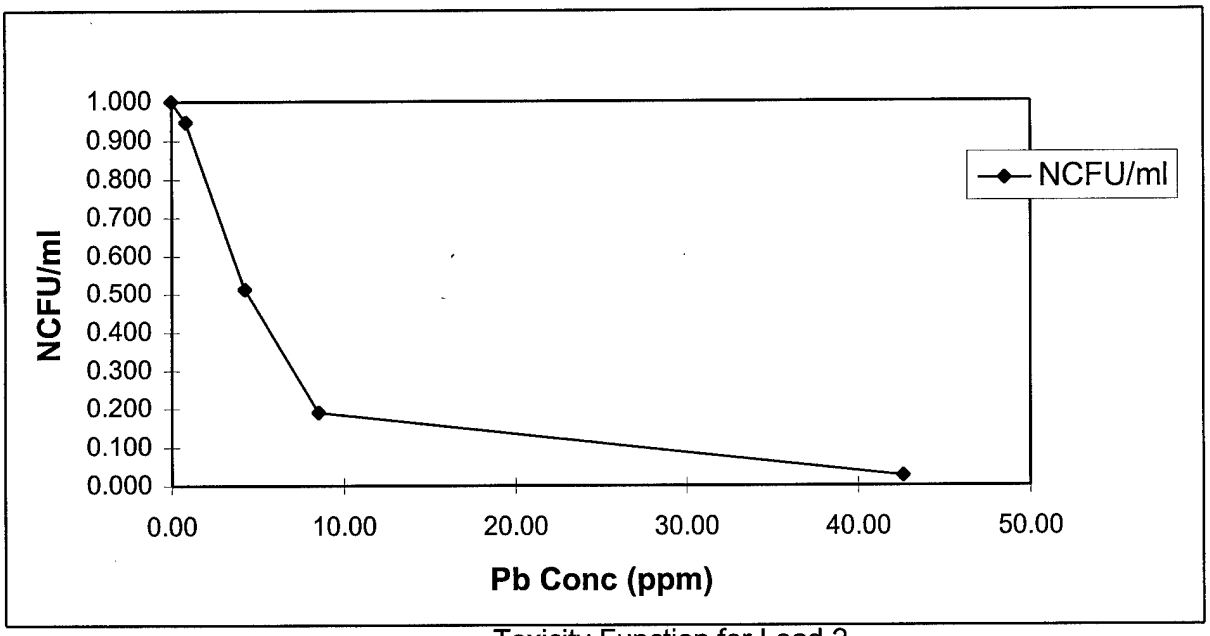
Pb Treatment 2

9/14/97

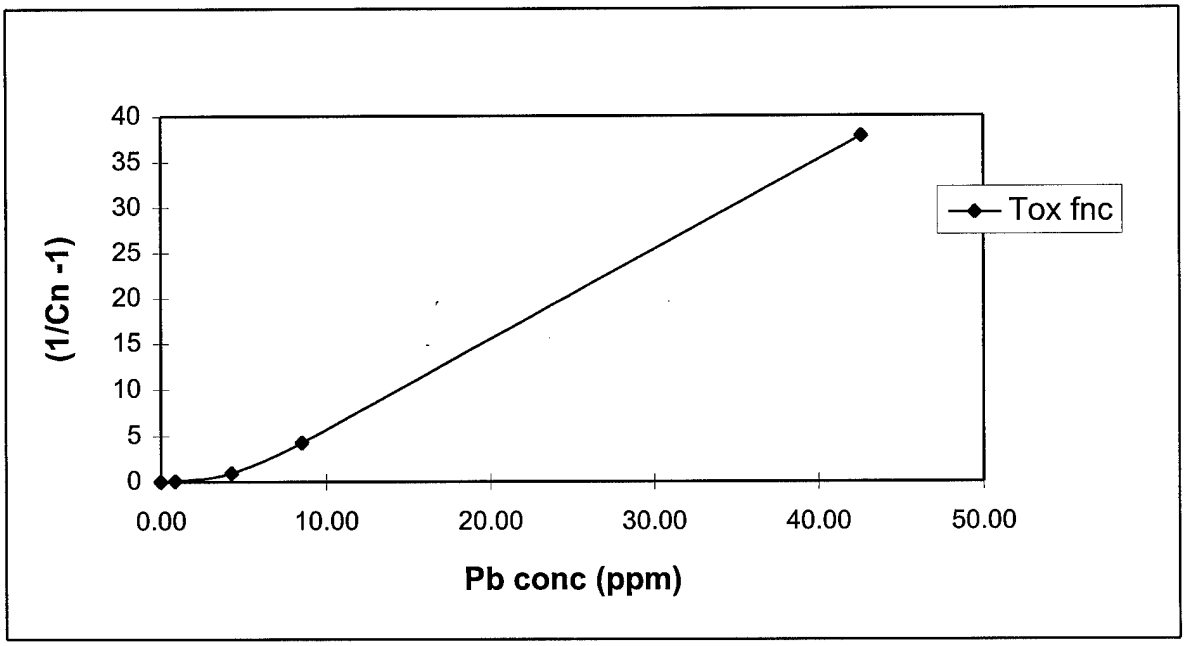
Culture	cfu/ml 1	cfu/ml 2	ave cfu/ml
42.62	18750	20000	19375
8.52	143750	142500	143125
4.26	391667	379167	385417
0.85	800000	625000	712500
0.00	785000	720000	752500

NCFU/ml	Tox fnc
0.026	37.8387
0.190	4.25764
0.512	0.95243
0.947	0.05614
1.000	0

NCFU/ml for Lead Treatment 2



Toxicity Function for Lead 2



Dilution Test

Treated

Micro cosm (ml)	Dilutn water (ml)	Total Volume	Sample Dilution	Total dilution %
20	280	305	0.06557	0.006557
30	270	306	0.09804	0.009804
40	260	307	0.13029	0.013029
50	250	308	0.16234	0.016234
60	240	309	0.19417	0.019417

9/2/97

	innoc (ml)	dil water (ml)	dilution %
microcsm	0.10	100.00	0.1

9/3/97

	Culture	Dilutn %	Count	cfu/ml	Comments
1	20.00	0.006557	97	970000	
2	20.00	0.006557	90	900000	
3	20.00	0.006557	92	920000	
4	30.00	0.009804	128	1280000	
5	30.00	0.009804	130	1300000	
6	30.00	0.009804	129	1290000	
7	40.00	0.013029	175	1750000	
8	40.00	0.013029	184	1840000	
9	40.00	0.013029	>300	#VALUE!	
10	50.00	0.016234	202	2020000	
11	50.00	0.016234	>300	#VALUE!	
12	50.00	0.016234	230	2300000	
13	60.00	0.019417	254	2540000	
14	60.00	0.019417	240	2400000	
15	60.00	0.019417	>300	#VALUE!	
16	Blank		3		

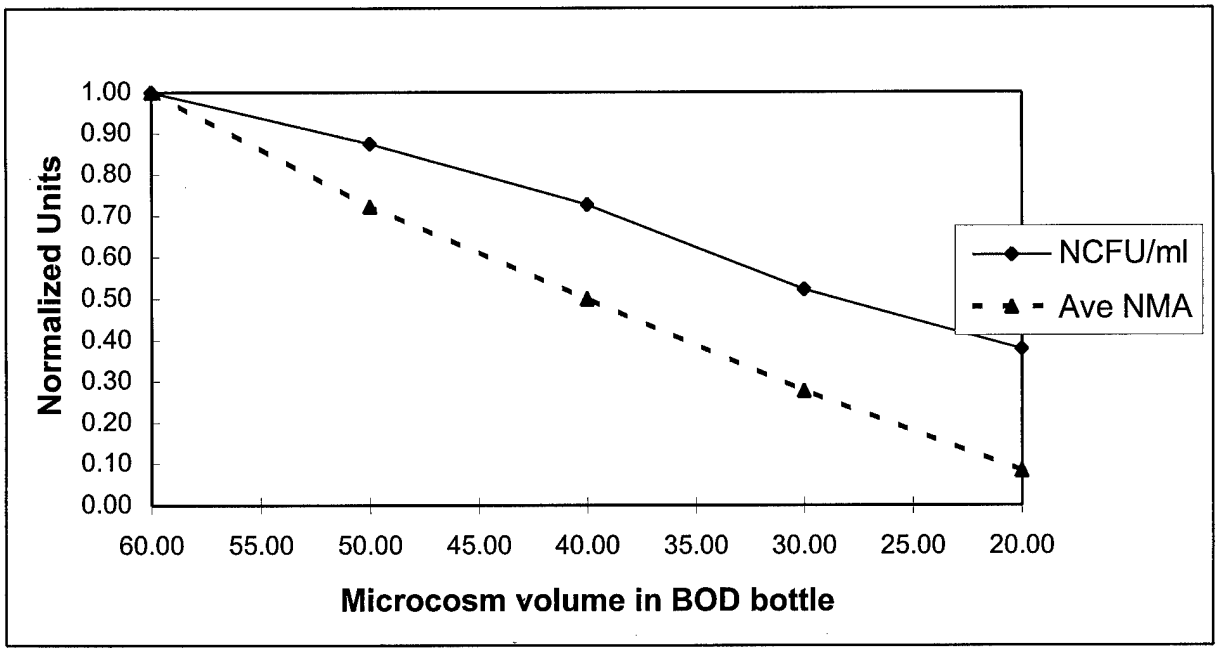
Dilution Test

9/3/97

Micro	cfu/ml 1	cfu/ml 2	ave cfu/ml
20.00	970000	900000	935000
30.00	1280000	1300000	1290000
40.00	1750000	1840000	1795000
50.00	2020000	2300000	2160000
60.00	2540000	2400000	2470000

NCFU /ml	DO Rate
0.38	0.003
0.52	0.01
0.73	0.018
0.87	0.026
1.00	0.036

NCFU/ml for Dilutions



Micro cosm (ml)	Treated Dilutn	Total Volume	Sample Dilution
40	260	300	0.13333

Metal Conc	16	11.00	5.00	2.00	10/7/97
Actual conc	13.87	9.53	4.33	1.73	

	innoc (ml)	dil water (ml)	dilution %	innoc (ml)	2nd Dil (ml)	2nd Dil %	innoc (ml)	3rd Dil (ml)	3rd Dil %
metals	0.50	50.00	0.13333	6.00	20.00	0.04	1.50	20.00	0.01
blanks	0.20	50.00	0.05333	6.00	20.00	0.016	1.5	20	0.004

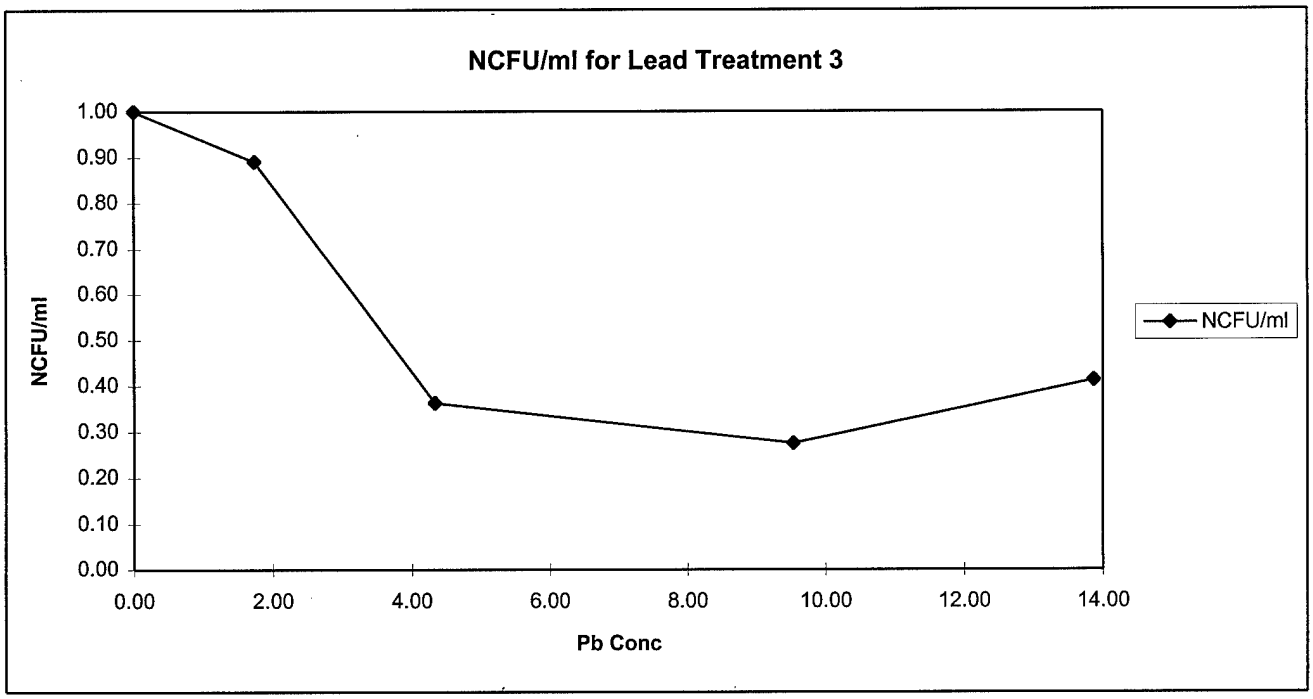
	Culture	Dilutn %	Count	cfu/ml	Comments	10/8/97
1	M-blank	0.053333	30	75000	Stir bars displace @ 5mls	
2	M-blank	0.053333	34	85000		
3	M-blank	0.016	17	141667		
4	M-blank	0.016	15	125000		
5	M-blank	0.004	7	233333		
6	M-blank	0.004	6	200000		
7	BR-blank	0.053333	26	65000		
8	BR-blank	0.053333	31	77500		
9	BR-blank	0.016	8	66667		
10	BR-blank	0.016	7	58333		
11	BR-blank	0.004	9	300000		
12	BR-blank	0.004	3	100000		
13	13.87	0.133333	31	31000		
14		0.133333	35	35000		
15		0.04	14	46667		
16		0.04	8	26667		
17		0.01	3	40000		
18		0.01	8	106667		
19	9.53	0.133333	20	20000		
20		0.133333	24	24000		
21		0.04	9	30000		
22		0.04	11	36667		
23		0.01	1	13333		
24		0.01	6	80000		
25	4.33	0.133333	34	34000		
26		0.133333	24	24000		
27		0.04	115	383333		
28		0.04	13	43333		
29		0.01	3	40000		
30		0.01	6	80000		
31	1.73	0.133333	39	39000		
32		0.133333	32	32000		
33		0.04	11	36667		
34		0.04	12	40000		
35		0.01	7	93333		
36		0.01	3	40000		
37	Blank		3			

Pb Treatment 3

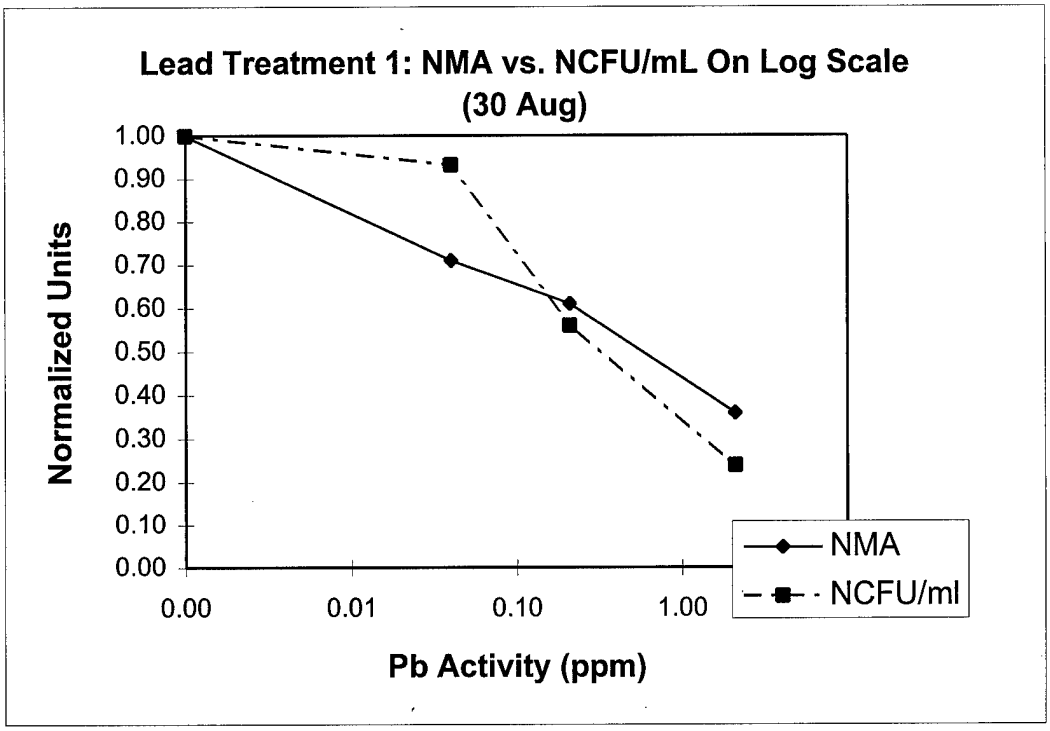
10/8/97

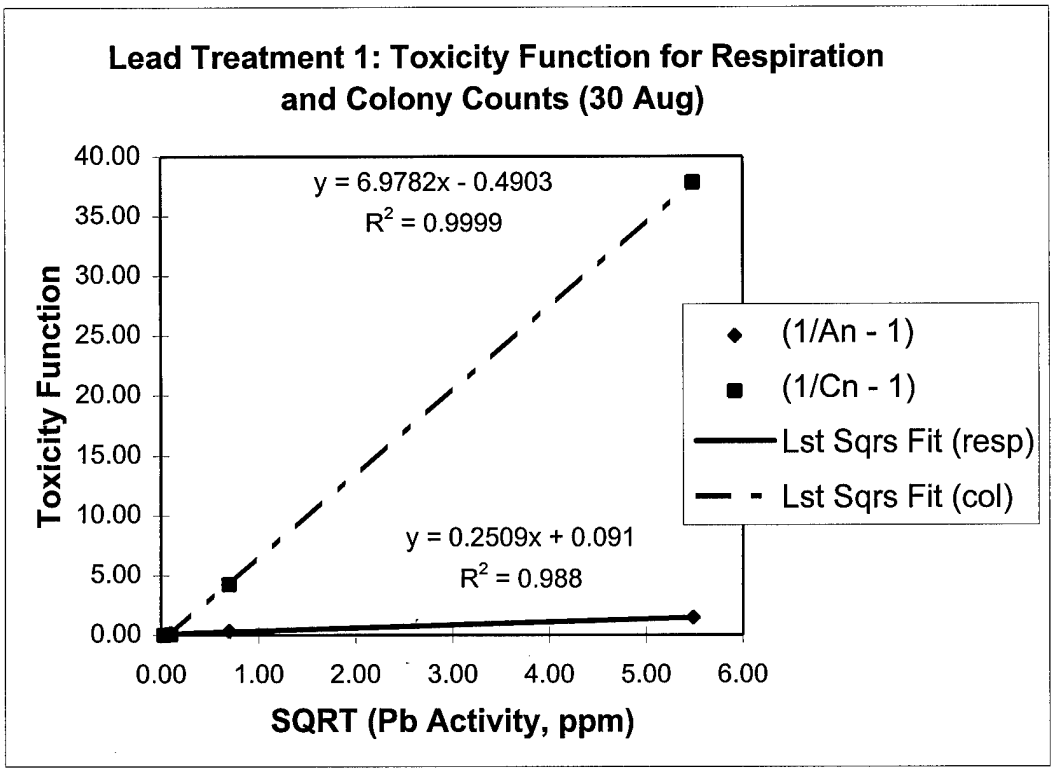
Culture	cfu/ml 1	cfu/ml 2	ave cfu/ml
13.87	31000	35000	33000
9.53	20000	24000	22000
4.33	34000	24000	29000
1.73	65000	77500	71250
0.00	75000	85000	80000

NCFU/ml	Tox fnc
0.41	1.4242
0.28	2.6364
0.36	1.7586
0.89	0.1228
1.00	0



Appendix F: Lead Treatment 1 Charts





Morphology		Dimensions (um	Counter Stain G Stain	Light stain	Med stain	Dark stain	Fluorescence y/g 02	y/g 09	blue 02	blue 09	Colony types Orange	Orange 2	Translucent	White	Cream
Bacillus	Small rods	1.2-1.5 x 0.5	-	X			X	X							
	rods	2 - 4 x 0.5 - 0.8	-	X			X	X					Cd, Pb	MB, Cu	Cu
	rods	2 - 3 x 0.5 - 1	-		X		X								
	rods	2 - 3 x 0.5 - 1	-		X		X			X					
	double rods	2 - 3 x 0.8 - 1	-		X		X								
	rods	1.5 - 2 x 1	-			X		None					MB, Pb*		MB, Pb, Pb*
	double rods	1.5 - 2 x 1	-			X		None					MB, Pb*		MB, Pb, Pb*
	rods	2 x 1	+												
	rods	2 x 1	-								Cd				
	long slender	10 - 50 x 1	-								MB				
	Rods	2 - 4 x 1	-								MB, Cu, Pb, Pb*			Cd	
	long slender	3 - 5 x 0.8 - 1	-										MB		
	long slender	7 -10 x .5 - 0.8	-										MB		
	long slender	6 - 10 x 0.8	-											MB	
	oval	1 x 0.5	-												MB
	slender	5 - 7 x 1	-											Cd	Cu, Pb
	oval	3.5 - 1.5	-												Cd
	spotted	4 - 12 x 0.8	-								Pb				
Cocci	chain	0.3 - 0.5	+					X							
	chain	1.2	-									Cu			
	single	0.5	-												
	single	1 - 1.5	+						X						
	single med sta	1 - 1.5	-												
	single	0.8 - 0.5	-											MB, Cd	
	single	1.2	-									Cu			

Characteristic Not Observed

Fluorescence

y/g	yellow /green
O2	Zeiss filter for DAPI fluorochrome
O9	Zeiss filter for Sytox Green nucleic acid stain
NONE	No emission w hen observed

		dilution %	ave cfu/ml
MB	Untreated Sample (Pb # 2 - 13 Sep)	0.00787	753000
Cu	5 ppm copper treatment	0.05	18000
Cd	8.9 ppm cadmium treatment	0.0157	181000
Pb	8.5 ppm lead treatment	0.0315	143000
Pb*	42 ppm lead treatment	0.105	19000

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Vita

Lieutenant Jason Goodbody was bornin Barstow, California on 9 December, 1970. He graduated from Woodbridge Senior High School in Woodbridge, Virginia in 1989 and entered undergraduate studies at the University of Virginia in Charlottesville, Virginia. He earned a Bachelor of Science degree in Engineering Science and a distinguished graduate commission from the Reserve Officer Training Corps in December of 1993.

In March of 1994 he entered active duty, serving his first assignment at Wright-Patterson Air Force Base, Ohio as the T-1A Systems Project Officer at the Flight Training System Program Office in Air Force Materiel Command (AFMC). He served at Wright-Patterson AFB until May 1996 when he entered the Graduate School of Engineering of the Air Force Institute of Technology. Lt. Goodbody married Staci Lynn Martin in September of 1997. *Their* follow-on assignment was Pope AFB.

Permanent Address: 12015 Skipjack Ct
Woodbridge VA 22192

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Jason B. Goodbody, First Lieutenant, USAF

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WPAFB OH 45433-7765

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13. ABSTRACT *(Maximum 200 words)*

This research employed a variety of microscopy and spread plating techniques to observe the effects of heavy metal treatments on a toluene-selected bacterial population.

Microbial colonies were cultured on spread plates and the resulting numbers were compared to respiration data. The mechanisms of reproduction were demonstrated to be more sensitive to metal treatments than were the mechanisms of respiration. Phase contrast, Gram stain, and fluorescent microscopy, were used to compare and document a wide variety of bacteria resulting from different metal treatments as well as from environmental changes within the source bioreactor. The removal of sensitive bacteria and the selection of metal tolerant species resulting from metal treatments was observed. Species that were initially unobserved within the bioreactor appeared to dominate when competing types of bacteria were removed and more agreeable environmental conditions were present. The use of fluorescent stains to differentiate between live and dead bacteria when treated with heavy metals proved to be impractical as the bacteria exhibited auto-fluorescence. Such new findings, however, did aid in the characterization of different types of bacteria and offered new techniques for potential heavy metal toxicity measurements as well as differentiation methods.

14. SUBJECT TERMS

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